Targeted Disruption of the ABP-120 Gene Leads to Cells with Altered Motility

D. Cox,* J. Condeelis,* D. Wessels,† D. Soll,‡ H. Kern,§ and D. A. Knecht§

*Albert Einstein College of Medicine, Yeshiva University, Bronx, New York; †University of Iowa, Iowa City, Iowa; and §University of Connecticut, Storrs, Connecticut

Abstract. The actin-binding protein ABP-120 has been proposed to play a role in cross-linking F-actin filaments during pseudopod formation in motile Dictyostelium amoebas. We have tested this hypothesis by analyzing the phenotype of mutant cell lines which do not produce ABP-120. Two different transformation vectors capable of targeted disruption of the ABP-120 gene locus have been constructed using a portion of an ABP-120 cDNA clone. Three independent cell lines with different disruption events have been obtained after transformation of amoebas with these vectors. The disruption of the ABP-120 gene by vector sequences results in either the production of a small amount of truncated ABP-120 or no detectable protein at all. The phenotypes of two different clones lacking ABP-120, generated in strains AX3 and AX4, have been characterized and show identical results. ABP-120− cells tend to remain rounder before and after cAMP stimulation, and do not reextend pseudopods normally after rapid addition of cAMP. In addition, ABP-120− cells translocating in buffer exhibit defects in both the rate and extent of pseudopod formation. The amount of F-actin cross-linked into the cytoskeleton after cAMP stimulation of ABP-120− cells is reduced at times when ABP-120 has been shown to be incorporated into the cytoskeleton, and this correlates temporally with the absence of reextension of pseudopods after cAMP stimulation. The instantaneous velocity is significantly reduced both before and after cAMP stimulation in the ABP-120− cells, and the cells show decreased chemotactic efficiency compared to ABP-120+ controls. This phenotype is consistent with a role for ABP-120 in pseudopod extension by cross-linking actin filaments as proposed by the “cortical expansion model” (Condeelis, J., A. Bresnick, M. Demma, C. Dharmawardhane, R. Eddy, A. L. Hall, R. Sauterer, and V. Warren. 1990. Dev. Genet. 11:333-340).

ABP-120 is an F-actin cross-linking protein found in Dictyostelium discoideum cells (Condeelis et al., 1982). The molecular structure of ABP-120 has been analyzed in detail. It is a 35-40-nm-long rod consisting of two identical subunits each with a molecular mass of 120,000 D on SDS-PAGE (Condeelis et al., 1984). The subunits are packed in an antiparallel orientation (Brink et al., 1990). Analysis of the cDNA sequence suggests that the COOH-terminal two thirds of each subunit is composed of six beta-sheet motifs and that the NH2-terminal 250 amino acids contain stretches of sequence which are conserved in a variety of dimeric actin cross-linking proteins (Noegel et al., 1989).

Controlled proteolysis of ABP-120 demonstrates that it can be divided into head and tail domains with discrete properties (Bresnick et al., 1990). The tail is degraded into a ladder of peptides and the sizes of these peptides are consistent with the presence of six beta-sheet repeats as proposed from sequence analysis (Noegel et al., 1989). The head contains the actin binding site. Controlled proteolysis of the head implicates a 27-amino acid sequence as essential for actin-binding activity (Bresnick et al., 1990). Recent experiments using synthetic peptides demonstrate that this 27-amino acid sequence is part of the actin binding site of ABP-120 (Bresnick et al., 1991). Because of the high degree of conservation of this 27-amino acid sequence in a variety of actin-binding proteins it has been proposed as an archetypical binding site for a broad family of proteins that bind to the side of the actin filament (Bresnick et al., 1990).

The structural properties of ABP-120 suggest that it is a mini-ABP (mini-filamin). The tails of both ABP-120 and ABP contain the repetitive beta-sheet motif, and the NH2-terminal actin-binding domains contain the conserved 27-amino acid sequence (Gorlin et al., 1990). In addition, ABP-120 shares with macrophase ABP (Niederman et al., 1983) the ability to promote orthogonal interactions between actin filaments in vitro (Condeelis, 1981; Wolosewick and Condeelis, 1986) and ABP-120 resides in such networks in situ (Ogihara et al., 1988).

ABP-120 has been implicated in pseudopod extension. Immunofluorescence analysis demonstrates that ABP-120 is concentrated in the cell cortex, and in lamellipods and pseudopods, during cell spreading and locomotion (Condeelis, 1981) and preferentially in pseudopods during cAMP stimu-
loration (Condeelis et al., 1988). Furthermore, ABP-120 becomes localized to Con A–induced pseudopods that are extended from the cell surface opposite to Con A caps whereas alpha-actinin and myosin II are found in Con A caps but not in Con A–induced pseudopods (Carboni and Condeelis, 1985). In addition, incorporation of ABP-120 into the actin cytoskeleton after cAMP stimulation peaks at times when actin is cross-linked into the cytoskeleton (Dharmawardhane et al., 1989) and when pseudopods are extended (Hall et al., 1988). ABP-120 cross-links actin filaments into networks similar to those observed in pseudopods (Wolosewick and Condeelis, 1986) and is concentrated in pseudopodal networks in situ (Ogihara et al., 1988).

These observations have led to the proposal that ABP-120 functions in the cross-linking of actin filaments to form networks in pseudopods (Dharmawardhane et al., 1989) thereby contributing to pseudopod extension as described by the "cortical expansion model" (Condeelis et al., 1990). Dictyostelium is an ideal organism in which to test this hypothesis. Genetic manipulation is possible, mutations are always dominant in the haploid ameboid stage, and cell behavior after stimulation is sufficiently synchronous to permit the correlation of changes in morphology with biochemistry. Such experiments have been performed successfully to study the effects of deletion or underexpression of myosin II (De Lozanne and Spudich, 1987; Knecht and Loomis, 1987) and myosin I (Jung and Hammer, 1990). The observed phenotypes implicate myosin II in cytokinesis, cell polarity, intracellular motility, and locomotion (Wessels et al., 1988; Wessels and Soll, 1990; Soll et al., 1990), and myosin I in phagocytosis and regulation of the frequency of pseudopod extension (Jung and Hammer, 1990; Wessels et al., 1991).

However, in several studies the deletion of other actin-binding proteins has not resulted in a measurable phenotype (e.g., severin, Andre et al., 1989; alpha-actinin, Wallraff et al., 1986) or has resulted in only subtle changes in biochemical properties (e.g., ABP-120, Brink et al., 1990). The failure to measure a phenotype in such cases might result from a redundancy of function with other actin-binding proteins (Bresnick et al., 1990). In the case of ABP-120, its loss may be compensated by ABP-240, a filament-like protein found to coexist with ABP-120 in Dictyostelium amebas (Hock and Condeelis, 1987; Condeelis et al., 1988). Alternatively, lack of a measurable phenotype may be due to the resolving power of the method used. Changes in the dynamics of pseudopod extension, turning, shape, and the rate of cellular translocation can not be resolved by qualitative methods alone, and for that reason computer-assisted, two-dimensional image analyzing systems have been developed (Soll, 1988; Soll et al., 1988). These systems have been effective in demonstrating the subtleties of normal behavior (Wessels et al., 1989) as well as the defects in cytoskeletal mutants still capable of translocation (Wessels et al., 1988; Wessels and Soll, 1990; Soll et al., 1990; Wessels et al., 1991).

In this study, we have analyzed the effects of disrupting the ABP-120 gene by homologous recombination. Our results demonstrate that the absence of ABP-120 results in defects in the incorporation of F-actin into the cytoskeleton in response to the rapid addition of cAMP, and in behavioral defects in both pseudopod extension and motility.

Materials and Methods

Cells and Growth Conditions

The different strains of Dictyostelium discoideum used in this study are listed in Table I. Cell culture was performed as described in Hall et al. (1988). G418-resistant cell lines were propagated in HL5 containing 20 μg/ml of G418 (geneticin from Sigma Chemical Co., St. Louis, MO) except where noted.

Transformation of Dictyostelium

Transformation of the axenic strains AX3 and AX4 was done using calcium phosphate–precipitated DNA as described previously (Knecht et al., 1990). DNA was either supercoiled (p20-N3 and p20-N1) or a linear Sacl–XhoI fragment containing only the disruption fragment from p20-N1. The linear fragment was treated with T4 DNA polymerase as described by Manstein et al. (1989) to try to promote single-vector insertions. Transformants were cloned by limiting dilution on SM bacterial plates (Sussman, 1987) and amebas from individual colonies were picked back into HL5 medium containing G418.

Whole Cell Lysates

Cells were grown to midlog phase in HL5 medium and harvested by centrifugation in a Sorvall (Newtown, CT) GLC-100 centrifuge for 4 min at 185 g. The cells were washed in a solution containing 14.8 mM NaH2PO4 and 5.2 mM K2HPO4, pH 6.6 (20 mM Na/K/PO4 buffer) and resuspended to a final density of 5 × 10^6 cells/ml in ice-cold 20 mM Na/K/PO4 buffer containing 2 mM EGTA, 2 mM EDTA, 0.04 μg/ml Aprotinin (Sigma Chemical Co.), and 20 μg/ml each of chymostatin and leupeptin. An equal volume of boiling 2× Laemmli sample buffer was added; the samples were vortexed for 10 s and heated to 100°C for 5 min. Samples were immediately loaded (10 μl/well) and run on 8% SDS-PAGE gels (Laemmli, 1970). The gels were subsequently Western blotted or stained with Coomasie brilliant blue.

Western Blots

Gels were run and transferred to nitrocellulose by electroblotting for 1,800 milliamp hours, these conditions have been shown to insure quantitative transfer of ABP-120 (Dharmawardhane et al., 1989). Immunoblots were performed as described by Bennett and Condeelis (1988). Nitrocellulose blots were incubated with 0.1 μg/ml of an affinity-purified polyclonal ABP-120 antibody and then labeled with 125I-protein A.

Southern and Northern Blots

RNA was prepared from vegetative cells by guanidinium isothiocyanate lysis and CsCl centrifugation as described in Maniatis et al. (1982). DNA was prepared using a DNA miniprep procedure (Knecht et al., 1990). RNA was electrophoresed on agarose gels containing 1.4% formaldehyde, partially degraded with 0.4 M NaOH, and transferred to Nytran membrane (Scheicher & Schnell, Inc., Keene, NH) as described in Maniatis et al. (1982). DNA was digested with restriction enzymes as indicated, electro-
Table I. Transformant and Control Designations

| Homologous recombinants | Controls |
|-------------------------|---------|
| AX3-1D-4’-AX3 transformed with linear p120N-1, fourth clone | AX3-1S-2’-AX3 transformed with circular p120N-1, fourth clone |
| AX4-3S-1’-AX4 transformed with circular p120N-3, clone 1 | AX4-TF’-AX4 transformed with “control vector” lacking ABP-120 insert |

The homologous recombinants were generated by transforming strain AX3 and AX4, respectively, with the linear and circular versions of the p120N vector. The controls were generated by transforming strain AX3 and AX4 with only the vector lacking the insert.

Computational Behavioral Analyses

To analyze behavior in buffer, aggregation-competent cells were inoculated into a Sykes-Moore chamber (Belco Glass Inc., Vineland, NJ) and perfused with buffer according to methods previously described in detail (Varnum et al., 1985; Wessels et al., 1988). Cellular behavior was recorded in real time on 0.5-in. video tape at 250x magnification and automatically digitized with a VP110 real-time contour digitizer (Motion Analysis Corp., Santa Rosa, CA) into the data file of a SUN II computer and then analyzed with Dynamic Morphology System software (Soll, 1988; Soll et al., 1988).

To analyze behavior before and after the addition of cAMP, cells were inoculated into a Dvorak-Stoker chamber (Nicholson Precision Instruments, Inc., Gaithersburg, MD), perfused with buffer for 40 s, and then perfused with buffer containing 10^{-6} M cAMP for an additional 80 s according to methods previously described in detail (Wessels et al., 1989, 1991; Wessels and Soll, 1990). Cellular behavior was recorded in real time on 0.75-in. video tape at 620x magnification and manually digitized into the SUN II data file with a manual digitizer and accompanying software for distortion compensation (Nystrom, 1989; Wessels and Soll, 1990). To analyze behavior in a spatial gradient of cAMP, cells grown and developed in the absence of G418 were inoculated onto the bridge of a gradient chamber (Zigmund, 1977). One border trough was filled with buffer (sink) and the other with 10^{-2} M cAMP according to methods previously described (Varnum and Soll, 1984; Varnum-Finney et al., 1987b). Amebas were incubated in the chamber for 5 min, and then behavior was recorded on 0.75-in. video tape. Cell perimeters were then automatically digitized into the SUN II data file. In all cases, the centroid location in each analyzed frame was computed from the x,y coordinates of the pixels at the perimeter of the digitized cell image, and instantaneous “velocity” was determined according to the central difference method (Maron, 1982). “Directional change” was calculated as the absolute difference in centroid direction between two consecutive frames. Cell “roundness” parameter was computed as (4π x area/perimeter^2) x 100 resulting in a value of 100 for a perfect sphere and 0 for a line. Differences pictures were generated by superimposing successive frames at 4-s intervals. An expansion zone was considered a pseudopod if

(a) the zone was elongate and composed of 3.5% or more of the total area at the time of initial appearance whether or not it continued to expand, or
(b) the zone was less than 3.5%, but continued to expand into an elongate extension. Parameters of pseudopod extension were calculated according to formulae previously described (Wessels et al., 1988). The “chemotactic index” was calculated by dividing directional distance (net distance towards source) by total distance (McCutcheon, 1946; Varnum and Soll, 1984).

In initial experiments, ABP-120^- and ABP-120^+ cells were analyzed in a single blind fashion.

Preparation of ABP-120^- Transformsants

Two different parental strains of Dictyostelium, strain AX3 and AX4, were each transformed with both of the constructs shown in Fig. 1 to determine if the transformation efficiency or the phenotype were vector or strain dependent. Supercoiled plasmid DNA of both constructs or a double-cut linear disruption fragment from pl20-NI were used to transform cells by the calcium phosphate precipitate method. Selected G418-resistant (G418^+) clones were then assayed for the presence of ABP-120 by Western blotting using an ABP-120^-specific affinity-purified polyclonal antibody (Carboni and Condeelis, 1985). This antibody recognizes multiple epitopes in both the head and tail domains of ABP-120 (Bresnick et al., 1990). Therefore, any truncations of ABP-120 being produced should be detected.

Cell lines from nine independent transformations were screened for the absence of ABP-120. The percentage of homologous recombination for G418^+ colonies tested was 19 and 7% when using supercoiled plasmid and the linear disruption fragment, respectively. However, the frequency of gene disruption was highly variable from transformation to transformation, ranging from 0-100%, and did not correlate with the parental strain or the transformation vector.

Fig. 2 shows a Western blot of whole cell lysates of ABP-120^- transformants and their controls as defined in Table I. In Western blots of control cell lines a band is seen at ~120 kD, whereas no such band is present in any of the ABP-120^- cell lines. When the blot is exposed for a longer period of time a lower molecular mass band of ~60 kD is seen in AX4-3S-1^- (Fig. 2 c) and AX3-ID-4^- but not in AX3-ID-4^+. The size of this peptide is expected from a single crossover insertion of the vector into the ABP-120 gene (data not shown). The NH2-terminal 60-kD fragment of ABP-120 is highly antigenic and the intensity of staining in Western blots is approximately the same as intact ABP-120, as determined by partial proteolytic digests (Bresnick et al., 1990). Using this information it is possible to estimate the amount of the truncated protein present in ABP-120^- cells compared to wild-type levels by scanning densitometry of Western blots. In AX3-ID-4^-, the cell line which produces the highest amount of the 60-kD peptide, the truncated protein is present at 3.5% of the wild-type level of ABP-120 (data not shown).
Northern blot analysis of the ABP-120<sup>−</sup> cell lines is shown in Fig. 3. The blot was probed with a 1.5-kb antisense transcript corresponding to the 5′ end of the gene. In untransformed and control transformed cell lines, mRNA of 2.8 kb is detected (Fig. 3, lanes 1-3). In cell line AX3-IS-2<sup>+</sup> an additional band is seen that migrates at a higher molecular weight. This represents a message that is produced from the vector inserted at the nonhomologous integration site. No message is detected in the AX4-IS-4<sup>−</sup> cell line and an altered message size is seen in both AX3-IS-1<sup>−</sup> and AX3-ID-4<sup>−</sup>.

Southern blots were performed to determine if the ABP-120 gene locus was altered in the ABP-120<sup>−</sup> cell lines. Genomic DNA was digested with NdeI and hybridized with a probe made from the ABP-120 fragment contained within the transformation vector. This probe should hybridize to both the native ABP-120 locus and any site into which the
vector has inserted nonhomologously. NdeI cuts once upstream of the gene and once near the 3' end of the gene giving rise to a 2.8-kb fragment (Fig. 1a). NdeI does not cut the transformation vectors. A multicopy insertion at the native gene locus should result in the absence of the 2.8-kb fragment and the appearance of a high molecular weight band corresponding to the multicopy repeat. The Southern blots demonstrate (Fig. 4) that a 2.8-kb fragment is present in all of the ABP-120+ lines but is absent in the ABP-120- lines. An intensely stained high molecular weight restriction fragment is seen in the ABP-120- lines as well as in AX3-IS-2+, where the integration was nonhomologous (representing the integration at a second site) consistent with a multicopy insertion of the plasmid. Southern blot analysis has confirmed that the genomic organization of the DNA in the ABP-120- cell lines corresponds to that expected from a homologous single crossover insertion of the vector into the ABP-120 locus (Knecht, D. A., and H. Kern, unpublished observations). There is no indication from Southern blots of any stray copies of the vector inserted at other sites in the ABP-120- transformants (Fig. 4 and unpublished results).
Figure 5. Comparison of the kinetics of changes in relative actin content present in Triton-insoluble cytoskeletons after cAMP stimulation of cells in suspension. Means ± SEM for 2–9 experiments are shown. (○) ABP-120+ (AX4-TF+); (●) ABP-120− (AX4-3S-1−).

Phenotype Analysis of the ABP-120− Cell Lines

On agar plate cultures, ABP-120− cell lines were capable of clearing bacterial lawns and forming fruiting bodies. Furthermore, ABP-120+ and ABP-120− strains grew in liquid HL5 culture with approximately the same doubling time.

ABP-120 is postulated to function in actin filament cross-linking in pseudopods during cAMP stimulation (Condeelis et al., 1988) and in pseudopod extension (Condeelis et al., 1990). Therefore, detailed analysis of ABP-120− cells was carried out regarding cAMP-stimulated incorporation of actin into the cytoskeleton, the efficiency of cAMP-stimulated pseudopod extension, cellular locomotion, and chemotaxis.

Incorporation of Actin into the Cytoskeleton

Incorporation of actin into the cytoskeleton results from a combination of actin polymerization and filament cross-linking (Dharmawardhane et al., 1989; Condeelis et al., 1990). If the cross-linking activity by ABP-120 is partially responsible for the incorporation of actin into the cytoskeleton after cAMP stimulation, the ABP-120− cells should show altered actin incorporation after being stimulated. Therefore, Triton-insoluble cytoskeletons were isolated from both ABP-120+ and ABP-120− cells (AX4-TF+ and AX4-3S-1−, respectively) and the relative actin content was determined. The assay was done rapidly on ice to retard actin polymerization after lysis and with low speed centrifugation immediately after lysis under conditions where only F-actin associated with the cytoskeleton would pellet to increase the sensitivity of the assay to cross-linking activity (Galvin et al., 1984; Dharmawardhane et al., 1989; Condeelis and Hall, 1990).

AX4-TF+ cells incorporate actin into the cytoskeleton with kinetics similar to those of wild-type cells (Fig. 5, solid line) and ABP-120 is also incorporated into the cytoskeleton as in wild-type cells (data not shown). AX4-3S-1− cells demonstrate reduced actin incorporation into the cytoskeleton at the times when ABP-120 is normally incorporated, i.e., 40 and 60 s (Fig. 5, dotted line). As shown by t test, P values of 0.0138 at 40 s and 0.0187 at 60 s indicate a significant difference in the actin incorporation at these times. There is no difference between the ABP-120+ and ABP-120− cells in the first peak of actin incorporation (P value of 0.8945), a time when ABP-120 is not normally associated with the cytoskeleton in wild-type cells (Table II). Cells grown in the presence or absence of G418 gave similar results (data not shown).

Immunofluorescence Analysis

Incorporation of ABP-120 into the cytoskeleton occurs at the same time as the appearance of pseudopods, and ABP-120 has been localized to newly formed pseudopods after cAMP stimulation (Condeelis et al., 1988; Hall et al., 1988). Immunofluorescence microscopy was done on both ABP-120+ and ABP-120− cells before and after cAMP stimulation. Fig. 6 shows AX4-TF+ cells before and after stimulation at a time where pseudopods are normally extended. The images demonstrate the presence of ABP-120, its cortical location, and colocalization with F-actin in cAMP-stimulated pseudopods. Fig. 7 shows AX4-3S-1− cells under the same conditions as the AX4-TF+ cells in Fig. 6. ABP-120 is absent in the ABP-120− cells, and the cells exhibit a rounder appearance when viewed with phase-contrast optics than ABP-120+ cells.

Motion Analysis

To test whether the absence of ABP-120 affected single cell motility, the behavior of ABP-120+, and ABP-120− cells were compared in (a) buffer, (b) after the rapid addition of cAMP, and (c) in a spatial gradient of cAMP.

Behavior in Buffer. Amebas were inoculated onto the glass chamber wall of a Sykes-Moore chamber and perfused with buffered salts solution at a rate which precluded any conditioning of the microenvironment. Cellular behavior was then videorecorded and analyzed as described in Materials and Methods. 13 ABP-120+ (AX3-IS-2+) and 15 ABP-120− (AX3-IS-4−) amebas were monitored for 10 min, and motility and dynamic morphology parameters computed at 4-s intervals, and then averaged over the 10-min period. The mean values of these averaged parameters computed for the

Table II. Quantitation of Relative Actin Content in Triton-insoluble Cytoskeletons

| Time | Actin | SEM  | n  | Actin | SEM  | n  |
|------|------|------|----|------|------|----|
| s    |      |      |    |      |      |    |
| 5    | 1.24 | 0.18 | 5  | 1.07 | 0.06 | 5  |
| 7    | 1.15 | 0.15 | 8  | 1.11 | 0.06 | 7  |
| 8    | 1.21 | 0.14 | 3  | 1.12 | 0.06 | 2  |
| 10   | 1.56 | 0.11 | 3  | 1.58 | 0.09 | 4  |
| 11   | 1.29 | 1    | 1  | 1.20 | 0.11 | 2  |
| 12   | 1.10 | 1    | 1  | 1.17 | 0.04 | 4  |
| 15   | 1.23 | 0.11 | 6  | 1.20 | 0.05 | 4  |
| 20   | 1.28 | 0.15 | 5  | 1.22 | 0.08 | 4  |
| 22   | 1.16 | 0.12 | 3  | 1.21 | 1    | 1  |
| 30   | 1.50 | 0.29 | 2  | 1.03 | 0.06 | 4  |
| 40   | 1.44 | 0.12 | 7  | 1.05 | 0.07 | 7  |
| 50   | 1.29 | 0.01 | 2  | 1.20 | 0.06 | 3  |
| 60   | 1.38 | 0.10 | 9  | 1.10 | 0.04 | 9  |
| 80   | 1.07 | 1    | 0.91 | 0.07 | 4  |

Quantitation of relative actin content in Triton-insoluble cytoskeletons isolated from ABP-120+ (AX4-TF+) and ABP-120− (AX4-3S-1−) cells ± SEM at various times after stimulation with 2' deoxy cAMP. These values were used for the graph in Fig. 5.
Figure 6. Immunofluorescence photographs of ABP-120+ (AX4-TF+) cells before (0 s) and after (40 s) a 1-µM 2‘ deoxy cAMP upshift. (Top row) 60× phase; (middle row) anti-ABP-120 (fluorescein channel); (bottom row) rhodamine phalloidin. Bar, 10 µm.

13 ABP-120+ and 15 ABP-120- cells are presented in Table III. ABP-120+ cells moved with a mean instantaneous velocity roughly three times that of ABP-120- cells. In addition, mean directional change for ABP-120+ cells was half that of ABP-120- cells, demonstrating that ABP-120+ cells moved more persistently than ABP-120- cells. Although the mean areas of ABP-120+ and ABP-120- cells were almost identical, the mean length was greater and the mean roundness parameter less for ABP-120+ cells, demonstrating that they were, on average, more elongate. An analysis of 5 AX4-TF+ and 5 AX4-3S-l- cells gave similar results (data not shown).

The Dynamics of Pseudopod Extension. To analyze pseudopod dynamics, amebas were again perfused with buffer in a Sykes-Moore chamber and cellular behavior was video-recorded. During 10 min of analysis, 13 ABP-120+ (AX3-IS-2+) cells formed 156 pseudopods (12.0 ± 5.0 pseudopods per 10 min per cell) while 15 ABP-120- (AX3-IS-4-) cells formed 60 pseudopods (4.0 ± 2.0 pseudopods per 10 min per cell). Both average initial and average maximum pseudopod area of ABP-120+ amebas were more than two times that of ABP-120- cells. Average pseudopod area of ABP-120+ cells, computed by dividing the sum of pseudopod areas measured at 4-s intervals by the total time the pseudopod existed was three times that of ABP-120- amebas (Table IV). These data demonstrate that the frequency, rate, and extent of pseudopod growth in ABP-120- cells were dramatically depressed. Similar results were obtained with the AX4-derived strains AX4-TF+ and AX4-3S-l- (data not shown).

Behavior after Rapid Addition of cAMP. When amebas of strains AX3 or AX4 are treated with the rapid addition of cAMP (0–10–6 M in 8 s), cells stop translocating, exhibit a transient freeze in cell morphology for 10 s, and then partially rebound by extending pseudopods in a nonpolar fashion (Wessels et al., 1989; Wessels and Soll, 1990). To test whether ABP-120- cells exhibited a normal response to the rapid addition of cAMP, ABP-120+ and ABP-120- cells were inoculated onto the glass wall of a Dvorak-Stodler chamber, perfused with buffer alone for 40 s, and then with buffer plus 10–6 M cAMP for a subsequent 80 s. In Fig. 8, the average
Figure 7. Immunofluorescence photographs of ABP-120' (AX4-3S-1') cells before (0 s) and after (40 s) a 1-μM 2'-deoxy cAMP upshift. Top, middle, and bottom rows as in Fig. 6. Bar, 10 μm.

Table III. Motility and Dynamic Morphology Parameters for ABP-120' and BP-120' Amebas

| Strain     | Cells | Mean | Mean | Mean | Mean | Mean | Mean | Mean |
|------------|-------|------|------|------|------|------|------|------|
|            |       | inst. Vel | dir. change | length | width | area | roundness |
| AX3-1S-2'  | 13    | 16.5 ± 6.4 | 24.1 ± 9.6 | 21.5 ± 3.9 | 5.2 ± 1.0 | 109.7 ± 29.5 | 47.0 ± 9.0 |
| AX3-1S-4'  | 15    | 4.6 ± 1.6 | 55.9 ± 9.6 | 16.3 ± 4.8 | 6.4 ± 1.3 | 106.1 ± 43.4 | 68.9 ± 14.0 |

Motility and dynamic morphology parameters averaged over 10 min for ABP-120' and ABP-120' amebas.

Table IV. The Dynamics of Pseudopod Extension by ABP-120' and ABP-120' Cells

| Strain     | Cells | # pseudopods | Average # pseudopods per 10 min | Average initial pseudopod area | Average maximum pseudopod area | Average time maximum pseudopod area | Average area* |
|------------|-------|--------------|---------------------------------|-------------------------------|-------------------------------|------------------------------------|--------------|
|            |       | n            | n                              | n                             | n                             | n                                  | n            |
| AX3-1S2    | 13    | 156          | 12.0 ± 5.0                     | 12.6 ± 3.9                    | 17.2 ± 7.1                    | 0.33 ± 0.19                        | 16.1 ± 5.3   |
| AX3-1S-4   | 15    | 60           | 4.0 ± 2.0                      | 5.3 ± 2.6                     | 6.4 ± 1.8                     | 0.47 ± 0.21                        | 4.9 ± 2.4    |

* Average pseudopod area was computed by dividing the sum of pseudopod area, measured at 4 s intervals during the time of expansion, by the number of time intervals.
ABP-120+ cells show a reduced rate of locomotion before cAMP stimulation and maintain this low level after stimulation. The average instantaneous velocity for AX3-IS-2+ cells (■) and AX3-IS-4- cells (○) before and after an upshift of 1 μM cAMP is shown (n = 10). Instantaneous velocity was averaged at 4-s intervals. The estimated concentration of cAMP in the Stodler chamber is plotted at the top of the graph.

The average instantaneous velocity for 10 ABP-120+ (AX3-IS-2+) and 10 ABP-120- (AX3-IS-4-) cells is plotted as a function of time before (−40−0 s) and after (0−+80 s) the addition of cAMP. The average instantaneous velocity plot for ABP-120+ cells before and after the addition of cAMP was similar to that previously published for AX3 cells (Wessels et al., 1989). Average instantaneous velocity was high before the addition of cAMP, but rapidly began decreasing within seconds after cAMP entered the chamber, reaching a minimum value 12 s after cAMP first entered the chamber. A partial rebound in instantaneous velocity began 20 s after the addition of cAMP, but the instantaneous velocity never attained the high values observed in cells translocating in buffer, in the absence of chemoattractant (−40–0 s).

The average instantaneous velocity of ABP-120- cells was less than half that of ABP-120+ cells before the addition of cAMP (Fig. 8). Within seconds after cAMP first entered the chamber, the average instantaneous velocity began to decrease, reaching roughly the same minimum value as ABP-120+ cells at 12 s. However, unlike ABP-120+ cells, there was little if any rebound after 20 s. It should be noted that this absence of a velocity rebound coincides with the absence of the second increase in cytoskeletal actin content (Fig. 5) in the ABP-120- cells. An analysis of 5 AX4-TF+ and 5 AX4-3S-1- cells gave similar results (data not shown).

Differences in the actin cytoskeleton are evident in the difference photographs of representative AX3 (A) ABP-120+ cell (AX3-IS-2+) and (B) ABP-120- cell (AX3-IS-4-) before and after the rapid addition of 1 μM cAMP. Expansion zones (pseudopods) are filled, retraction zones are hatched, common zones are unfilled. In each difference picture, the direction of cell centroid translocation between the earlier and later images is present and noted by an arrow. Zero time (0 s) indicates the estimated time, ± 1 s at which 1 μM cAMP was added.
after 28 s. The representative ABP-120+ cell (Fig. 9 B) had formed a pseudopod towards the lower right-hand corner before the addition of cAMP (-12---4 s). Upon addition of cAMP, the cell remained relatively fixed in shape with very little, random expansion during the subsequent 48 s. No significant rebound in expansion zones or change in shape like that observed in ABP-120+ cells (Fig. 9 A) was apparent.

Similar results were obtained for the AX4-derived strains AX4-TF+ and AX4-3S-1-. The representative ABP-120+ cell in Fig. 10 A was elongate, translocating to the top of the panel before cAMP addition. During the 8 s after the addition of cAMP, there was a freeze in shape with little detectable expansion. This was followed by random expansion zones leading to a loss of the elongate cell shape after 20 s. The dynamics of behavior before and after the addition of cAMP were similar in AX3- and AX4-derived ABP-120+ cells (n = 15 and 20, respectively). The representative ABP-120+ AX4 cell (AX4-3S-1-) in Fig. 10 B was translocating towards the bottom of the panel just before cAMP addition, but exhibited only slight vertical polarity. After the addition of cAMP, there was minor random expansion with some loss in original cell shape. Again there was little evidence of any rebound in expansion after 20 s. The general behavior of expansion zones before and after the addition of cAMP was similar in ABP-120+ AX3 and AX4 cells (n = 15 and 20, respectively).

Chemotaxis in a Spatial Gradient. To test whether the absence of ABP-120 affects the capacity of single cells to assess the direction of a spatial gradient, ABP-120+ and ABP-120- cells were subjected to spatial gradients of cAMP generated in a gradient chamber and the chemotactic index (CI) was computed according to methods previously described (Varnum and Soll, 1984; Varnum-Finney et al., 1987b). In Fig. 11, histograms are presented of the CI of ABP-120+ and ABP-120- cells derived from strains AX3 (A) and AX4 (B). 80% of ABP-120+ AX3 cells exhibited positive CIs, and the average CI was +0.39. 68% of ABP-120+ AX3 cells exhibited positive CIs and the average CI was +0.09. 96% of ABP-120+ AX4 cells exhibited positive CIs and the average CI was +0.48 (± SD 0.24). 53% of ABP-120- AX4 cells exhibited positive CIs and the average CI was +0.11 (± SD 0.19). Therefore, the absence of ABP-120 results in a decrease in the average chemotactic index of both AX3 and AX4 cells in spatial gradients of cAMP.

Discussion

Disruption of the ABP-120 Gene Locus

Transformation of either strain AX3 or AX4 with uncut or doubly cut plasmids containing the 1.5-kb coding sequence resulted in G418-resistant cells which no longer expressed ABP-120. Southern blot analysis of these clones indicates that a multicopy insertion of the transforming plasmid occurred at the native gene locus resulting in the appearance of high molecular weight restriction fragments that are recognized by the probe. Northern blots are consistent with this interpretation since in all ABP-120- clones mRNA transcripts were either of altered size or absent.

Some ABP-120- clones expressed a truncated ABP-120 fragment of 60 kD as expected after homologous recombination with the plasmids used. These truncated proteins were expressed at very low levels compared with wild-type amounts of ABP-120 and are unlikely to influence the phenotype of cells as measured in this study. However, the phenotype analysis was done using two independently derived clones, one producing a small amount and the other with undetectable levels of the truncated protein and both clones showed identical ABP-120- phenotypes.

1. Abbreviation used in this paper: CI, chemotactic index.
ABP-120- Cells Exhibit Diminished Pseudopod Extension Activity

Ameboid movement probably results from the superimposition of a number of force-producing mechanisms involving myosin I, myosin II, and actin polymerization (Condeelis et al., 1990). In addition, ameboid movement is composed of a number of discrete events such as cell gliding, pseudopod extension, surface retraction, cytoplasmic streaming, cell-substrate adhesion, and membrane flow (Taylor and Condeelis, 1979). Given the complexity of ameboid movement one would anticipate that the deletion of a single protein involved in only one of these events would not lead to the inhibition of the general process of cellular translocation, and in the case of ABP-120, this is the case. ABP-120- mutants are capable of cellular translocation, and the retention of this ability explains why they are also capable of chemotaxis and multicellular morphogenesis. However, ABP-120- cells are not normal. They move on average at a depressed rate of translocation, turn more often, and are on average rounder than wild-type cells. More importantly, although ABP-120- cells form actin-filled pseudopods when translocating in the absence of chemoattractant, the dynamics of pseudopod extension are altered. The number of pseudopods and maximum area of an extended pseudopod is less than that of ABP-120+ cells and the average time necessary for maximum extension is greater than that of ABP-120+ cells, demonstrating that the absence of ABP-120 from pseudopods decreases the efficiency of extension. This defect may be the basis for the defects observed in the general rate of cellular translocation.

Although ABP-120- cells exhibit a decrease in the instantaneous velocity of translocation in buffer, they still respond to the rapid addition of 10^-6 M cAMP with a rapid cessation of cellular translocation, as has been demonstrated for wild-type AX3 (Wessels et al., 1989) and wild-type AX4 (Wessels and Soll, 1990) amebas and shown here for ABP-120- transformants. The kinetics of the decrease in motility are identical to wild-type cells. In the case of wild-type cells and ABP-120+ transformants, there is a freeze in cell shape during the first 10-15 s after a rapid addition of cAMP, followed by a partial rebound in the instantaneous velocity of the cell centroid, the result of an increase in randomly oriented pseudopod extension. The rebound closely coincides with the second peak of incorporation of actin into the cyto-

Figure 11. ABP-120+ cells show reduced chemotaxis efficiency in response to cAMP compared with control ABP-120+ cells. Histograms of the chemotactic indices of (A) AX3-derived strains AX3-1S-2° (○) and AX3-1S-4° (■) and (B) AX4-derived strains AX4-TF° (□) bars and AX4-3S-1° (■) bars. The mean chemotactic index for ABP-120+ and ABP-120- cells is noted in each panel by white and black arrows, respectively.
skeleton. In the case of ABP-120− cells, there is an apparent freeze in cell shape, but no partial rebound in the instantaneous velocity of the cell centroid consistent with the diminished pseudopod extension activity of these cells. The absence of a velocity rebound correlates with the absence of the second peak of actin incorporation into the cytoskeleton in ABP-120− cells indicating a role for ABP-120 in actin filament crosslinking and pseudopod extension (Condeelis et al., 1990).

The depression in instantaneous cell velocity and the rate and extent of pseudopod extension may also be the reason that ABP-120− cells exhibit a decrease in average chemotactic index measured in vitro in a gradient chamber. Under conditions of a stable spatial gradient, it appears that wild-type amebas use lateral pseudopodial extension in order to assess the direction of the spatial gradient, and that direction may be assessed by a temporal mechanism which computes the change in concentration with time (Varnum-Finney et al., 1987a,b). Therefore, a decrease in the rate of pseudopod extension, as observed in ABP-120− cells, would dampen the temporal sensing system.

**Comparisons of ABP-120− Transformants with a Chemically Mutagenized ABP-120− Cell Line**

It is important to compare the properties of the ABP-120− cells generated by homologous recombination described here with 1-methyl-3-nitro-1-nitrosoguanidine mutagenized cells lacking ABP-120 described recently (Brink et al., 1990). Four assays were done in common in these two studies and can be compared directly: the ability of cells to complete development, the measurement of cAMP-stimulated incorporation of actin into the cytoskeleton, and the measurement of velocity and chemotaxis in a stable spatial gradient of cAMP.

The results presented here with the ABP-120− transformants are in agreement with those for the 1-methyl-3-nitro-1-nitrosoguanidine mutant (HGI264) in that the ability of ABP-120− cells to complete development and grow in liquid culture is similar in both cases. However, significant differences between the phenotypes of the HGI264 mutant and the ABP-120− homologous recombinants were observed in the three other assays. In the mutant HGI264 the incorporation of actin into the cytoskeleton was reported to be affected in only the first peak, which showed an increased incorporation of actin while a second broad peak, encompassing the second and third peaks in our assays, was unaffected (Brink et al., 1990). This is opposite to the result with the ABP-120− transformants reported here, where only the second and third peaks are affected by deletion of ABP-120 and the result is a decrease in actin incorporation.

These differences may be explained by the fact that two different assays were used to measure the incorporation of actin into the cytoskeleton in the two studies. In the experiment using HGI264, cells were lysed at high density resulting in a final actin concentration of 4−8 μM deduced from total actin content measurements reported previously (Dhar-mawardhane et al., 1989) and the lysates were incubated for as long as 45 min before centrifugation. These conditions (10 mM KCl, 8 mM NaPO₄) are sufficient to cause significant polymerization of actin in cell lysates where the critical concentration would be 1 μM or less (Rickard and Sheterline, 1986). Therefore, the assay used with HGI264 would be influenced by actin polymerization occurring after lysis.

The assay used with the ABP-120− transformants described here generates cell lysates with a final actin concentration of 0.26 μM, and the lysates are immediately centrifuged to avoid postlysis polymerization. This assay is less influenced by actin polymerization and is more sensitive to actin cross-linking.

The third and fourth assays which can be compared between these two studies are the measurement of velocity of locomotion and chemotaxis in a stable spatial gradient of cAMP. HGI264 cells showed no variation from wild-type cells in a spatial gradient chamber (Fisher et al., 1989) for motility, analyzed by a processing system, or in chemotaxis (Brink et al., 1990). The average CIs for HGI264 and wild-type AX2 cells, in this case, were 0.34 and 0.29, respectively. In contrast, the CIs of ABP-120− AX3 and AX4 cells were 0.34 and 0.48, respectively, and ABP-120− AX3 and AX4 cells were 0.09 and 0.11, respectively. The fact that we have observed the same depression in two independently isolated ABP-120− transformants prepared in two different cell strains indicates that ABP-120 is indeed necessary for a strong chemotactic response under the in vitro conditions which we have used.

The difference in the way in which mutants were obtained in these two studies may contribute to such different results. Chemically mutagenized cells may have multiple genetic alterations which are compensatory, while transformants prepared by homologous recombination should have few uncharacterized changes since transformation vectors usually insert into the genome as a single multicycop tandem repeat (Nellen et al., 1984; Knecht et al., 1986).

**The ABP-120− Phenotype and Cortical Expansion**

It has been proposed that actin polymerization and subsequent cross-linking by what may be a broad family of actin-binding proteins (Bresnick et al., 1990) contributes to the force for pseudopod extension in a mechanism termed the cortical expansion model (Condeelis et al., 1990). The results of the present study demonstrate that phenotypes resulting from the deletion of ABP-120 implicate this protein in the cross-linking of actin filaments after cAMP stimulation when pseudopods are synchronously extended. Furthermore, the defect in the dynamics of pseudopod extension in buffer and the absence of a rebound in pseudopod formation after cAMP stimulation in ABP-120− cells suggest that actin filament cross-linking, in general, might play a direct role in pseudopod extension as proposed by the cortical expansion model (Condeelis et al., 1990) where filament cross-linking is a necessary step for pseudopod extension.

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