Three actin cross-linking proteins, the 34 kDa actin-bundling protein, α-actinin and gelation factor (ABP-120), have both unique and redundant roles in the growth and development of *Dictyostelium*

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SUMMARY

The contribution of three actin cross-linking proteins, α-actinin (αA), gelation factor (ABP-120), and the 34 kDa actin-bundling protein to cellular functions has been studied in three single mutant (αA−, 120−, and 34−) and three double mutant (αA−/120−, 34−/αA−, 34−/120−) strains of *Dictyostelium* generated by homologous recombination. Strains αA−/120− and 34−/αA− exhibited a reduced rate of pinocytosis, grew to lower saturation densities, and produced small cells in shaking cultures. All strains grew normally in bacterial suspensions and on agar plates with a bacterial lawn. Slow growth under conditions of reduced temperature and increased osmolarity was observed in single mutants 34− and αA−, respectively, as well as in some of the double mutant strains. Motility, chemotaxis, and development were largely unaltered in 34−/αA− and 34−/120− cells. However, 34−/αA− cells showed enhanced aggregation when starved in suspension. Moreover, morphogenesis was impaired in both double mutant strains and fruiting bodies of aberrant morphology were observed. These defects were reverted by re-expression of one of the lacking cross-linking proteins. The additive and synthetic phenotypes of these mutations indicate that actin cross-linking proteins serve both unique and overlapping functions in the actin cytoskeleton.

Key words: Actin cross-linking protein, Gene replacement, *Dictyostelium*, Endocytosis, Synthetic phenotype

INTRODUCTION

The microfilament system composed of actin, myosins, and actin-associated proteins has been implicated in a variety of key cellular processes including locomotion, endocytosis, cytokinesis, and morphogenesis (Fukui, 1993; Noegel and Luna, 1995). Among the actin-associated proteins, F-actin cross-linking proteins are believed to play an essential role in the regulation of cell structure and motility, since they participate in the rearrangement and localization of actin filaments through the formation and stabilization of actin bundles and three-dimensional networks (Furukawa and Fechheimer, 1997; Matsudaira, 1991; Otto, 1994). To date, eleven different 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), ABP120 (also termed gelation factor; Condeelis et al., 1981; Noegel et al., 1989; Fucini et al., 1997), α-actinin (Fechheimer et al., 1982; Condeelis and Vahey, 1982; Brier et al., 1983; Noegel et al., 1987), elongation factor 1α (Demma et al., 1990), comitin (Weiner et al., 1993), a 30 kDa bundling protein (Brown, 1985), a 34 kDa protein (Fechheimer and Taylor, 1984; Fechheimer, 1987; Fechheimer et al., 1991), cortexillins I and II (Faix et al., 1996), and fimbrin (Prassler et al., 1997).

One of the major advantages of *Dictyostelium* as a model organism for studying the microfilament system is that a variety of genetic approaches can be used to generate mutants with defects in cytoskeletal components (Schleicher et al., 1995). Homologous recombination has been employed to create cells with mutations in one or two actin cross-linking proteins. Single mutants lacking α-actinin (Schleicher et al., 1988), gelation factor (Brink et al., 1990; Cox et al., 1992), the 34 kDa actin-bundling protein (Rivero et al., 1996a), and cortexillin I or II (Faix et al., 1996) are either normal or display moderate defects in the ability to grow, locomote, and complete the developmental cycle. A model of functional redundancy has been previously proposed to explain the limited severity of phenotypes observed in single mutants (Witke et al., 1992; Rivero et al., 1996a,b). In support of this model, double mutants lacking α-actinin and gelation factor (Witke et al., 1992; Rivero et al., 1996b), and double mutants lacking cortexillin I and II (Faix et al., 1996) have been shown to exhibit severe synthetic phenotypes. These results suggest that much remains to be learned about the functions of the multiple actin cross-linking proteins, and that some of the functions of
the actin cross-linking proteins may be deduced only by analysis of synthetic phenotypes.

In this study, we have generated double mutant strains of Dictyostelium lacking the 34 kDa actin bundling protein and either α-actinin (34-αA/αA) or gelation factor (34-120-), and performed additional characterization of the αA/120- strain described previously (Rivero et al., 1996b). The effects of single and double mutations in these cross-linking proteins have been examined in a wide range of cellular functions including growth, endocytosis, growth under physiological stress, locomotion, and morphogenesis. The expanded range of growth conditions employed reveals that the 34- and αA- single mutants show impaired growth under conditions of reduced temperature and hyperosmotic stress, respectively. In addition, the three double mutant strains (αA/120-, 34-αA/, 34-120- ) all show synthetic phenotypes. Moreover, the three strains show a distinct but overlapping subset of phenotypes consistent with both shared and unique capabilities of the actin cross-linking proteins as proposed in the model of partial functional redundancy.

MATERIALS AND METHODS

Dictyostelium strains and growth conditions

Dictyostelium discoideum strain AX2-214, an axenically growing derivative of wild-type strain NC-4, is the parent strain for all mutants. The isolation of single mutants lacking the 34 kda actin bundling protein (Rivero et al., 1996a), α-actinin, or gelation factor (Eichinger et al., 1996), and double mutants lacking both α-actinin and gelation factor (Rivero et al., 1996b) has been previously described. Cells of strain AX2-214 (referred to as wild-type) and mutant strains were grown in liquid nutrient medium at 21°C with shaking at 160 rpm unless otherwise indicated (Claviez et al., 1982). Growth on plates was either on SM agar with Klebsiella aerogenes (Williams and Newell, 1976), or on nutrient agar plates with E. coli B/r (Bonner, 1947).

Generation of mutants

Double mutants 34-αA/αA and 34-120- were generated by disrupting either the α-actinin gene or the gelation factor gene in the 34 kDa actin bundling protein deficient strain (Rivero et al., 1996a) via homologous recombination using the vectors pDab α·1.2 and pDgel 1.5, respectively (Witke et al., 1992). pDab α·1.2 contains the N-terminal 1.2 kb EcoRI fragment of the α-actinin cDNA (Noegel et al., 1987), and pDgel 1.5 contains an N-terminal 1.5 kb EcoRI fragment of the gelation factor cDNA (Noegel et al., 1989), both under the control of an actin-6 promoter and an actin-8 terminator. Both vectors contain a G418 resistance cassette.

To express native or mutated forms of α-actinin in the 34-αA/αA strain, plasmids pαM, pMI, and pMII (Witke et al., 1993) were introduced by co-transformation with pUCBsrABam (Adachi et al., 1994), a vector containing a blasticidin resistance cassette. Plasmids pMI and pMII harbor α-actinin genes mutated in EF-hand I or II, respectively. To express the 34 kDa actin bundling protein in the 34-120- and 34-αA/αA strains, plasmid p34 (Rivero et al., 1996a) was introduced by co-transformation with pUCBsrABam.

All vectors were introduced as supercoiled DNA by electroporation (Mann et al., 1994). Blasticidin (ICN Biomedicals Inc., Aurora, OH) and G418 (Sigma, St Louis, MO) were used for selection of transformants. Mutants were identified by colony blotting (Wallraff et al., 1986) using α-actinin specific monoclonal antibody 47-62-17 (Schleicher et al., 1988), gelation factor specific monoclonal antibody 82-471-17 (Brink et al., 1990), or 34 kDa protein specific monoclonal antibody B2C (Furukawa et al., 1992).

Western, southern, and northern blotting

SDS-polyacrylamide gel electrophoresis and western blotting were performed as described (Laemmli, 1970; Towbin et al., 1979) using either iodinated sheep anti-mouse antibodies or an enhanced chemiluminescence detection system (Amersham Buchler GmbH & Co. KG, Germany). DNA and RNA were isolated, transferred to Hybond N filters (Amersham) and hybridized as described (Noegel et al., 1985).

Pinocytosis

Quantitative studies of pinocytosis were performed essentially as described previously using the soluble fluorescent marker Lucifer Yellow (Swanson et al., 1985; Rivero et al., 1996a).

Osmotic and temperature stress

For the analysis of growth in shaking suspension under stress conditions, cells were grown either: (a) in axenic medium at 15°C; (b) in axenic medium supplemented with 30 mM NaCl at 20°C; or (c) in axenic medium supplemented with 115 mM sorbitol at 20°C. To test resistance against osmotic shock, the cells were shaken for 2 hours in Soerensen phosphate buffer with 0.4 M sorbitol as previously described, and viability assayed after dilution and plating on SM agar with K. aerogenes (Rivero et al., 1996b).

Immunofluorescence microscopy

Cells were fixed in cold methanol (~20°C). Contact site A and α-actinin were detected using monoclonal antibodies 33-249-17 (Berthold et al., 1985) and 47-62-17 (Schleicher et al., 1988) followed by incubation with Cy3-labeled anti-mouse IgG (Weiner et al., 1993). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma Chemical Co.).

Motility and chemotaxis

For quantitative analysis of cell motility and chemotaxis, cells were grown to a density of 2 to 3x10⁶ cells/ml, washed in Soerensen phosphate buffer, resuspended at a density of 1x10⁷ cells/ml and starved for 6 hours with shaking. Analysis was performed using an image processing system (Segall et al., 1987) and a chemotaxis chamber (Fischer et al., 1989) with a maximum cAMP concentration of 5x10⁻³ M (Brink et al., 1990).

Cell size and cell adhesion

Cell size was measured as described (Rivero et al., 1996a). To analyze adhesion, the cells were adjusted to the same total cell volume by resuspending them at an OD₆₀₀ of 0.9 (roughly equivalent to a density of 1x10⁷/ml AX2 cells) in 10 ml Soerensen phosphate buffer and were shaken at 160 rpm. As an estimate of the time course of adhesion, changes in optical density (600 nm wavelength) were monitored using a LKB Ultraspec III spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Adhesion was also assayed by measuring light scattering using the method of Beug and Gerisch (1972) under standardized shear forces.

Development of Dictyostelium discoideum

Cells were grown to a density of 2 to 3x10⁶ cells/ml, washed in 17 mM Soerensen phosphate buffer, pH 6.0, and resuspended at a density of 1x10⁶ cells/ml in the same buffer. Morphology was studied by allowing 1x10⁹ cells to develop on 1.2% (w/v) water agar or phosphate-buffered agar plates at 21°C. For the analysis of developmentally regulated genes, 0.8x10⁸ cells were allowed to develop on nitrocellulose filters (Millipore type HA, Millipore, Molsheim, France) at 21°C as described (Newell et al., 1969).
RESULTS

Mutants in F-actin cross-linking proteins

The 34–/αA− and 34–/120− double mutant strains were generated as described in Materials and Methods. The gene disruption events were confirmed using Southern blot analyses (not shown). At the RNA level, shortened transcripts for α-actinin and ABP 120 were detected in the 34–/αA− and 34–/120− double mutants, in contrast to the ~3 kb messages in the wild-type AX2 (not shown). Absence of either α-actinin or gelation factor was confirmed by western blot analyses using monoclonal antibodies 47-62-17 or 82-471-17 specific for α-actinin or gelation factor, respectively (Fig. 1). The antibodies against both proteins have binding sites located in the amino terminal halves of the respective molecules. Thus, the absence of labeling in the mutant strains suggests that products that might result from translation of the truncated transcripts apparent in the northern analyses do not accumulate.

In addition to these new double mutant strains, we have analyzed the following cell lines in this study: AX2, the wild-type parent strain; 34−, the 34 kDa actin bundling protein deficient hygromycin resistant mutant (Rivero et al., 1996a); αA−, the α-actinin deficient mutant (Eichinger et al., 1996; Rivero et al., 1996b); 120−, the gelation factor minus mutant (Eichinger et al., 1996; Rivero et al., 1996b); and αA−/120−, the double mutant deficient in α-actinin and gelation factor (Rivero et al., 1996b). The 34 kDa actin bundling protein deficient cell line 34 kD−/ura and its parent strain DH1 (Rivero et al., 1996a) have also been included in certain experiments. Table 1 provides information on genotype and nomenclature of the strains used in this study, as well as the strains in which the cross-linking proteins have been re-expressed in the mutants. The absence of a robust sexual genetic system in Dictyostelium does not allow rigorous demonstration that the phenotype segregates with and results from the gene replacement. Reversion of the phenotype in these ‘rescue’ strains provides formal evidence that the phenotype is closely correlated with the designated null mutations.

Growth, cell size distribution, and endocytosis of mutant strains

Cell growth is the result of the interplay between a variety of cellular processes including fluid or receptor mediated endocytosis, phagocytosis, and cytokinesis. Since the actin cytoskeleton has been shown to be involved in these processes, we have determined growth rates of AX2 and mutant strains under several conditions (Fig. 2). Under standard laboratory conditions (shaking at 160 rpm at 20°C), double mutants 34−/αA− and 34−/120− saturated at lower densities than cultures of AX2; αA−/120− cells grew with a prolonged doubling time. At 220 rpm, all strains grew with a comparable doubling time and reached high cell densities. For 34−, αA− and 120− single mutants, curves similar to that of AX2 were obtained. For all curves shown in this Fig. each point represents the average of three determinations.

Fig. 1. Western blot analysis of double mutant strains 34−/αA− (upper panel) and 34−/120− (lower panel). Total protein from 2x10^5 cells was resolved by 10% SDS-PAGE, blotted onto nitrocellulose, and probed with α-actinin specific mAb 47-62-17 or gelation factor specific mAb 82-471-14. Clones 34−/αA−#5 and 34−/120−#4 were chosen for further analyses.

Fig. 2. Growth of wild-type and double mutant strains in axenic medium. Cultures were inoculated with 5x10^4 cells/ml and grown at 21°C with shaking at 160 rpm. (A), 100 rpm. (B), or 220 rpm. (C). At 160 and 100 rpm, cultures of 34−/αA− and αA−/120− saturated at lower densities than cultures of AX2; αA−/120− cells grew with a prolonged doubling time. At 220 rpm, all strains grew with a comparable doubling time and reached high cell densities. For 34−, αA− and 120− single mutants, curves similar to that of AX2 were obtained. For all curves shown in this Fig. each point represents the average of three determinations.
Table 1. Dictyostelium strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AX2</td>
<td>N/A</td>
<td>Wild-type strain AX2-214, derivative of NC-4. Parent strain of single mutants 34−, αA− and 120−.</td>
</tr>
<tr>
<td>34−</td>
<td>abpB−/hygR</td>
<td>34 kD actin bundling protein deficient cell line generated from AX2 by gene replacement.</td>
</tr>
<tr>
<td>αA−</td>
<td>abpA−/G418R</td>
<td>α-Actinin deficient cell line generated from AX2 by gene replacement. Named AHR in previous papers (Eichinger et al., 1996b; Rivero et al., 1996a).</td>
</tr>
<tr>
<td>120−</td>
<td>abpC−/G418R</td>
<td>Gelation factor (ABP-120) deficient cell line generated from AX2 by gene disruption. Named GHR in previous papers (Eichinger et al., 1996b; Rivero et al., 1996a).</td>
</tr>
<tr>
<td>34−/αA−</td>
<td>abpA−/abpB−/hygR/G418R</td>
<td>34 kDa protein and α-actinin deficient cell line generated from 34− by gene disruption.</td>
</tr>
<tr>
<td>34−/120−</td>
<td>abpB−/abpC−/hygR/G418R</td>
<td>34 kDa protein and gelation factor deficient cell line generated from 34− by gene disruption.</td>
</tr>
<tr>
<td>αA−/120−</td>
<td>abpA−/abpC−/ble/G418R</td>
<td>α-Actinin and gelation factor deficient cell line generated from αA− by gene disruption. Previously named AGHR (Rivero et al., 1996a).</td>
</tr>
<tr>
<td>34−/120−R34</td>
<td>abpB−/+abpC−/hygR/G418R/bleR</td>
<td>Rescued cell line generated by reexpression of the 34 kDa protein in the 34−/120− double mutant.</td>
</tr>
<tr>
<td>34−/αA−R34</td>
<td>abpA−/+abpB−/hygR/G418R/bleR</td>
<td>Rescued cell line generated by reexpression of the 34 kDa protein in the 34−/αA− double mutant.</td>
</tr>
<tr>
<td>34−/αA−RαA−WT</td>
<td>abpA−/+abpB−/hygR/G418R/bleR</td>
<td>Rescued cell line generated by reexpression of α-actinin in the 34−/αA− double mutant.</td>
</tr>
<tr>
<td>34−/αA−RαA−M1</td>
<td>abpA−/+M1/abpB−/hygR/G418R/bleR</td>
<td>Rescued cell line generated by reexpression of α-actinin (mutated in EF-hand I) in the 34−/αA− double mutant.</td>
</tr>
<tr>
<td>34−/αA−RαA−M2</td>
<td>abpA−/+M2/abpB−/hygR/G418R/bleR</td>
<td>Rescued cell line generated by reexpression of α-actinin (mutated in EF-hand II) in the 34−/αA− double mutant.</td>
</tr>
<tr>
<td>DH1</td>
<td>ura−</td>
<td>AX3-derived cell line containing a deletion in the pyr5-6 locus encoding UMP synthase (Caterina et al., 1994). Parent strain of 34 kD−/ura.</td>
</tr>
<tr>
<td>34 kD−/ura</td>
<td>abpB−/ura−</td>
<td>34 kD actin bundling protein deficient cell line generated from DH1 by gene replacement (Rivero et al., 1996b).</td>
</tr>
</tbody>
</table>

The nomenclature of Loomis et al. (1995) has been followed for the loci corresponding to α-actinin (abpA), 34 kDa actin bundling protein (abpB) and gelation factor (abpC).

doubling time for double mutants 34−/αA− was slightly prolonged (13 hours), and reached saturation at a lower density of 6×10⁶ cells/ml. The double mutant αA−/120− exhibited a prolonged doubling time (20 hours), and saturated at a lower cell density of 6×10⁶ cells/ml as previously reported (Rivero et al., 1996b) (Fig. 2A). With shaking at 100 rpm (Fig. 2B), similar doubling times were obtained for all strains except for the αA−/120− cells where the doubling time was prolonged to 32.5 hours. The cultures reached saturation at slightly lower cell densities with shaking at 100 rpm as compared to 160 rpm, 1.0×10⁷ cells/ml for AX2 and 34−/120− and 4.0×10⁶ cells/ml for 34−/αA− and αA−/120−. When cells were grown with shaking at 220 rpm (Fig. 2C), all strains showed a doubling time in the range of 9 to 13 hours and reached densities of up to 1.5×10⁷ cells/ml. Under all conditions in these experiments, growth of the single mutants αA−, 120− and 34− was similar to the wild-type AX2 (not shown). The effects of shaking speed may arise from effects on aeration, oxygen availability and energetics in the cultures. Additional analyses are needed to understand this phenomenon in detail.
The distribution of the number of nuclei revealed by DAPI staining was comparable among AX2, the single mutants 34⁻, αA⁻, and 120⁻, and the double mutants 34⁻/120⁻ and 34⁻/αA⁻ with most cells being uni- or bi-nucleated (not shown). Abundant nucleus-free particles such as were present in the αA⁻/120⁻ double mutant (Rivero et al., 1996b) were not observed in the other strains.

Qualitative examination of cultures of the 34⁻/αA⁻ mutants grown in axenic medium suggested that the cells were smaller in size than cultures of AX2. In a quantitative analysis, the distributions of cell sizes between strains AX2 and 34⁻/120⁻ were indistinguishable, with diameters of 10.8±1.7 µm and 10.6±1.5 µm, respectively (Fig. 3). In contrast, 34⁻/αA⁻ cells were significantly smaller with a diameter of 9.4±1.4 µm (P<0.001, ANOVA), a characteristic previously reported for the αA⁻/120⁻ double mutant (Rivero et al., 1996b). This difference is more pronounced when cell surface or cell volume is considered, with 34⁻/αA⁻ cells possessing a surface area approximately 76% and a volume approximately 66% that of AX2 cells.

The ability of mutant strains to perform pinocytosis was assessed by the uptake of the fluid phase marker Lucifer Yellow. Quantitative studies revealed little difference between wild type, the single mutants 120⁻, αA⁻, 34⁻, and the double mutant 34⁻/120⁻ (Fig. 4 and Rivero et al., 1996a). By contrast, double mutants 34⁻/αA⁻ and αA⁻/120⁻ were clearly deficient in fluid phase endocytosis. The alterations in growth in axenic medium of the double mutants 34⁻/αA⁻ and αA⁻/120⁻ were closely correlated with the reduced rate of pinocytosis.

Since prior work implicates the three cross-linking proteins in this study in the process of phagocytosis (Furukawa et al., 1992; Furukawa and Fechheimer, 1994; Cox et al., 1996; Rivero et al., 1996b), we have analyzed this process in the single and double mutants. Growth curves of Dictyostelium in shaking cultures with E. coli B/r as food source showed a similar doubling time of 3.3 to 3.8 hours for all strains tested (Fig. 5A). In addition, growth rates on SM-agar plates with K. aerogenes as a food source were similar for all strains tested in this study, with the exception of αA⁻/120⁻, which grew significantly faster (Fig. 5B). This could result from the delayed developmental pattern of αA⁻/120⁻ which favors spreading on the bacterial lawn instead of aggregation and development. These results suggest that there is not a major impediment to phagocytosis in these strains. Yet, the rate of growth in the presence of bacteria as a food source may be a rather insensitive test of phagocytosis, since αA⁻/120⁻ was impaired in a quantitative assay of bacterial uptake (Rivero et al., 1996b). The presence of an excess of bacterial particles can overcome even a defect in the adhesion of bacteria to the cell surface, as shown by mutants defective in talin (Niewöhner et al., 1997). Thus, an impairment of phagocytosis in the 34⁻/αA⁻ and 34⁻/120⁻ mutants is by no means ruled out by these findings. Prior results indicating a deficiency of phagocytosis in the 120⁻ cell line (Cox et al., 1996) is a clear difference, and is likely a result of the genetic background of the strains (Rivero et al., 1996a,b).
Growth with physiological stress

Changes in temperature, humidity, and osmolarity pose significant physiological challenges to growth and survival of free living organisms. It is conceivable that a role of a protein that is not essential under optimal circumstances in the laboratory may be revealed under certain stress conditions. For this reason, we tested the capability of single and double mutants to survive or grow under conditions of low temperature or high osmolarity.

Cultures of wild-type cells grown at 15°C in suspension in axenic medium exhibited a doubling time of 13.3 hours and reached cell densities of $3 \times 10^6$ cells/ml (Fig. 6A). Similar cell densities at saturation but slightly longer doubling times of 17.3 and 18.5 hours were observed for $\alpha A^-$ and $120^-$ single mutants, respectively. The $34^-$ single mutants were clearly impaired, showing a prolonged doubling time of 23.1 hours and reduced cell density at saturation. The impairment was even more pronounced in double mutant strains $34^-/120^-$ and $34^-/\alpha A^-$, where growth was almost completely abolished. Growth of the $\alpha A^-/120^-$ strain was less severely impaired than the other double mutants showing growth similar to the single mutant $34^-$ strain.

We have previously reported that the double mutant $\alpha A^-/120^-$ exhibited an increased sensitivity towards acute osmotic shock (Rivero et al., 1996b). In contrast, osmotic stress did not affect cell viability for $34^-$, $34^-/\alpha A^-$, and $34^-/120^-$ (not shown). Since the role of a particular actin cross-linking protein in the cytoskeletal rearrangements elicited by acute osmotic stress could differ from its role in the adaptation to sustained altered osmolarity, we tested the ability of the mutant cells to grow in the presence of an increased osmotic content. This was achieved by supplementing the axenic medium with either 30 mM NaCl or 115 mM sorbitol.

In the presence of 30 mM NaCl, AX2 as well as single mutants $34^-$ and $120^-$ were able to grow to a density of $5 \times 10^6$ cells/ml at saturation with a doubling time of about 18 hours (Fig. 6B). Similar results were obtained with the $34^-/ura$ mutant and its parent strain DH1 (not shown). The $\alpha A^-$ cells and double mutants $\alpha A^-/120^-$, $34^-/\alpha A^-$ and $34^-/120^-$ grew only to cell densities approximately half of the wild-type strain, the doubling time being slightly prolonged to 20 hours.

In the presence of 115 mM sorbitol, AX2 cultures grew with a doubling time of 13.3 hours and reached densities of $3 \times 10^6$ cells/ml. With the exception of $\alpha A^-$ and the double mutant $34^-/\alpha A^-$, all other strains grew to similar densities, but with prolonged doubling times (16 to 20 hours). The $\alpha A^-$ grew slowly and to a slightly lower density. The double mutant $34^-/\alpha A^-$ was severely impaired, with a doubling time of 28.9 hours and saturation at around $1 \times 10^6$ cells/ml. Growth of $34^-/ura$ cells was only slightly affected in the presence of sorbitol when compared to the AX3-derived parent strain DH1 (not shown).

![Graphs showing growth of single and double mutants under stress conditions.](image)

**Fig. 6.** Growth of single and double mutants under stress conditions. Cultures were inoculated with $5 \times 10^4$ cells/ml and grown with shaking at 15°C (left panel), in the presence of 30 mM NaCl at 20°C (middle panel), or in the presence of 115 mM sorbitol at 20°C (right panel). When cells were grown at 15°C, double mutants $34^-/\alpha A^-$ and $34^-/120^-$ were the most severely affected. The cells lacking $\alpha$-actinin and all the double mutant strains were the most severely affected by the presence of NaCl. In the presence of sorbitol, double mutant $34^-/\alpha A^-$ was the most severely affected. For all curves shown, each point represents an average of three determinations.

**Table 2. Motility and chemotactic orientation of wild-type and double mutant strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Speed (µm/minute)</th>
<th>Orientation (cos $\theta$)</th>
<th>Average turn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AX2</td>
<td>8.65±1.25</td>
<td>-0.05±0.10</td>
<td>44.87±6.91</td>
</tr>
<tr>
<td>$34^-/120^-$</td>
<td>8.08±1.60</td>
<td>-0.04±0.06</td>
<td>35.82±4.68*</td>
</tr>
<tr>
<td>$34^-/\alpha A^-$</td>
<td>9.10±1.57</td>
<td>0.01±0.05</td>
<td>36.33±4.88*</td>
</tr>
<tr>
<td>Gradient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AX2</td>
<td>13.50±1.17</td>
<td>0.20±0.07</td>
<td>37.12±1.71</td>
</tr>
<tr>
<td>$34^-/120^-$</td>
<td>10.20±1.17</td>
<td>0.20±0.05</td>
<td>35.30±3.83</td>
</tr>
<tr>
<td>$34^-/\alpha A^-$</td>
<td>11.76±0.76</td>
<td>0.21±0.05</td>
<td>33.79±2.63</td>
</tr>
</tbody>
</table>

Cell tracks were recorded over four 30 minute periods. In each period 41 images were taken with a time lapse of 45 seconds 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 with a steepness of $2.5 \times 10^{-8}$ M cAMP/mm was established. 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 the original to final position and the total path length, multiplied by the cosine of the angle which 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. Values are mean ± standard deviation of 7 to 8 independent experiments.

*P<0.05; ‡P<0.001 relative to AX2 (ANOVA).
Motility and chemotactic behavior of double mutant strains

Single mutants $\alpha A^-$, 120$^-\,$, and 34$^-$ are able to locomote and orient properly in the presence of a cAMP gradient, whereas double mutant $\alpha A^-/120^-$ show a reduced speed and fail to orient in the direction of the cAMP gradient (Rivero et al., 1996a,b). Quantitative analysis of motility and chemotactic behavior of the double mutants 34$^-/\alpha A^-$ and 34$^-/120^-$ using a chemotaxis chamber and a computer controlled image processing system (Table 2) showed that AX2 and both double mutant strains exhibit a similar rate of locomotion (8-9 $\mu$m/minute) in the absence of cAMP. In the presence of a cAMP gradient, both double mutants showed a slight but significant reduction in speed (24.5% and 12.9% for 34$^-/120^-$ and 34$^-/\alpha A^-$, respectively) as compared to the wild-type strain. The ability to orient in the direction of the cAMP gradient was unaffected. The average turn between successive images, an estimation of the persistence of motility, was significantly lower in 34$^-/120^-$ and 34$^-/\alpha A^-$ mutants only during migration in the absence of a chemotactic gradient. This phenotype resembles that of the parent strain 34$^-$ which also exhibited a decrease in the average turn reflecting an increase in the persistence of motility (Rivero et al., 1996a).

Cell-to-cell adhesion of mutant strains

Dictyostelium cells starved in shaking suspension adhere
through both calcium-dependent and EDTA-stable intercellular contacts. The adhesion protein gp24/DdCAD1 mediates formation of calcium-requiring contacts (Gao et al., 1992; Knecht et al., 1987; Wong et al., 1996), while EDTA resistant associations are formed by csA glycoprotein (gp80) (Müller and Gerisch, 1978; Noegel et al., 1986) and gp150. Adhesion assays were performed to determine whether the cells lacking one or two of the cross-linking proteins would show a defect that might arise from an alteration in the membrane cytoskeleton at the contact sites. Agglutination of cells was assessed by measuring the decrease in the light scattering at 600 nm (Fig. 7A). Neither single mutants 34−, αA−, and 120−, nor double mutant 34−/120− differed significantly from the wild type. Double mutant αA−/120− showed a delay in the time course of agglutination that correlates with the overall delay in the developmental pattern already described for this strain (Rivero et al., 1996b). Surprisingly, double mutant 34−/αA− exhibited a pattern of significantly stronger agglutination than that of the wild-type strain AX2 (P<0.005 between 6 and 12 hours, ANOVA). Examination under the microscope revealed that aggregates of 34−/αA− were clearly bigger than aggregates of AX2. Western analysis of the accumulation of csA glycoprotein showed that changes in the amount of this cell adhesion molecule were not responsible for this agglutination pattern (Fig. 7B).

The behavior of aggregation competent 34−/αA− cells was further confirmed using an agglutinometer so that standardized shear forces are applied to the cells (Beug and Gerisch, 1972). Cell suspensions were adjusted to the same total cell mass to correct for differences in cell size, since the 34−/αA− cells are smaller (Fig. 3). After 1 hour in the agglutinometer, when equilibrium is reached, 34−/αA− cells showed lower light scattering values than AX2 cells in the absence of EDTA (Fig. 7C), indicating the formation of larger aggregates (Fig. 7D). In the presence of EDTA, aggregates of 34−/αA− were only slightly bigger than that of AX2. Thus, the larger aggregate size of the 34−/αA− may be due to enhanced activity of a calcium-dependent adhesion mechanism. A similar behavior was observed when the cell suspensions were adjusted to the same cell density instead of to the same total cell mass, indicating that the pattern observed arises from an intrinsic property of the cells.

**Development of double mutant strains**

The contribution of α-actinin, gelation factor, and the 34 kDa actin bundling protein to the developmental process has been studied in the double mutants 34−/αA− and 34−/120−. The developmental pattern of the wild-type and mutant cells was examined on phosphate buffered agar, on water-agar, and on nitrocellulose filters. Under all conditions, early development was unaltered in both mutant strains, with the exception that mounds and slugs were usually of smaller size than that of wild type. However, as development proceeded, aberrant fruiting bodies formed (Fig. 8, left panels). In 34−/αA− double mutants, sori were only partially lifted from the substrate, and frequently, the stalk continued to extend upward with a second small sorus at the tip. Fruiting bodies with two sori are occasionally observed in wild-type cells. However, few normal-looking fruiting bodies can be observed among the culminants formed by the 34−/αA− strain. In 34−/120− mutants, fully developed fruiting bodies were short and squat and possessed a thick stalk. A small proportion of 34−/120− cells do not develop beyond the mound stage. In

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**Fig. 8.** Development of AX2 and double mutant strains 34−/αA− and 34−/120−. On the left, morphology of fully developed fruiting bodies. Cells were plated on phosphate agar and allowed to develop at 21°C. Pictures were taken 40 hours after plating the cells. In fruiting bodies of 34−/αA− sori are only partially lifted from the surface. Fruiting bodies of 34−/120− are short and squat compared to AX2. Bar, 0.1 mm. On the right, expression of developmentally regulated genes. 0.75·108 cells were allowed to develop on nitrocellulose filters at 21°C for the times indicated. 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. The nomenclature of Loomis et al. (1995) has been followed for the developmentally regulated genes.
both double mutant strains, the developmental cycle ends with the formation of viable spores, although at a lower yield than in AX2.

The timing and levels of expression of developmentally regulated genes was examined to assess the progress of distinct stages of morphogenesis. AX2 and double mutant cells were allowed to develop synchronously on nitrocellulose filters, and RNA was isolated every four hours throughout the developmental cycle (Fig. 8, right panels). The message for the cAMP receptor 1 (carA) (Klein et al., 1988) and contact site A gene (csaA) (Noegel et al., 1986) were used as probes for the early phases of development. AX2 and 34−/120− did not differ in the pattern of expression of car1 and csaA genes. By contrast, premature and prolonged expression of genes specific for early development was observed in the 34−/αA− double mutant.

Terminal differentiation into spore and stalk cells was assessed by the expression of prespore and prestalk specific genes psaA (Early et al., 1988) and ecmB (Jermyn et al., 1987), respectively. The onset of expression was accelerated in 34−/αA− by comparison to AX2. In addition, both psaA and ecmB genes were weakly expressed in 34−/120− cells compared to the wild-type strain perhaps due to the failure of a significant proportion of cells to differentiate.

![Fig. 9. Rescue of 34+/αA− and 34−/120−cells. Both double mutants were transformed with vectors allowing the re-expression of the 34 kDa actin bundling protein. Mutant 34+/αA− was transformed with vectors allowing the expression of wild-type or mutated forms of α-actinin. See text and Table 1 for details on the nomenclature of strains. (A) Western blot analysis of AX2 (1,4) and rescue strains 34+/αA−R34 (2), 34−/120−R34 (3), 34−/αA−RαA-WT (5), 34−/αA−RαA-M2 (6) and 34−/αA−RαA-M1 (7). Western blots were performed as described in the legend to Fig. 1 and incubated with 34 kDa protein specific mAb B2C (upper panel) or α-actinin specific mAb 47-62-17 (lower panel). (B) Growth of AX2 and rescue strains of 34+/αA− in axenic suspension cultures. Growth is restored in all rescue strains. (C) Cell size distribution of AX2, 34+/αA−, and rescue strains of 34−/αA−. Cell size is completely restored in 34+/αA− R34 and RαA-M2, almost completely in RαA-WT and only partially in RαA-M1. (D) Growth of AX2, 34−/120−, and 34−/120−R34 in axenic suspension cultures at 15°C. Expression of the 34 kDa protein in the 34−/120− double mutant rescues the cold-sensitive growth phenotype to a level nearly that of AX2 as observed for the 120− single mutant strain (compare to Fig. 6A).]
Rescue of double mutants by re-expression of the 34 kDa protein or α-actinin

The 34-/120− and 34-/αA− double mutant cells were transformed with a plasmid that allowed expression of the 34 kDa actin bundling protein to verify that the phenotypes arise from the gene replacement event and not from a capricious mutation arising in the strain. Moreover, the 34-/αA− mutants were also transformed with plasmids that allowed expression of wild-type or altered forms of α-actinin with mutations in the first or second of the calcium binding EF hands (Witke et al., 1993). The actin 15 promoter in the plasmids constructed for the rescue experiments is expressed in cells growing vegetatively in axenic culture, but not in vegetative cells growing on bacteria, and only transiently during early development (Knecht and Loomis, 1987). Thus, we focused on analysis of rescue of the phenotypes observed in axenic vegetative cells.

Strains 34-/αA−R34 and 34-/120−R34 re-expressed the 34 kDa protein at levels slightly lower than the AX2 strain (Fig. 9A). Similarly, strains 34-/αA−RαA-WT and 34-/αA−RαA-M2 expressed α-actinin at levels 40% and 69% of AX2 respectively, whereas 34-/αA−RαA-M1 expressed α-actinin at levels similar to AX2 (Fig. 9A). Expression of the 34 kDa protein, or wild-type α-actinin, or the α-actinins mutated in one or the other of the two EF hands resulted in restoration of the growth properties of the 34-/αA− double mutant to wild-type levels in all cases (Fig. 9B). Cell size distribution was also quantified (Fig. 9C). Re-expression of the 34 kDa protein, wild-type α-actinin, or α-actinin mutated in EF-hand II resulted in a cell size distribution similar to AX2. Re-expression of α-actinin mutated in EF-hand I, on the contrary, only partially restored the cell size of 34-/αA− mutants, yielding a bimodal distribution curve with peaks of similar magnitude corresponding to cells of the size of the double mutant or the wild-type strain.

The possibility that the restoration of cell growth and size in 34-/αA−RαA-M1 and M2 strains was due to integration of the α-actinin expressing vector into the endogenous gene, thereby leading to the production of wild-type α-actinin, was ruled out by Southern blot analysis. Restriction fragments corresponding to the disrupted endogenous gene and the expression vector could be demonstrated in both strains (not shown). Furthermore, immunofluorescence using an α-actinin specific monoclonal antibody was performed in strains 34-/αA−RαA-WT, M1, and M2 to check whether non-uniform levels of expression of the reintroduced protein could account for the partial rescue of the phenotype described above. In all strains rescued by expression of either wild-type or mutated α-actinin, the α-actinin was uniformly expressed in the cell population. Striking differences from cell to cell were not apparent. Strain 34-/120−R34 expressed the 34 kDa protein at levels similar to AX2 (Fig. 9A). The slow growth of the 34-/120− at 15°C was rescued in the 34-/120−R34 cells (Fig. 9D), indicating that this phenotype did arise from absence of the two actin binding proteins, and was not caused by an extraneous mutation in the double mutant strain.

DISCUSSION

The actin based cytoskeleton, comprised of multiple actins, myosins, and actin associated proteins, is a primary regulator of cell shape and movement in eucaryotic cells. At least 11 different actin cross-linking proteins have been identified in Dictyostelium discoideum, and null mutations in single highly conserved proteins have been reported to result in little or no phenotype. We have analyzed the phenotypes of single mutant strains 34−, 120−, and αA−, and the three double mutant strains 34−/αA−, 34−/120−, and αA−/120− in order to dissect the contributions of the actin cross-linking proteins to cell structure and movement in Dictyostelium. The findings have been combined with results from other recent studies of these strains (Fisher et al., 1997; Rivero et al., 1996a,b) and collected in Table 3. We first describe the defects in actin dependent functions in vegetative and developing Dictyostelium cells from a cell biological viewpoint, and then discuss our findings regarding the unique and partially overlapping contributions of actin cross-linking proteins from a genetic and evolutionary perspective.

Contributions of the actin cross-linking proteins to specific processes mediated by the actin cytoskeleton in vegetative cells

The actin cytoskeleton has been implicated in endocytosis and/or vesicle transport in previous studies in a number of species including plants (Lichtscheidl and Foissner, 1996), yeast (Kübler and Riezman, 1993; Mulholland et al., 1997), and mammals (Gottlieb et al., 1993; Fath and Burgess, 1996). An actin-dependent component of fluid phase uptake termed macropinocytosis was first described in macrophages (Swanson and Watts, 1995), and subsequently associated with crown shaped structures on the surface of Dictyostelium (Hacker et al., 1997). In Dictyostelium discoideum, actin has been found to be associated with biochemically purified endocytic vesicles (Adessi et al., 1995), and treatment of cells with cytochalasin A inhibits fluid-phase uptake in a dose-dependent manner (Hacker et al., 1997). Genetic evidence for participation of the actin cross-linking proteins in endocytosis is provided by synthetic phenotypes of the double mutants described in this report (Table 3). A number of cytoskeletal components have been described in the crown-like structures in Dictyostelium including the 34 kDa actin-bundling protein (Furukawa and Fechheimer, 1994), coronin (de Hostos et al., 1991), and myosin IB (Novak et al., 1995), suggesting a participation in the endocytic process. Detailed examination of the endosomal and lysosomal pathways in the myosin I mutants suggests that the action of myosin I is limited to the internalization step since intracellular movement of the vesicles is not impaired in these mutants (Temesvari et al., 1996). In addition, mutations in coronin (Hacker et al., 1997) or multiple myosin isoforms result in cell lines with strongly impaired rates of pinocytosis (Novak et al., 1995; Jung et al., 1996). This impairment of fluid phase endocytosis, in the range of 40 to 60%, is similar to that reported here for the double mutants 34−/αA− and αA−/120− mutants (Fig. 4). The correlation between a decreased pinocytotic activity and decreased growth rates and cell density saturation observed for 34−/αA− and αA−/120− was also observed on myosin I mutants (Novak et al., 1995; Jung et al., 1996; Novak and Titus, 1997) suggesting that these traits may both reflect a central role of the actin cytoskeleton in these processes.
temperature or hyperosmotic conditions revealed additional roles of the actin cross-linking proteins in the growth of vegetative cells. Absence of the 34 kDa protein was detrimental to growth at reduced temperature (Fig. 6A and Table 3), an observation that correlates with the enhanced actin cross-linking activity of the 34 kDa protein at low temperatures (Hellewell and Taylor, 1979). Thus, the 34 kDa protein may maintain structure in the cell cortex at reduced temperature. Cells lacking α-actinin exhibited growth defects under hyperosmotic conditions. These phenotypes were even more profound in the double mutant strains αA−/34−, and αA−/120− (Fig. 6B and Table 3). Localization of α-actinin at the contractile vacuole (Furukawa and Fechheimer, 1994) is consistent with slow growth of α-actinin mutants under conditions of altered osmolarity. This model is consistent with the earlier observation that myosin IC is localized at the contractile vacuole and is essential for resistance to hypoosmotic shock (Dobrstein et al., 1993). In addition, amoebae challenged by hyperosmotic shock form a circumferential ring composed of a contractile cross-linked bundle of actin filaments (Aizawa et al., 1998) which may provide a structural explanation for the role of α-actinin in response to elevated osmotic conditions described in this paper as well as the previously described role of myosin II in this process (Kuwayama et al., 1996). Osmotic homeostasis is also reported to involve effects on the endocytic pathway (Steck et al., 1997), so the salt and sorbitol sensitivity may also reflect roles of the actin cross-linking proteins in vesicle uptake and/or traffic. Finally, slow growth of the 34−/120− strain in hyperosmotic media clearly reveals the roles of cross-linking proteins other than α-actinin in adaptation to this form of physiological stress. The synthetic phenotype reveals a function not apparent in either the 34− or 120− single mutant strains.

**Alterations in adhesion, development, and morphogenesis**

One of the most remarkable features of the Dictyostelium life cycle is the formation of a multicellular fruiting body from single amoebae. This cycle is triggered by starvation and involves cell adhesion, differentiation, and sorting into spore and stalk cells. The actin cytoskeleton is associated with the plasma membrane in vegetative cells, and contributes to formation of specialized cell to cell contact regions in Dictyostelium during development (Luna et al., 1990). A number of distinct mechanisms of the association of the cytoskeleton with the membrane have been defined (Noegel and Luna, 1995). Biochemical and immunochemical studies have shown that in aggregation-competent Dictyostelium cells, the regions of cell-to-cell contact are highly enriched in gp80 (Choi and Siu, 1987) and the 34 kDa protein but not α-actinin (Fechheimer et al., 1994). Surprisingly, 34− cells and αA− cells agglutinated like the wild type, but 34−/αA− displayed a pattern of agglutination significantly stronger than AX2. The enhanced adhesion was less pronounced in EDTA (Fig. 7C and D) so that adhesion was less pronounced in EDTA (Fig. 7C and D) so that the calcium-dependent adhesion system such as p24/DdCAD1 may be involved (Knecht et al., 1987; Wong et al., 1996). Aggregation competent cells respond to a CAMP stimulus with a transient increase in intracellular free calcium (Yumura et al., 1996) that is presumably connected with the rearrangements of the actin cytoskeleton preceding the chemotactic movement. Lack of two calcium-regulated actin cross-linking proteins may lead to imperfect regulation, greater stability of actin networks, and more stable cell-to-cell contacts if calcium-insensitive actin cross-linking proteins are recruited to substitute.

Evidence on the role of components of the actin cytoskeleton during morphogenesis has been obtained from the study of mutants lacking one or more actin binding proteins. For example, mutants defective in myosin II heavy chain (Springer et al., 1994) and double mutants defective in profilin I and II (Haugwitz et al., 1994), or α-actinin and gelation factor (Witke et al., 1992; Rivero et al., 1996b) are able to differentiate into prespore and prestalk cells, but development beyond the mound stage does not occur. In both myosin heavy chain minus and αA−/120− mutants, the defect can be attributed to a weakening of the cortical cytoskeleton that renders the cells unable to generate the protrusive force necessary for sorting out of the populations of differentiated cells (Shelden and Knecht, 1995; Knecht and Shelden, 1995; Xu et al., 1996; Rivero et al., 1996b). In the double mutants 34−/αA− and 34−/120− described here, differentiation into prespore and prestalk cells was not impaired, and development proceeded beyond the mound stage. However, the fruiting bodies formed exhibited aberrant morphologies. The phenotype of the 34−/120− mutant resembles to some extent that of mutants

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**Table 3. Partial summary of unique, additive, and synthetic phenotypes observed in single and double mutant strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>34°</th>
<th>αA−</th>
<th>120°</th>
<th>34°/αA−</th>
<th>34°/120°</th>
<th>αA−/120°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth 20°C</td>
<td>wt*</td>
<td>wt‡</td>
<td>wt‡</td>
<td>Slow; ↓ density</td>
<td>wt</td>
<td>Slow; ↓ density‡</td>
</tr>
<tr>
<td>Pinocytosis</td>
<td>wt*</td>
<td>wt</td>
<td>wt</td>
<td>Slow</td>
<td>wt</td>
<td>Slow</td>
</tr>
<tr>
<td>Cell size</td>
<td>wt*</td>
<td>wt‡</td>
<td>wt‡</td>
<td>Small</td>
<td>wt</td>
<td>Small‡</td>
</tr>
<tr>
<td>Growth 15°C</td>
<td>34°/αA−</td>
<td>34°/120°</td>
<td>αA−/120°</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth in NaCl</td>
<td>wt</td>
<td>Slow; ↓ density</td>
<td>wt</td>
<td>Slow; ↓ density</td>
<td>wt</td>
<td>Slow; ↓ density</td>
</tr>
<tr>
<td>Growth in Sorbitol</td>
<td>wt</td>
<td>Slow; ↓ density</td>
<td>wt</td>
<td>Slow; ↓ density</td>
<td>wt</td>
<td>Slow; ↓ density</td>
</tr>
<tr>
<td>Motility</td>
<td>‡</td>
<td>Persistence*</td>
<td>‡</td>
<td>Persistence</td>
<td>‡</td>
<td>Persistence</td>
</tr>
<tr>
<td>Adhesion</td>
<td>wt*</td>
<td>wt</td>
<td>wt</td>
<td>Fast; large aggregates</td>
<td>wt</td>
<td>Slow</td>
</tr>
<tr>
<td>Gene expression</td>
<td>wt*</td>
<td>wt†</td>
<td>wt†</td>
<td>Accelerated</td>
<td>wt</td>
<td>Slow</td>
</tr>
<tr>
<td>Morphogenesis</td>
<td>wt*</td>
<td>wt‡</td>
<td>wt‡</td>
<td>Stacked sori</td>
<td>Short squat stalks</td>
<td>Development arrested‡</td>
</tr>
</tbody>
</table>

Phenotypes are color coded as unique, additive, or redundant.

*Data from Rivero et al., 1996a.
‡Data from Rivero et al., 1996b.
§Data from Fisher et al., 1997.
defective in myosin essential light chain (Chen et al., 1995) as a fraction of the aggregates do not progress beyond the aggregation stage, whereas the remainder form fruiting bodies with short and thick stalks. Tracking the motility of single labeled cells in aggregates composed of mutant cells, or mixtures of mutant and wild-type cells may reveal the steps in morphogenesis that require participation of specific combinations of actin cross-linking proteins.

Calcium signaling to the cytoskeleton: role of the EF hands of α-actinin

Study of the free calcium distribution in Dictyostelium slugs (Cubitt et al., 1995; Yumura et al., 1996) suggests that calcium does act as a signal mediating changes in the cytoskeleton during development and morphogenesis. A number of possible targets of calcium including protein kinases and actin binding proteins such as the 34 kDa actin bundling protein and α-actinin have been identified (Noegel and Luna, 1995).

Dictyostelium α-actinin contains two EF-hands at the C terminus that are responsible for the calcium regulation. Biochemical studies indicate that both EF-hands possess different regulatory properties (Witke et al., 1993). Inactivation of EF-hand I abolished completely the F-actin cross-linking activity, whereas mutation of EF-hand II rendered a protein still able to cross-link actin in a Ca2+ dependent manner, although higher Ca2+ concentrations were necessary for inhibition. In a prior study, it was reported that calcium-insensitive forms of α-actinin were sufficient to provide α-actinin function in αA−/120− double mutants (Witke et al., 1993). These same calcium-insensitive forms of α-actinin were only partially able to supply α-actinin function to the 34−/αA− mutants. Expression of α-actinin mutated in EF-hand I in the double mutant 34−/αA− was not able to completely restore cell size. Expression of α-actinin mutated in EF-hand II restored cell size, despite the fact that the rescue mutant 34−/αA−/RM2 produced slightly less α-actinin than the wild-type strain. The same applies for the rescue with wild-type α-actinin, although in this case the comparatively lower levels of the expressed protein could well account for the shoulder towards cells of small size apparent in the distribution curve. It is interesting to note that expression of any of the α-actinin forms, either mutated or not, was sufficient to restore the growth rate in axenic medium, presumably overcoming the pinocytosis defect. This would imply that α-actinin mutated in EF-hand I displays some actin-binding activity, and in fact, residual binding has been described in isolated actin-binding regions of α-actinin (Witke et al., 1993; Way et al., 1992). One interpretation of these findings is that the essential contribution of calcium-regulated actin cross-linking was clearly revealed in the 34−/αA− mutants which lack both of the calcium-regulated cross-linking proteins. By contrast, calcium-regulated cross-linking of actin filaments could be supplied either by α-actinin or by the 34 kDa protein in wild type, single mutants, or αA−/120− double mutants. However, the requirement for calcium-regulated cross-linking was apparent due to the incomplete restoration of function in the 34−/αA− by expression of the α-actinin mutated in the first EF-hand.

Synthetic phenotypes reveal unique and redundant functions of genes

The simple paradigm of classical genetics is that mutations in genes yield phenotypes that reveal the function of the gene product. The paradigm suggests a product of no value or function if the null mutation produces no phenotype. In eucaryotes, this occurs with high frequency. For example, it has been estimated that only 5% of null mutations are lethal and that 66% of null mutations in the non-essential genes produce no phenotype (Oliver, 1992). The paradigm is resurrected if we posit that the phenotype observed reveals only the subset of the contributions of the gene product which are unique.

Cells lacking the 34 kDa protein, α-actinin, and gelation factor all show specific defects indicating the presence of some unique functions (Table 3). The 34− cells, αA− cells, and 120− cells show defects in cold sensitivity and persistence of motility, salt and sorbitol sensitivity, and phototaxis, respectively. These results indicate that the ability to overcome physical stress and/or to respond to environmental signals may provide a driving force for the evolution and maintenance of these diverse actin binding proteins.

Redundancy, operationally defined as synthetic phenotypes in the double mutants of these three actin cross-linking proteins, is observed in each of the three double mutant cell lines studied (Table 3). Redundancy is apparent in the 34−/αA− strain in the phenotypes of growth at 20°C, cell size, fluid phase uptake, sorbitol sensitivity, adhesion, gene expression, and morphogenesis. Redundancy is apparent in the 34−/120− strain in the phenotypes of salt sensitivity, and morphogenesis. Redundancy is apparent in the αA−/120− strain in the phenotypes of growth at 20°C, pinocytosis, cell size, motility, adhesion, gene expression, and morphogenesis. The three double mutant strains exhibit different combinations of redundant phenotypes so that the functions of the three actin cross-linking proteins would appear to overlap with each of the others in distinct arenas of actin-dependent processes. These data are evidence for both the unique and overlapping functions of the actin cross-linking proteins 34 kDa protein, α-actinin, and gelation factor, and provide a clear rationale for selection and maintenance of these multiple actin cross-linking proteins.

In a number of species including yeast, Drosophila, C. elegans, and mouse, it has been found that double and triple mutations produce synthetic phenotypes in which there is a synergy or greater than additive effect (reviewed by Guarente, 1993). This can arise due to mutations in molecules that have either only a very distant/indirect relationship, or molecules that contribute at different steps along a linear pathway, or that have similar and overlapping functions. In the latter case, the phenomenon is termed genetic redundancy (Thomas, 1993). Evolution of redundancy has been postulated to occur through gene duplication, and/or by a process whereby a molecule which performs one function is altered to acquire the ability to perform a new function overlapping with that of another gene product (Brookfield, 1997; Tautz, 1992). In this scenario, the most ancient processes are predicted to be among the most highly redundant. Redundant genes with complete overlap would not be predicted to be evolutionarily stable, since no selection against accumulation of deleterious mutations would be predicted to occur (Nowak et al., 1997). Redundant genes can be stably maintained if there is a difference in the genetic fitness or lack of complete overlap of the functions of the two genes. In addition, a driving force for maintenance of redundancy can arise from a high error rate during a given...
process such as development and morphogenesis (Nowak et al., 1997; Thomas, 1993).

Our studies reveal both unique and overlapping functions of the 34 kDa protein, α-actinin, and the gelation factor in Dictyostelium. In the broad context of evolutionary theory, the finding of partial redundancy among the actin cross-linking proteins of Dictyostelium is more expected than surprising. Moreover, virtually all eucaryotic cells contain multiple actin cross-linking proteins that may have partially overlapping functions. For example, in the budding yeast S. cerevisiae single mutants lacking actin cross-linking proteins Abp1p and Sac6p (fimbrin) are normal or mildly affected, while double mutants lacking both proteins are either inviable or grow extremely slowly (Adams et al., 1993). In the fission yeast S. pombe, α-actinin and fimbrin are both present along with actin in a medial ring at the site of division. Single mutants grow and divide normally, but double mutants lacking fimbrin and α-actinin are synthetically lethal with defects in medial ring formation and cytokinesis (J.-Q. Wu, J. Bähler and J. R. Pringle, personal communication). In the mammalian brush border, three different actin cross-linking proteins, villin, fimbrin, and small espin are all present in the microfilament core bundle (Bartles et al., 1998). There is likely some redundancy of function among these three cross-linking proteins, as homozygous villin-deficient mice are viable, fertile, and exhibit no gross abnormalities (Pinson et al., 1998). Finally, two actin cross-linking proteins, fascin and forked, both contribute to formation of actin bundles during bristle morphogenesis in Drosophila (Tilney et al., 1995, 1998). Here distinct functions of the two proteins have been assigned based primarily on the ultrastructure of the abnormal actin bundles in strains with alterations in expression of the fascin and/or forked proteins. Thus, synthetic phenotypes of actin cross-linking proteins are not necessarily evidence of complete redundancy and identical function. Additional investigations could potentially reveal distinct contributions to cell structure and function which are likely required for conservation of these proteins across extended evolutionary distances. Future studies of the unique and redundant roles of the multiple actin cross-linking proteins will be aided by generation of different single and multiple mutant strains, as well as by development and/or utilization of quantitative assays for other specific aspects of cell function.

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