Isolation of actin-associated proteins from *Caenorhabditis elegans* oocytes and their localization in the early embryo

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The actin cytoskeleton plays an important, but poorly understood, role in the development of multicellular organisms. To help illuminate this role, we used actin filament affinity chromatography to isolate actin binding proteins from large quantities of *Caenorhabditis elegans* oocytes. To examine how these proteins might be involved in early development, we prepared antibodies against some of them and determined their distribution in fixed embryos. Three of these proteins co-localize with different subsets of the embryonic actin cytoskeleton. One co-localizes with actin to all cell cortices. The second oscillates between the nucleus and cortex in a cell-cycle-dependent manner. The third is asymmetrically enriched at the anterior cortex of one-cell embryos, showing a temporal and spatial localization suggestive of a function in generating developmental asymmetry. We conclude that biochemistry is a feasible and useful approach in the study of early *C.elegans* development, and that the embryonic actin cytoskeleton is regulated in a complex fashion in order to carry out multiple, simultaneous functions.

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Introduction

The actin cytoskeleton has many different roles in the early development of multicellular organisms. For example, it has been shown to be involved in the delineation of cytoplasmic boundaries (Sullivan and Theurkauf, 1995), in the determination of embryonic axes (Jeffery and Swalla, 1990), in the anchoring of mRNAs to embryonic poles (Yisraeli *et al.*, 1990) and in facilitating cell shape changes and membrane polarity (Johnson and Maro, 1985; Gueth-Hallonet and Maro, 1992). For none of these roles are the molecular mechanisms known.

We have focused on the nematode, *Caenorhabditis elegans*, where the actin cytoskeleton is essential for the segregation of developmental potential. For example, one-cell *C.elegans* embryos divide into two cells of different sizes and different developmental fates. Brief treatment with an inhibitor of actin filament formation causes one-cell embryos to divide into two cells of equal size, with

a missegregation of developmental determinants (Hill and Strome, 1988, 1990). Non-muscle myosin II appears to play a role in the generation of this one-cell polarity since its elimination using antisense techniques results in a similar phenotype (Guo and Kemphues, 1996). The proper orientation of spindles also depends on intact actin filaments. In wild-type two-cell embryos, the smaller cell reorients its spindle along the anterior–posterior axis via a mechanism thought to involve an interaction between astral microtubules and a cortical site rich in actin and actin-capping protein (Hyman and White, 1987; Waddle *et al.*, 1994). Inhibition of actin filament formation prevents the spindle reorientation from occurring (Hyman and White, 1987).

Biochemistry has yet to be widely exploited as a tool for studying *C.elegans* development (for recent exceptions, see Stroehrer *et al.*, 1994; Lichtsteiner and Tjian, 1995). Because a molecular understanding of *C.elegans* development will require detailed characterization of the proteins that bind to the major cytoskeletal filaments and regulate their properties, we set out to expand the repertoire of biochemical techniques available for analyzing the *C.elegans* embryonic cytoskeleton.

The technique of actin filament (F-actin) affinity chromatography (FAAC) was specifically designed for studying the actin cytoskeleton (Miller and Alberts, 1989). In this approach, actin-associated proteins are isolated by their ability to bind to a column of actin filaments *in vitro*. This technique has previously been used to identify new actin binding proteins from *Drosophila* embryos and *Saccharomyces cerevisiae* (Drubin *et al.*, 1988; Miller *et al.*, 1989). In both organisms, subsequent cDNA cloning and characterization of these proteins have provided new insights into the function of the actin cytoskeleton (Drubin *et al.*, 1990; Mermall *et al.*, 1994; Field and Alberts, 1995). In this report, we have used FAAC to identify over a dozen *C.elegans* proteins from oocytes that associate with actin filaments. By raising mouse polyclonal antibodies against a number of these proteins, we have determined their distribution in the early *C.elegans* embryo. We found that three of the proteins examined co-localize with all or part of the actin cytoskeleton with spatial patterns that suggest each plays a distinct role in early development.

Results

Isolation of *C.elegans* actin-column binding proteins

Oocytes were judged to be an ideal source for isolating *C.elegans* actin binding proteins because they are well-defined cells that contain a reservoir of proteins important for early development. In addition, soluble proteins are needed for FAAC, and some of the proteins of interest might be more soluble in oocytes than in embryos (Wessel

Fig. 1. Protocol for isolating large quantities of *C.elegans* oocytes. This protocol was modified from Strocher *et al.* (1994). The step in which the hermaphrodites are physically disrupted has been omitted for simplicity. See Materials and methods for details.

and Chen, 1993; Bachman and McClay, 1995). We therefore developed a reproducible protocol for isolating large quantities of pure *C.elegans* oocytes, which is outlined in Figure 1. Hermaphrodites were treated with drugs to induce oocyte laying; this freed the fragile oocytes without harming them. Drug concentrations and buffer conditions were optimized to favor oocyte survival and to minimize salt inhibition of oocyte laying. A syringe was used to permit efficient and quick filtration of the oocytes away from hermaphrodites. In this manner, we routinely obtained between 2.5 and 4.3 ml packed oocytes containing 30–40 mg/ml of protein from a preparation. We found it practical to perform two such preparations per week.

Extract from the oocytes was prepared using conditions worked out earlier for *Drosophila* embryo extracts (Miller *et al.*, 1991). Reproducible purification of *C.elegans* actin-column binding proteins required keeping the oocytes in K buffer (even one quick rinse in water results in 50% lower protein yield) and breaking open the oocytes gently with low power sonication.

The clarified *C.elegans* oocyte extract was passed through three columns linked in series: an albumin column to control for proteins that bind non-specifically to the column matrix and then two F-actin columns. The second F-actin column was included to capture actin binding proteins unable to bind to the first F-actin column due to

a possible saturation of its binding sites. The three columns were separated, washed and then eluted with a mixture of 1 M KCl, 3 mM MgCl₂ and 1 mM ATP.

Elution profiles for the three columns loaded with *C.elegans* extract are shown in Figure 2A. The total amount of protein eluted from the first F-actin column was 11-fold the amount eluted from the albumin control column, demonstrating a high level of binding specificity to F-actin (from experiment to experiment this ratio ranged from 3- to 13-fold and was typically ~10-fold). The total amount of protein eluting from the second F-actin column was roughly half that from the first F-actin column, revealing that the binding sites on the first F-actin column may have been saturated, but that most of the actin binding proteins had been captured by the two columns.

Equal amounts of total protein from the column load, flow-through and each of the three eluates were electrophoresed on SDS–polyacrylamide gels and visualized using Coomassie stain (Figure 2B). As expected, the protein composition of oocyte extract was very complex (lane 1), and none of its proteins were visibly depleted in the column flow-through (lane 2). The protein profiles of eluates from the two actin columns were similar to each other (lanes 4 and 5), and they differed significantly from that of eluates from the albumin control column (lane 3). Seventeen protein bands were visible in the eluates from

Fig. 2. Actin affinity chromatography with *C.elegans* oocyte extracts. (A) Total protein eluted from two 10 ml actin columns and one 10 ml albumin column that had been loaded with 110 ml of oocyte extract (165 mg total protein). One ml fractions were collected and every third fraction was assayed for protein content. The total protein eluted from the albumin column, actin column 1 and actin column 2 was about 0.11, 1.3 and 0.58 mg respectively. (B) Coomassie Blue-stained proteins electrophoresed on a 5–15% polyacrylamide gradient gel. An estimated 20 µg of total protein was loaded in each lane. From left to right: lane 1, total oocyte extract; lane 2, column flow-through; lane 3, albumin column elution; lane 4, second actin column elution; lane 5, first actin column elution. The locations of the yolk proteins are noted with (>) marks to the left of lane 1. The strong actin band in actin column eluates is also marked. The location of six CABPs discussed in this paper are noted. CABPs that run as doublets here, such as CABP12/13 and 14/15, were completely resolved on non-gradient gels before excision and injection into mice. Molecular weight markers (in kDa) are shown on the left.

the F-actin columns that were not visible in either the total extract or the eluates from albumin columns. These proteins are referred to as CABP(1–17) for *C.elegans* actin-column binding proteins #1 (lowest apparent molecular weight) through to #17 (highest apparent molecular weight).

Of the 17 CABPs, CABP12 is the only one that is reproducibly eluted from actin columns in an ATP-dependent manner. We have identified CABP12 as a myosin II based on three criteria (data not shown): (i) it co-migrated with the myosin II marker band on SDS-polyacrylamide gels; (ii) it required ATP for its elution from actin columns; and (iii) it was recognized by an antibody that binds myosin IIs in a variety of organisms.

Production of mouse polyclonal antibodies and protein immunoblotting

Mouse polyclonal antibodies were raised against a number of the CABPs in order to investigate their distributions in the early embryo. Approximately 1.2 mg of protein from pooled F-actin column elutions were electrophoresed on preparative SDS-polyacrylamide gels. Individual CABPs were excised as gel slices and injected into individual mice.

The ascites from CABP-injected mice were used to probe protein blots. Sera from six mice strongly cross-reacted with a single band from actin column eluates (Figure 3A). In all cases, the serum recognized a band of the same molecular weight as the CABP injected; other bands recognized, if any, were minor. The relative quantity of each of the proteins, as detected by blotting, was greater in F-actin column eluates than in total

oocyte extract (compare the two immunoblots in Figures 3A and B), verifying that these six CABPs were highly enriched on these columns. None of the sera recognized proteins from albumin column eluates, and pre-immune sera from these mice did not cross-react with any proteins from actin column eluates or total extract (data not shown).

Immunofluorescence in fixed embryos

Double-label immunofluorescence with actin antibody and the CABP antibodies was performed using a modification of a technique that preserves the actin cytoskeleton (Barstead and Waterston, 1989; Waddle *et al.*, 1994). All steps—permeabilization, fixation and antibody incubation—were carried out in solution so that the embryos were treated uniformly. This eliminated concerns raised by the more traditional *C.elegans* technique of freeze-fracturing (e.g. Albertson, 1984); namely that squashing embryos and attaching them to a polylysine surface might affect the cytoskeleton and/or the attainment of uniform permeabilization.

We have characterized in detail the staining pattern associated with three of the six CABP antibodies available. These antibodies recognize structures that coincide with the actin cortex and are described below. The staining patterns for the other three antibodies are presently under investigation.

CABP1 is localized throughout the actin-rich cortex

CABP1 (20 kDa) localizes to cell cortices in a manner similar to actin. In one-cell embryos, CABP1 antigen is found in the entire cortex and, to a lesser extent, in the

Fig. 3. Characterization of CABP antibodies by Western blotting. Protein immunoblots were probed with diluted ascites fluid from mice that had been injected with individual CABPs. An equal amount (20 μ g) of total actin column eluate (**A**) and total oocyte extract (**B**) were each loaded as a single lane across the top of an 8 cm 8% SDS-polyacrylamide gel, electrophoresed and transferred to nitrocellulose under identical conditions. Equal loading/transfer of gels was verified by Ponceau S staining. Each blot was then probed with antibodies to each of the following CABPs (labeled above each lane): CABP14 at 1:200, CABP13 at 1:100, CABP12 (myosin II) at 1:100, CABP11 at 1:50, CABP9 at 1:100 and CABP1 at 1:100. Each blot was treated identically with secondary antibody conjugated to horseradish peroxidase, developed with ECL reagents (Amersham) and exposed to film for identical periods of time. Molecular weight markers (in kDa) are noted on the left of each blot. The fainter, lower molecular weight band below the main CABP14 band might represent a degradation product as overexposure of the blot reveals a faint ladder of such bands typical of protein degradation.

cytoplasm (Figure 4A; for actin staining, see Figure 6 below; co-staining with CABP1 and actin antibodies was not possible due to an incompatibility of fixation conditions). No anterior–posterior asymmetry is noticeable. Similarly, in two-cell embryos, CABP1 antigen localizes to the entire cortex of each cell (Figures 4B), although the membrane between cells shows reduced staining when compared with that of the actin pattern. This pattern persists at the four-cell stage and other early embryonic stages (Figure 4C). We have not seen any obvious cell-cycle regulation in CABP1 localization.

CABP14 cycles in and out of the actin cortex

CABP14 localizes to the actin-rich cortex of embryonic cells in a cell-cycle-dependent manner. It localizes to the nucleus of cells in prophase, to the nucleus and cortex of cells in prometaphase, to the cortex and cytoplasm of cells in metaphase and to the cleavage furrow of dividing cells.

These various cell-cycle-dependent patterns are obvious from three different early embryos co-stained with CABP14 and actin antibodies (Figure 5). In the first embryo (two-cell stage), the posterior cell, P_1 , is in prophase and the anterior cell, AB, is in prometaphase. Typical of any embryonic cell in prophase, CABP14 is localized to the nucleus of P_1 in this embryo and, typical of any embryonic cell in prometaphase, CABP14 is localized predominantly to the nucleus and cortex of AB (Figure 5A). In the second embryo (four-cell stage), the two daughters of AB (ABa and ABp) are in metaphase and the two daughters of P_1 (EMS and P_2) in prophase. The two metaphase cells show intense staining around the cortex of each cell and also some staining in the cytoplasm, whereas the two prophase cells show only nuclear staining

Fig. 4. CABP1 staining in early embryos. Anterior is to the left in each embryo. Scale bar, 10 μ m. (**A**) One-cell embryo at prometaphase. Compare this with the actin staining in Figure 6B. (**B**) Two-cell embryo with the anterior cell, AB, in metaphase and the posterior cell, P_1 , in prometaphase. The membrane between AB and P_1 is weakly stained. Compare this with the actin staining in Figure 6C. (**C**) Four-cell embryo (two views). To the left is a medial view with all four cells visible. Compare with actin staining in Figure 5B. To the right is a more lateral view of the same embryo with ABp in focus to show that CABP1 is localized around the entire cortex.

(Figure 5B). In the third embryo (eight-cell stage), P_2 is dividing into C and P_3 . Localization of CABP14 along the cleavage furrow between the forming cells is evident (Figure 5C). Two of the AB granddaughters, both in prometaphase, are in view and show nuclear and cortical CABP14 localization. The beginning of nuclear localization in the forming C and P_3 cells and the recently divided E cell is faintly visible. CABP14 staining at different cleavage furrows varies from robust to weak, suggesting either a temporal component to its localization at the cleavage furrow or its association with a labile structure at the cleavage furrow. CABP14 staining is not detected in interphase or anaphase cells (not shown).

CABP11 localizes asymmetrically in the one-cell cortex

CABP11 (140 kDa) localizes to the actin-rich cortex of the one-cell embryo in a dramatic fashion. Between the time when the pronuclei meet and the end of metaphase of the first cell cycle (~10 min), CABP11 is enriched in the anterior cortex of one-cell embryos (Figure 6A and B). This anterior enrichment occurs relative to cortical actin itself, which is no less abundant in the posterior than anterior (Figure 6A and B). There is some variability in the size of the anterior cap (compare Figure 6A and B) and CABP11 is not completely excluded from the posterior.

Two-cell embryos also show asymmetry in CABP11 localization. In the posterior cell, P_1 , CABP11 appears to be enriched in the anterior cortex (Figure 6C). Since this is also the region where AB and P_1 come together, we cannot rule out the possibility that this is the posterior cortex of AB. None the less, two pieces of evidence suggest that the enrichment seen is in the anterior of P_1 : (i) by carefully following the actin cortical staining in each cell, we appear to be able to discriminate between AB's and P_1 's cortex at the cell–cell boundary, and CABP11 is strongly present on P_1 's cortex; and (ii)

Fig. 5. Actin and CABP14 staining in early embryos. Actin antibody staining is shown in red; CABP14 antibody in green. Anterior is up, tilted to the left. Scale bar, 10 μ m. (A) Two-cell embryo. P₁ is in prophase; AB in prometaphase. (B) Four-cell embryo. The two AB daughters are in metaphase; EMS and P₂ are in prophase. (C) Eight-cell embryo. P₂ is dividing into C and P₃. The cleavage furrow is marked ($\wedge$). An asterisk has been placed in the nuclei of the two AB granddaughters that are in this field of view (both in prometaphase).

CABP11 localizes to the bright dot of actin that is thought to reside on the anterior cortex of P₁ (Figure 6C; Waddle *et al.*, 1994). The staining of CABP11 in the cortex of the anterior cell, AB, is more complex and apparently very dynamic (not shown).

Since CABP11 staining is very similar to that of PAR-3 (Etemad-Moghadam *et al.*, 1995), a protein required for asymmetry at the one-cell stage, we tested whether CABP11 localization is perturbed in *par-3* mutant embryos. Whereas CABP11 localization is normal in one-cell control (*unc-32*) embryos (Figure 7A), its localization is perturbed in one-cell *par-3 unc-32* embryos. Two types of staining are observed. In some *par-3* embryos, CABP11 staining is not present at the cortex and is often significantly reduced (Figure 7B; 6/15 embryos). In others, CABP11 staining is present at the cortex in reduced amounts relative to controls and is no longer anteriorly enriched (Figure 7C; 9/15 embryos). Actin cortical staining itself appears to be normal in all of the *par-3* mutant embryos. We do not know the reason for the two classes of *par-3* staining

Fig. 6. Actin and CABP11 staining in early embryos. Actin antibody staining is shown in red; CABP11 antibody in green. Anterior is up and to the left. Scale bar, 10 μ m. (A) One-cell embryo at the time of pronuclei convergence. (B) One-cell embryo at late prometaphase. (C) Two-cell embryo. AB is in metaphase; P₁ in prometaphase. The bright dot of actin staining found on the cortex of P₁ is marked with an arrowhead ($\blacktriangle$).

patterns, although there is known variability in some *par-3* mutant phenotypes, e.g. the orientation of spindles at second cleavage (Cheng *et al.*, 1995). A variability in CABP11 fixation is another possibility.

Discussion

We used FAAC to expand our knowledge of the *C.elegans* early embryonic actin cytoskeleton. Although proteins of very minor abundance would have escaped our notice, by developing methods for preparing large quantities of *C.elegans* oocyte extracts, we were able to identify 17 proteins that associate with F-actin *in vitro*. Mouse polyclonal antibodies were generated that specifically recognize six of these *C.elegans* CABPs. Using these antibodies, we succeeded in localizing three CABPs to specific parts of the actin cytoskeleton in fixed early embryos. In addition, the methods of oocyte isolation and extract preparation that we worked out are quite general ones that should enable biochemical approaches to be taken to many other aspects of early *C.elegans* development.

CABPs in early embryonic development

What do the localizations of the three CABPs analyzed here tell us about their possible functions and the roles of

Fig. 7. Actin and CABP11 staining in control and *par-3* mutant embryos. All three images have been collected under identical confocal settings and exposures to allow direct comparison. Actin antibody staining is shown in red; CABP11 antibody in green. Anterior is to the left. Scale bar, 10 μ m. (A) One-cell *unc-32(e189)* control embryo at the time of pronuclei convergence. The increase in cytoplasmic staining (compare with Figure 6A and B) probably represents a decrease in the signal:noise ratio caused by alterations in the antibody fixation/incubation procedure required to handle small numbers of *par-3* embryos (see Materials and methods). (B) One-cell *par-3(it71) unc-32(e189)* embryo at pronuclei convergence. At this exposure (chosen to give a good balance of actin and CABP11 staining in all three embryos) no staining is visible, although faint cytoplasmic staining was detected. (C) One-cell *par-3(it71) unc-32(e189)* embryo at prometaphase. The lone dot of CABP11 staining in the anterior cortex is the polar body, which is recognized by both actin and CABP11 antibodies.

the actin cytoskeleton in early development? The CABP1 antigen localizes to all cortices of early embryonic cells in a manner similar to actin, suggesting that this 20 kDa protein is involved in some non-specialized actin function. Its localization also serves as an important control for CABP11, showing that not all of the associated components of the actin cytoskeleton localize asymmetrically at the one-cell stage. CABP1 is readily detected by blotting in unfractionated cell extracts (Figure 3), consistent with its being a relatively abundant protein with a general function in regulating the actin cytoskeleton.

The localization of CABP14 (260 kDa) to the actin-rich cortex is dependent on the cell cycle. The CABP14 antibody stains the nuclei of cells early in the mitotic cycle (prophase and prometaphase), the cortices of cells later in the cell cycle (prometaphase and metaphase), and the cleavage furrows of cells during cytokinesis. Our data suggest that there is a significant change (in either protein levels or antibody recognition) in CABP14 at the metaphase to anaphase transition, since staining disappears at this time. There is no detectable CABP14 staining in interphase cells, suggesting that it functions only in actively dividing cells.

Based on its cell-cycle-dependent variation and its localization to the cleavage furrow, we speculate that CABP14 plays a role in cytokinesis, although we cannot rule out a role for CABP14 in the nucleus since it is also sometimes found there. It is interesting that CABP14 displays a localization pattern strikingly similar to that of the *Drosophila* protein anillin (190 kDa), which also oscillates from the nucleus to the cortex to the cleavage furrow. Anillin, identified in *Drosophila* extracts by FAAC, shows no significant homology to other known proteins,

and it binds to actin filaments *in vitro* (Field and Alberts, 1995). To our knowledge, these are the only two actin-binding proteins that localize in this way. Further study of CABP14 and anillin may be important for understanding cell-cycle control of the actin cytoskeleton.

In the one-cell embryo, CABP11 antigen (140 kDa) co-localizes with the actin cortex but is highly enriched in the anterior. We suggest that CABP11 is involved in generating the asymmetry at the one-cell stage. Consistent with this hypothesis, the presence of normal levels of CABP11 at the cortex, as well as its anterior localization, require PAR-3, a protein known to be involved in generating asymmetry in the one-cell embryo. Like CABP11, PAR-3 is enriched at the anterior cortex of one-cell embryos (Etemad-Moghadam *et al.*, 1995; our partial sequence of a CABP11 clone demonstrates that the two proteins are different). Furthermore, PAR-3 and CABP11 are asymmetrically localized at the two-cell stage to the anterior cortex of P₁, although CABP11's localization in AB is different from that reported for PAR-3 (Etemad-Moghadam *et al.*, 1995).

In conjunction with other recent results, our CABP11 data suggest that the actin cytoskeleton is used at multiple points during the generation of one-cell asymmetry. Non-muscle myosin II appears to be required upstream of PAR-1, PAR-2 and PAR-3 in the establishment of asymmetry since the normal localization of these proteins all depend on non-muscle myosin function, whereas the localization of myosin is not perturbed in any *par* mutant (Guo and Kemphues, 1996). CABP11 probably also acts downstream of myosin since its distribution is disturbed in a *par-3* mutant (which is downstream of myosin). This result suggests that CABP11 could be operating either in

conjunction with PAR-3 (marking the anterior of the cell) or downstream of PAR-3, perhaps to help reinforce asymmetric cues. Such a reinforcing role for the actin cytoskeleton has been seen in the generation of asymmetries in budding yeast and mammalian epithelial cells (Drubin and Nelson, 1996).

Our results raise the issue of whether cortical actin itself is asymmetrically localized. Previous studies, utilizing a different permeabilization technique and fixative, have shown that cortical actin itself may be anteriorly enriched in one-cell embryos (Strome, 1986; Kirby *et al.*, 1990), although this asymmetrical actin localization appears to be functionally unimportant (Rose *et al.*, 1995). We note that in these studies there was little actin present in the posterior cortex. Using our fixation techniques, actin is localized in a robust fashion to the anterior and posterior cortex, and we find no evidence for a consistent enrichment of actin foci in the anterior cortex (see Figures 6 and 7).

In conclusion, the methods used in this work should complement the molecular-genetic approach that has been traditionally used to examine *C.elegans* development by facilitating biochemical- and cell biological-oriented studies of the cytoskeleton. The success of FAAC relies on having sufficient quantities of starting material and on maintaining the native conditions of proteins so that they will bind and elute from F-actin columns. It is independent of a mutant phenotype or prior knowledge of a protein of interest, although the use of antisense RNA, which works well in *C.elegans* (Guo and Kemphues, 1996), allows one later to test genetic function of cloned genes *in vivo*. The type of biochemical technique adopted here should therefore be useful in identifying components missed by the various genetic screens and in expanding our knowledge of early embryogenesis.

Materials and methods

Preparation of *C.elegans* oocytes [modified from Stroehrer *et al.* (1994)]

For standard *C.elegans* reagents and protocols, see Wood (1988). Preparation of NG agar–chicken egg trays is described in Stroehrer *et al.* (1994). Because optimal yields are critically dependent on moisture content, the egg mixture on the tray should be dried to the point where it just becomes a gel.

Large quantities of synchronous, oocyte-rich hermaphrodites were grown on NG agar–egg trays as follows. Two freshly starved 6 cm plates of *fer-1(ba576)* hermaphrodites, grown at 18°C, were used to seed each of two egg trays. The trays were put at 18°C for 7 days until the second generation of gravid hermaphrodites appeared. The hermaphrodites were washed off the trays, rinsed and treated with standard alkaline hypochlorite (0.5% NaOCl, 0.25 N KOH) for 5 min. All but embryos dissolve. These were collected and allowed to hatch-off in standard M9 solution overnight. The hatched, synchronous larvae were pelleted, seeded on six newly prepared egg trays (10⁶ larvae per tray) and grown at 25–26°C (restrictive temperature) for 5 days. At this temperature, *fer-1(ba576)* hermaphrodites make defective sperm and accumulate large numbers of fertile oocytes (Ward and Miwa, 1978).

The oocyte-rich hermaphrodites were washed off the trays with cold EN (10 mM Na₃EDTA, pH 7.9, 100 mM NaCl) and cleaned by standard sucrose flotation in a 250 ml conical tube. To remove larvae, the hermaphrodites were washed several times in K buffer (40 mM KCl, 10 mM K-HEPES pH 7.5), resuspended in 250 ml K buffer and allowed to settle for 8 min at room temperature. The supernatant was aspirated off, removing the small larvae which float (gravid adults settle to the bottom). This gravity enrichment was repeated twice more. Finally, the gravid hermaphrodites were transferred to a beaker, adjusted to 5 mg/ml serotonin (Sigma cat. No. H-7752) in K buffer to induce oocyte laying and stirred gently for 30 min (the composition of K buffer is critical,

since higher salt concentrations inhibit serotonin-induced egg laying and lower salt concentrations decrease the protein content of the oocytes, presumably via osmotic stress). The mixture was taken up in a 60 ml syringe and squirted four times onto a 45 µm Nytex mesh stretched over a 6 inch embroidery hoop. Oocytes pass through this mesh into a beaker placed below, but adults do not. The hermaphrodites were collected, treated again with serotonin and oocytes were collected by filtration as described. The hermaphrodites were then incubated in 0.25 mg/ml levamisole (Sigma cat. No. L-9756) in K buffer, which causes hermaphrodites to constrict and forces out more oocytes, which were collected as above. Finally, the hermaphrodites were resuspended in 200 ml of K buffer, transferred to a household blender, homogenized for 10 s at the lowest setting and filtered again (this step physically breaks open the hermaphrodites, freeing some of the remaining oocytes). This homogenization procedure was repeated twice more.

To remove drugs and small debris, the oocyte-containing filtrate from the 45 µm Nytex was poured onto a 20 µm Nytex screen and dipped repeatedly in cold K buffer. At the end, the 20 µm screen contained mostly pure oocytes and a few larvae (estimated to be <1% by volume). Typical yields were 2.5–4.3 ml of packed oocytes per preparation.

Preparation of extract and isolation of actin column-binding proteins

Oocyte high speed extract was prepared under actin depolymerization conditions as described (Miller *et al.*, 1991), except that the oocytes were broken open by sonication using a miniprobe at low setting (three 1 min bursts). The protein concentration of the extract was typically 1.5 mg/ml. If not used immediately, glycerol was added to 10%, the extract was frozen in liquid nitrogen and stored at –80°C. Before use, the frozen extract was thawed and spun at 80 000 g for 45 min to remove denatured proteins (~10% loss).

Actin affinity chromatography (including all reagents) was performed as described (Miller *et al.*, 1991). Two 10 ml F-actin columns (from rabbit skeletal muscle G-actin prepared as described by Pardee and Spudis, 1982) and one 10 ml bovine serum albumin (BSA) column were prepared in 20 ml syringes. The columns were connected in series such that oocyte extract (110 ml) first passed through the control albumin column, next through actin column #1 and then through actin column #2. After loading, the columns were uncoupled, washed separately with column buffer (>10 column volumes) and eluted with column buffer plus 1 M KCl, 1 mM ATP and 3 mM MgCl₂. One millilitre fractions were collected and protein concentrations were determined using Bradford Reagent (Bio-Rad) calibrated with albumin standards. Fractions containing protein were pooled, frozen in 20 µg aliquots in liquid nitrogen and stored at –80°C. For analysis, aliquots were thawed, precipitated with 10% trichloroacetic acid, washed with acetone and resuspended in SDS sample loading buffer. Samples were boiled, electrophoresed through 5–15% SDS–polyacrylamide linear gradient gels and the proteins visualized by staining with Coomassie Blue. The experiment was repeated independently once at the same scale as above (110 ml extract with 10 ml columns) and many times at a smaller scale (30 ml extract with 3 ml columns) and in each case comparable results were obtained.

Generation of mouse polyclonal antibodies from gel-separated proteins

The proteins in pooled actin column eluates (0.6 mg) were electrophoresed through either 5% or 11% preparative SDS–polyacrylamide gels to optimize separation for high or low molecular weight proteins respectively. Ten protein bands of 100–300 kDa, selected because they were greatly enriched in actin column elutions compared with BSA column elutions and the total extract, were individually excised from the 5% gel. Likewise, seven proteins of 20–75 kDa were excised from the 11% gel. To date, 11 of these proteins have been injected into mice subcutaneously. Polyclonal ascites were generated using established protocols with the assistance of the University of California at Berkeley Hybridoma Facility (Karu, 1993; Ou *et al.*, 1993), and the quality of each serum was evaluated using protein immunoblotting (see below).

Protein immunoblotting

Protein mixtures (20 µg total) were electrophoresed in a single lane on 8 cm mini-SDS–polyacrylamide gels and transferred onto nitrocellulose at 80 V for 3 h in a Bio-Rad minigel transfer apparatus. Antibody incubations were performed using a Miniblotter 28 (Immunitics, Cambridge, MA). The yolk proteins were identified by their size and by immunoblotting with yolk-specific antibodies (gift of Peg MacMorris).

The myosin II band was identified with affinity-purified antibodies to a peptide conserved in all myosin IIs (T.J.Mitchison, unpublished data).

Immunofluorescence in whole, fixed embryos

Permeabilization, fixation and antibody staining of wild-type N2 embryos were performed by modifications of the techniques given in Barstead and Waterston (1989) and Waddle *et al.* (1994). For formaldehyde fixation (used with actin, CABP11 and CABP14 antibodies), N2 embryos were grown to the young adult stage for 3 days at 20°C on high growth plates. These adults were washed off with water into a 1.5 ml microfuge tube and collected by a brief spin in a microfuge at 3000 r.p.m. for 10–20 s. Excess *Escherichia coli* were removed by washing in water and embryos were isolated by treatment with 1.5 ml alkaline hypochlorite (see above) for 7 min. Hypochlorite was removed by four 0.8 ml washes in egg salts with spinning as above. An aliquot of embryos was taken at the fourth wash to ascertain viability immediately prior to fixation (>95%). Washed embryos were fixed for 10 min in 1.5 ml 3.5% formaldehyde, 1× cytoskeletal buffer and 11% sucrose (Cramer and Mitchison, 1993). Embryos were spun, washed once with 0.8 ml phosphate buffered saline (PBS) (Harlow and Lane, 1988) and post-fixed for 5 min in 1.5 ml of –20°C methanol. The embryos were again spun, washed in 0.8 ml PBS and permeabilized for 12 min in 0.8 ml 1× PBS plus 0.5% detergent (Tween 20 for actin-CABP11 and Triton X-100 for actin-CABP14). Embryos were incubated in 0.5 ml blocking solution (1× PBS, 10% normal donkey serum, 1% BSA, 0.1% detergent) for 20 min and then incubated in 0.25 ml blocking solution plus antibody (CABP11 at 1:50 and CABP14 at 1:100) for 2 h. Antibody was washed out with three 10 min washes of 0.8 ml 1× PBS, 0.1% detergent and one 10 min wash of blocking solution. Embryos were then incubated for 1 h in 0.25 ml block plus 1:200 cyanine 5 (cy5)-labeled anti-mouse secondary antibody (715-175-150 from Jackson Labs). Washes were performed as for the primary antibody with 5% normal mouse serum added to the third wash to block any free secondary antibody-binding sites. Fluorescein (FITC)-labeled actin antibody (mAbC4 from ICN, which recognizes cytoplasmic actin in a wide variety of organisms including *C.elegans* and gives a staining pattern almost indistinguishable from that of phalloidin; Waddle *et al.*, 1994) was diluted 1:10 in blocking solution (0.1 ml) and added to the embryos for 2 h (antibody labeling is described in Oegema, Zheng and Alberts, submitted). Washes were performed as above, except that the third wash included DAPI to stain DNA. DAPI fluorescence allows determination of the cell cycle status of cells from the appearance of the chromosomes, and it also marks the anterior pole in one-cell embryos by staining the polar body (Wood, 1988). We were not able to ascertain reproducible antibody staining patterns of one-cell embryos prior to pronuclear convergence since these very early staged embryos are susceptible to damage by the bleach treatment.

CABP1 antibody staining, which is not preserved by formaldehyde fixation, was carried out by fixing egg-salt-washed embryos for 3 min in room temperature methanol. Permeabilization (with Triton X-100), blocking, antibody incubation (at 1:500) and washing were performed as above. Co-staining with actin antibody was not possible after methanol fixation.

To stain *par-3* mutant embryos, ~300 uncoordinated L4 or young adult hermaphrodites from the strain KK653 [*par-3(it71) unc-32(e189)/qC1*; Etemad-Moghadam *et al.*, 1995] were picked onto a 6 cm NG plate and allowed to develop to the young adult stage overnight. *par-3(it71)* is thought to be a null allele (Cheng *et al.*, 1995). Staining was as above with the following changes due to the limited number of embryos: incubations were in 0.65 ml microfuge tubes; spin times were increased to 1 min; the number of consecutive washes decreased from four to three; PBS washes were eliminated altogether; and volumes were decreased 2.5- to 3-fold. These changes reduced the loss of embryos, although the quality of staining was somewhat less. As a control, *unc-32(e189)* hermaphrodites were identically stained in parallel with the *par-3 unc-32(e189)* hermaphrodites and identical confocal settings were used.

Pre-immune sera from the mice injected with any of these CABPs showed no staining, with the exception of variable centrosomal staining associated with CABP11 pre-immune sera. Secondary antibody staining alone resulted in a low background glow. We verified by single labeling that there was no bleed-through between the cy5 and FITC channels.

Immunofluorescence was observed with a Bio-Rad MRC 600 confocal microscope, using a 60× Nikon Plan Apo lens (NA 1.4). For a given embryo, images of actin and CABP co-staining were collected by Kalman averaging 5–10 scans of an identical, single optical section (thickness of section varied from embryo to embryo between 1.5 and

3.0 µm). DAPI for each embryo was visualized using a mercury lamp and UV cube mounted in the Nikon Optiphot associated with the confocal apparatus. The DAPI stain could not be excited by the confocal itself, which lacks the proper laser line.

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