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Identification of *Drosophila* cytoskeletal proteins by induction of abnormal cell shape in fission yeast

(actin/ezrin/cofilin/profilin/morphogenesis)

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ABSTRACT **To clone metazoan genes encoding regulators of cell shape, we have developed a functional assay for proteins that affect the morphology of a simple organism, the fission yeast *Schizosaccharomyces pombe*. A *Drosophila melanogaster* cDNA library was constructed in an inducible expression vector and transformed into *S. pombe*. When expression of the *Drosophila* sequences was induced, aberrant cell shapes were found in 0.2% of the transformed colonies. Four severe phenotypes representing defects in cytokinesis and/or cell shape maintenance were examined further. Each displayed drastic and specific reorganizations of the actin cytoskeleton. Three of the cDNAs responsible for these defects appear to encode cytoskeletal components: the actin binding proteins profilin and cofilin/actin depolymerizing factor and a membrane-cytoskeleton linker of the ezrin/merlin family. These results demonstrate that a yeast phenotypic screen efficiently identifies conserved genes from more complex organisms and sheds light on their potential *in vivo* functions.**

The mechanism by which cells change shape in response to developmental and cell cycle cues remains a mystery. The location, architecture, and dynamics of the actin-rich cytoskeleton are consistent with a key role for this structure in cell shape change and maintenance. To understand how the actin cytoskeleton performs its diverse functions, we need to identify its constituents and learn how they interact in molecular detail. Reverse genetic strategies (the analysis of mutations generated in known proteins) are invaluable for establishing whether specific proteins are determinants of cell shape. For example, in *Drosophila*, reverse genetic studies implicate nonmuscle myosin II as a motor for cell shape change during both cytokinesis (1) and morphogenetic movements during development (2, 3). Despite these and other successes, reverse genetic strategies are at best cumbersome for the identification of candidate proteins that are associated with a given process. Thus, little is known about the proteins that localize the myosin motor or its partner actin in transient structures capable of transmitting force to, and through, the actin-rich cell cortex and the plasma membrane with which it associates.

To identify genes whose products are involved in a specific developmental process, phenotypic or genetic interaction screens in *Drosophila* are extremely powerful (see references in refs. 4–6). However, some classes of proteins are difficult to target with such screens because they (*i*) are required for cell viability, (*ii*) have general or unknown expression patterns, (*iii*) have multiple or overlapping functions, or (*iv*) are maternally loaded into the egg. One such class includes elements of the actin cytoskeleton. Genes encoding cytoskeletal components have been fortuitously identified in phenotypic screens for defects in a variety of processes—e.g., embryonic development and larval cell division [nonmuscle myosin II subunit genes zipper (2, 7) and spaghetti squash (1), respectively], oogenesis [profilin encoded by chickadee (8)], and bristle morphogenesis [fascin encoded by singed (9)]. Systematic genetic approaches, such as those applied to embryonic pattern formation (e.g., ref. 7), have not been available.

We have devised a technique that detects cytoskeletal proteins by their ability to perturb cell shape when overexpressed in fission yeast. This method simplifies potential phenotypes, is not biased against redundant or generally required proteins, allows immediate determination of the sequence of recovered genes, and yields a phenotype that is informative and potentially useful in genetic interaction screens. In a pilot screen, we transformed *Schizosaccharomyces pombe* cells with *Drosophila* cDNAs in an inducible expression library, induced high-level transcription of the cDNAs, and selected those that altered cell morphology. Three out of four such clones disrupted the yeast cell's normal actin structures and were found to encode proteins with strong similarity to actin binding proteins identified in other phyla. Thus, this screen appears to select evolutionarily conserved proteins by their ability to interact with endogenous yeast proteins.

MATERIALS AND METHODS

cDNA Library Construction and Sequence Analysis. Molecular biological manipulations were executed using standard protocols (10) unless otherwise noted. First-strand cDNA was synthesized from 0- to 24-h *Drosophila melanogaster* embryo RNA as described (11), except that poly(A)$^+$ mRNA was selected by two cycles of binding to an oligo(dT)-cellulose column (New England Biolabs), and reverse transcription was primed with *Bam*HI/*Not* I-poly(dT) linker-primers. DNA polymerase I (Stratagene) was used to generate the second strand in the presence of methyl-dCTP. The cDNAs were blunted with T4 polymerase (New England Biolabs) and then ligated to *Sal* I adapters containing a *Pac* I site. cDNAs were sized on Sephacryl S-400 (Pharmacia), cleaved with *Bam*HI, and then cloned into the REP3Xho (pMBS36Leu) *S. pombe*/*Escherichia coli* shuttle vector (12), prepared with *Sal* I and *Bam*HI. Insert sequences were determined using the Sequenase 2.0 kit (United States Bio-

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; F-actin, filamentous actin; MER, moesin, ezrin, and radixin; RP, ribosomal protein.

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chemical) and analyzed with LASERGENE software for Macintosh (DNAstar, Madison, WI). BLASTP (13) was used to query the combined sequence data bases. Computer-generated alignments from the LASERGENE multialignment program were edited by eye.

Yeast Cell Shape Screen. Transformed *Leu*$^-$ cells were plated onto Leu$^-$ plates with thiamine (to select for the plasmid and repress the *nmt1* promoter) and then twice replica plated to Leu$^-$ plates without thiamine (to remove dead cells and derepress the *nmt1* promoter) at 10,000 colonies per 9-cm plate. We screened ≈10% of the complexity of the library for colonies whose cells had aberrant shapes. Of the ≈100 observed, ≈50 were picked and then grown on Leu$^-$ plates with thiamine (10–20% of the colonies selected by cell shape phenotype did not survive replating). Plasmid DNA was purified from the colonies (14) and used to transform *E. coli*, and *E. coli*-grown plasmid was used to retransform *S. pombe* by using the alkali cation method (14).

Microscopy. We evaluated cell morphology and the distribution of actin as a function of time after derepression of the *nmt1* promoter. The promoter is derepressed by washing the cells into thiamine-free medium and incubating them at 31°C. Several cell doublings are required before intracellular thiamine is sufficiently diluted to cause activation of the promoter (12). Here, we report the terminal phenotypes that were displayed after 24–36 h. In each case, this terminal phenotype appeared to be a natural progression from phenotypes seen as early as 20 h. Cell numbers plateaued as the phenotypes developed although the cultures were not near saturation, except D9 cells that continue to grow and divide while induced. For staining, expression of the cDNA clones was derepressed, and cells were incubated for various times, fixed, and then stained with rhodamine-phalloidin (Molecular Probes) by standard protocols (15–17). Cells were mounted on glass slides and coverslips in 4′,6-diamidino-2-phenylindole (DAPI; Sigma) at 5 μg/ml with 0.1% *n*-propyl gallate and 0.5 mM dithiothreitol as antifade (Sigma) and were observed with a 100× 1.3 n.a. objective on a Zeiss Axiophot microscope equipped for differential interference and epifluorescence with standard Zeiss components and filter sets. Digital images were acquired with a Star 1 cooled charge-coupled device camera (Photometrics, Tucson, AZ), driven by IP Lab software (Signal Analytics, Vienna, VA) on a Quadra 950 (Apple Computer, Cupertino, CA). The 12-bit 576 × 384 pixel images were captured with an IEEE 488 GPIB communications board (National Instruments, Austin, TX) and recorded onto a Magneto optical disk (Sony, Park Ridge, NJ) mounted in an optical disk drive (Microtech Instramentational, East Haven, CT). Images were processed with PHOTOSHOP (Adobe Systems, Mountain View, CA), assembled into finished composite panels in CANVAS (Deneba Software, Miami), and then outputted to a Kodak dye-sublimation printer (Eastman Kodak) for hard copy. To quantitatively compare the level of actin in different cells, exposure times and image processing were standardized so that each image was handled in an identical fashion.

RESULTS AND DISCUSSION

A small fraction of fission yeast transformed with random *Drosophila* cDNAs displayed aberrant morphologies. An embryonic *Drosophila* cDNA library was constructed in the pREP vector (12). The cDNAs are oriented and can be inducibly expressed in *S. pombe* from the thiamine-repressible *nmt1* promoter. The library was used to transform *S. pombe* cells by the spheroplast method (14). Transformed colonies were visually screened for aberrant morphology. In ≈100 of the 50,000 colonies inspected, the cells deviated from the normal capsule shape (Fig. 1*a*), displaying a variety of phenotypes. We chose to analyze four severe phenotypes

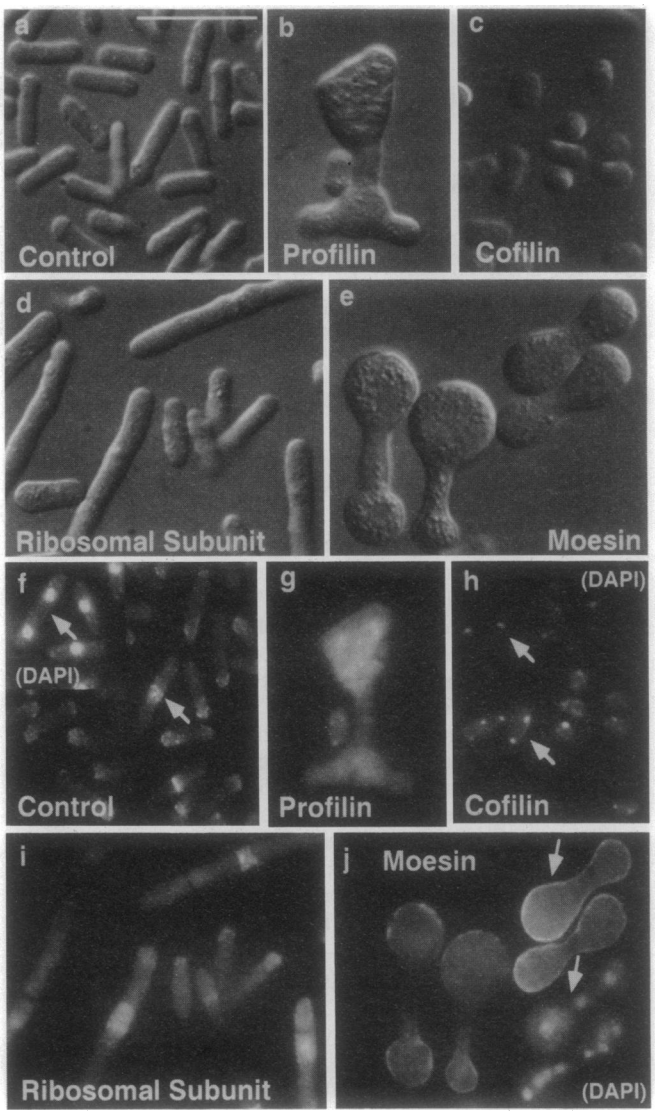

FIG. 1. Select *Drosophila* cDNAs induce aberrant cell shape and disrupt F-actin distribution in *S. pombe*. Differential interference microscopy shows the wild-type morphology of *S. pombe* cells before cDNA induction (control) (*a*) and after induction of cDNAs D88 (profilin) (*b*), D61 (cofilin-like sequence) (*c*), D9 (RP S17) (*d*), and D17 (moesin-like fragment) (*e*). Epifluorescence microscopy reveals the distribution of F-actin indicated by rhodamine-phalloidin staining (*f–j*) and nuclear morphology is indicated by DAPI staining (*Insets* in *f*, *h*, and *j*, labeled DAPI) in the same cells shown in *a–e*. DAPI-stained nuclei are normally rounded but display chromatin-rich and -poor regions. Wild-type actin structures are entirely missing (*g*, *h*, and *j*) or mislocalized (*i*) in cDNA-induced cells. A small percentage of induced D17 cells contained rings or arcs of phalloidin staining (data not shown).

that appeared to reflect defects in cytokinesis and/or cell shape maintenance. The cDNA clones were isolated and retransformed into *S. pombe* to verify the reproducibility of the phenotypes. Visual inspection of 50,000 colonies required no more than 2–3 days, and inserts from the selected clones were rapidly analyzed using vector-specific primers for PCR amplification and sequencing. Thus, compared to traditional genetic and biochemical approaches, this screen requires minimal time to go from candidate phenotype or activity to sequenced gene product.

Each of the four cDNAs induced a characteristic and reproducible change in morphology (Fig. 1 *b–e*). To better characterize the mechanism by which the clones perturb cell shape, we analyzed the morphology of the actin cytoskeleton

and the distribution of DNA by staining fixed and permeabilized *S. pombe* cells with rhodamine-phalloidin and DAPI. Overexpression of each of the exogenous proteins disrupted the wild-type distribution of actin (Fig. 1 *g–j*). The actin cytoskeletons in transformants whose expression was repressed by the presence of thiamine (Fig. 1 *a* and *f*) and in wild-type *S. pombe* cells (972h$^-$, data not shown) were identical and had a cell-cycle-dependent distribution of actin characterized by foci or dots of filamentous (F) actin at the growing ends of the cells and a diffuse concentration of cortical actin at the cleavage planes associated with forming septa (identical to published patterns; refs. 15–17).

The sequences of the cDNA inserts from each strain were determined and used to search available protein data bases (Swiss-Prot, PIR, and GenBank releases of March 1992). All four cDNAs were readily identifiable by their close similarities to reported sequences (Table 1). Three of the four encode products highly similar to cytoskeletal protein families that are widely conserved throughout evolution and known to interact with actin. Below we summarize the salient features of each of these proteins and describe the corresponding overexpression phenotypes.

The D88 cDNA strongly inhibited cell division and caused complete disruption of cell shape and the actin cytoskeleton (Fig. 1 *b* and *g*). The enlarged, tortured, and branched cells had a uniformly diffuse distribution, but an overall greater concentration, of F-actin. No discrete actin structures could be resolved. D88 encodes a full-length profilin, a small ubiquitous protein that binds actin monomers (18). The *Drosophila* profilin gene, chickadee, has been cloned and shown to be required for regulated actin filament assembly (8). The D88 clone could rescue *S. pombe* cells with a lesion in the endogenous yeast profilin gene, *cdc3* (ref. 19; K.A.E. and D.P.K. in collaboration with M. Balasubramanian and K. Gould, Vanderbilt University), but only under conditions that caused a low level of expression of the *Drosophila* profilin sequence. Overexpression of *Drosophila* profilin also causes an aberrant cell shape phenotype in *cdc3* cells. These results suggest that the apparent increase in F-actin is due to the accumulation of an excess of a functional profilin, which correlates with the *in vivo* role of profilin in modulating actin filament assembly (8, 18).

The D61 cDNA strongly suppressed both cell growth and division, yielding small cells with irregularly shaped nuclei and large dots and cables of phalloidin staining (Fig. 1 *c* and *h*). The D61 cDNA encodes a member of the cofilin family (20–22), another group of small actin binding proteins (Fig. 2). The cellular role of these proteins is unknown, but they bind actin monomers and filaments *in vitro* and are found in a wide variety of cell types in which actin is being actively redistributed, such as developing muscle, amoebae, and pollen. In cultured cells, cofilin can redistribute to the nucleus, leading to speculation that it is responsible for delivering actin into the nucleus. Our interpretation of the

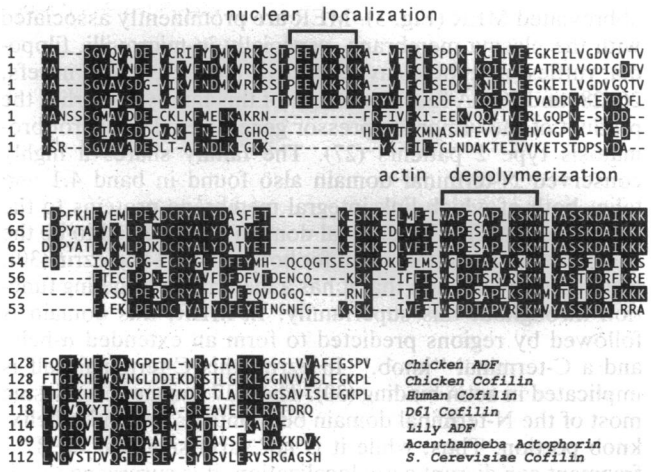

FIG. 2. D61 encodes a *Drosophila* member of the cofilin protein family that includes actin depolymerizing factor (ADF), destrin, depactin, and actophorin. Amino acid sequence alignments include six family members from a range of phyla (for references to sequences, see refs. 21 and 22; the actophorin sequence is in the GenBank data base and unpublished). Consensus residues (identical in at least four species or three phyla) are shown in white type on a black background. D61 cofilin is 34% identical to actin depolymerizing factor from lily pollen and 33% identical to human cofilin, although the plant and human proteins are only 26% identical. Two short functional sequences have been characterized in vertebrate cofilins (23–25). A 12-amino acid sequence, sufficient to depolymerize F-actin *in vitro*, is conserved throughout phylogeny, while a nuclear localization signal is present only in the vertebrate and *Drosophila* sequences (each is labeled).

overexpression phenotype is that *Drosophila* cofilin can cause actin filaments to aggregate and also distort the yeast nucleus, possibly by accumulating there either alone or in a complex with actin. Since nuclear actin–cofilin aggregates in vertebrates do not stain with phalloidin (23), this interpretation is consistent with the observed staining patterns. Recovery of this cDNA has allowed us to initiate a genetic analysis of cofilin, which, to our knowledge, has not previously been described in *Drosophila*. We have isolated genomic clones spanning the cofilin gene, confirmed the coding sequence (identical to the cDNA except for two small introns), and localized the gene to polytene chromosome position 60AB.

The D17 cDNA induced large multinucleate dumbbell-shaped cells. Unlike the other clones, D17 caused the recruitment of F-actin to a subplasmalemmal cortex (Fig. 1 *e* and *j*). Analysis of the time course of the D17 phenotype suggests that the localization of F-actin to the membrane just precedes the disruption of cell shape. D17 is a partial cDNA that encodes a protein fragment structurally similar to the C-terminal half of vertebrate moesin, ezrin, and radixin, here

Table 1. Summary of pREP/*Drosophila* cDNA clones that induced cell shape and cell division defects in fission yeast

Drosophila cDNA	Highest similarity	Function	Yeast actin phenotype	Size, aa	Polytene location
D88	Profilin	Regulation of actin filament assembly	Diffuse filaments	126	26A (ref. 8)
D61	Cofilin/ADF	(Actin depolymerization, nuclear translocation)	Aggregated filaments	148	60AB*
D17	Moesin/ezrin	(Membrane-cytoskeleton connection)	Cortical filaments	366 (of 580)	8B3-4*
D9	RP S17	General protein synthesis	Normal structures, mislocalized	131	67B (ref. 31)

D88 contains a 1-kb cDNA of a transcript from the constitutive chickadee promoter, slightly truncated in the 5′ untranslated region but containing the entire reported coding region (8). ADF, actin depolymerizing factor. Presumed functions are in parentheses.

*Genomic clones were isolated using D61 and D17 as probes and localized to polytene chromosomes by standard methods (1). GenBank accession numbers are as follows: U08217 for D61 and U08218 for D17.

abbreviated MER (Fig. 3). MER are prominently associated with the plasma membrane, especially in microvilli, filopodia, ruffles, and the cleavage furrow (see references in refs. 26, 28, and 29). A fourth member of this family is merlin, the product of the tumor suppressor gene deleted in neurofibromatosis type 2 patients (27). The family shares a highly conserved N-terminal domain also found in band 4.1 and talin, both of which link integral membrane proteins to the cytoskeleton. This N-terminal domain is suggested to be the site of membrane attachment for both band 4.1 and ezrin (30), leading to speculation that it has a membrane-targeting function throughout this superfamily. In MER, this domain is followed by regions predicted to form an extended α-helix and a C-terminal "knob." In ezrin, this C-terminal half is implicated in actin binding (30). The D17 fragment is missing most of the N-terminal domain but contains the entire helix-knob portion. Thus, while it is not surprising that the D17 fragment can disrupt actin localization, it is surprising that it can apparently target actin to the plasma membrane. The identification of a MER-like protein in a genetically tractable metazoan organism such as *Drosophila* should allow us to assess the function of these domains *in vivo*.

The fourth cDNA is derived from the previously cloned ribosomal protein (RP) S17 gene and contains the entire reported RP S17 coding region (31). It generates an elongated cell division cycle (*cdc*)-like phenotype, distinguishable from the cytoskeletal phenotypes because the induced cells maintain constant diameter, growing only at the ends (Fig. 1 *d* and *i*), branch very rarely, and can form colonies while induced. Remarkably, the distribution of actin was perturbed even in these cells. While the other proteins are likely to affect actin distribution directly, RP S17 is not reported to interact with the cytoskeleton and likely exerts its effects indirectly. Perturbation of the actin cytoskeleton through the overexpression of RP S17 nevertheless suggests that the overall premise of our screen is correct:

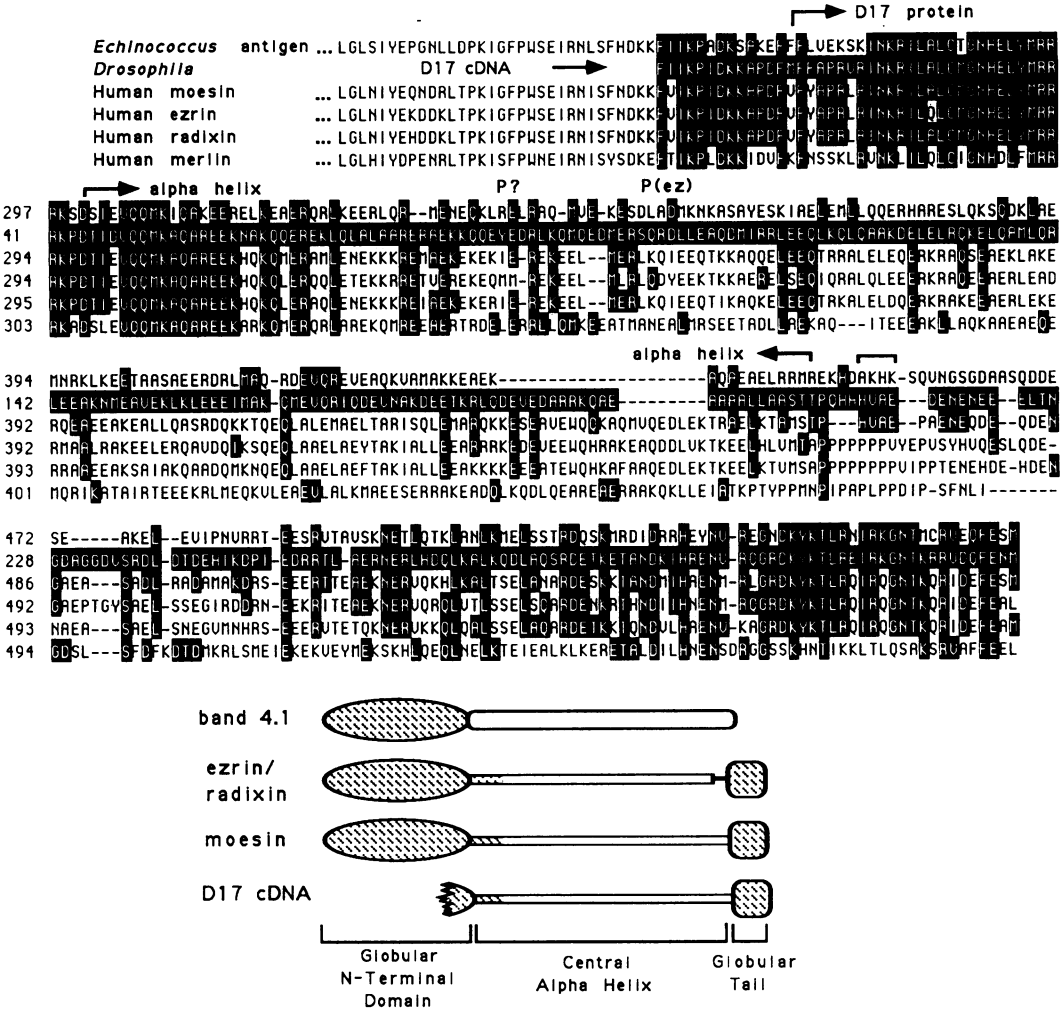

FIG. 3. D17 encodes a *Drosophila* member of the MER family of actin–membrane cross-linking proteins. Amino acid sequence alignment of six protein family members including representative MER family members from humans, the MER protein of the parasitic helminth *Echinococcus*, and the translated D17 cDNA begins at residue 222 of ezrin/moesin (26, 27) is shown at the top. The beginning of the D17-encoded sequence is labeled, and residues identical to it are shown in white type on a black background. The first potential translational start is shown with an arrow labeled D17 protein. Based on this alignment the D17 clone encodes just over 50% of an intact MER-like protein. Compared to human moesin, the first 59 amino acids of D17 are 100% similar and 92% identical, and the C-terminal 66 amino acids are 78% similar and 67% identical. The central predicted α-helical region of D17 (marked by arrows labeled α-helix) is poorly conserved at the sequence level, but its N-terminal half is very basic and its C-terminal half is very acidic, a feature strongly conserved even between merlin and D17, two of the most divergent MER-like proteins. Ezrin is multiply phosphorylated on serines and threonines and can be tyrosine-phosphorylated by the epidermal growth factor receptor; the only major site for epidermal growth factor phosphorylation in ezrin's C-terminal half is indicated as P(ez) (28). While this specific site is unique to ezrin, D17 has a single tyrosine in its helix (indicated by P?) surrounded by an identical arrangement of charged residues and, therefore, potentially phosphorylated. A horizontal bracket indicates a sequence identical between moesin and D17 that substitutes for the proline-rich region found in other MER proteins. The schematic representation of MER/band 4.1 superfamily members is shown at the bottom. The predicted domain structure is indicated and highly conserved regions are hatched.

alterations in cell shape are induced by proteins that can modulate the actin cytoskeleton.

In summary, we report the surprising specificity of a *Drosophila* cDNA screen performed in fission yeast. We recovered three cDNAs that are predicted to encode actin binding proteins that regulate cytoskeletal dynamics in complex organisms. This yield was obtained from ≈10% the complexity of the library, suggesting that a reasonably large number of other cDNAs encoding cytoskeletal proteins can be obtained from the library by this method. In addition to other sequences of similar known proteins, we expect that cDNAs defining previously uncharacterized cytoskeletal protein families can be recovered, though further biochemical characterization would be required to establish their function. The specificity we observed in the screen suggests the phenotypes are caused by evolutionarily conserved interactions of these *Drosophila* proteins with the yeast cytoskeleton. The screen appears to work in two general ways: (*i*) overexpression of a protein with normal function, such as profilin, analogous to a hypermorphic mutation, or (*ii*) a dominant negative effect due to a protein with partial or abnormal function, likely illustrated by the MER-like fragment. As a third possibility, an exogenous protein could be disruptive because it retains full activity but cannot be regulated by the yeast cell. A striking example of this was demonstrated by Superti-Furga *et al.* (32) who expressed chicken c-Src in *S. pombe* and found its tyrosine kinase activity was unregulated and toxic to cells. Interestingly, the terminal morphology of these cells was very similar to that of D17, suggesting that upstream regulatory proteins, in addition to cytoskeletal elements, could be identified and studied using this system (though cDNAs encoding regulatory proteins will likely be less abundant in this library).

We envision this cross-phylogenetic screen, which couples the relative simplicity of yeast genetics to cDNAs isolated from a more complicated organism, as a powerful functional cloning strategy that is generally useful and very flexible. By changing the selection criteria, this method can be used to clone genes of other classes. For example, a variety of additional phenotypes, including those characteristic of conventional *S. pombe cdc* (19), *cut* (33), and other mutations, were also observed. They can be selected and their cDNAs can be analyzed in a comparable fashion. We chose to use *Drosophila* cDNAs to facilitate further genetic analysis of the cell shape determinants that we identified in order to elucidate their roles in metazoan development; however, the striking structural and functional conservation we observed suggests that cDNAs from more complex organisms could be screened successfully.

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