Functional Analysis of a Human Homologue of the Drosophila Actin Binding Protein Anillin Suggests a Role in Cytokinesis

Karen Oegema,* Matthew S. Savoian,§ Timothy J. Mitchison,‡ and Christine M. Field†

*Cell Biology Program, European Molecular Biology Laboratory, Heidelberg, Germany D-69117; ‡Department of Cell Biology, Harvard Medical School, Boston, Massachusetts 02115; §Division of Molecular Medicine, New York State Department of Health, Wadsworth Center, Albany, New York 12201-0509

Abstract. We have characterized a human homologue of anillin, a Drosophila actin binding protein. Like Drosophila anillin, the human protein localizes to the nucleus during interphase, the cortex following nuclear envelope breakdown, and the cleavage furrow during cytokinesis. Anillin also localizes to ectopic cleavage furrows generated between two spindles in fused PtK1 cells. Microinjection of antianillin antibodies slows cleavage, leading to furrow regression and the generation of multinucleate cells. GFP fusions that contain the COOH-terminal 197 amino acids of anillin, which includes a pleckstrin homology (PH) domain, form ectopic cortical foci during interphase. The septin H cdc10 localizes to these ectopic foci, whereas myosin II and actin do not, suggesting that anillin interacts with the septins at the cortex. Robust cleavage furrow localization requires both this COOH-terminal domain and additional NH2-terminal sequences corresponding to an actin binding domain defined by in vitro cosedimentation assays. Endogenous anillin and H cdc10 colocalize to punctate foci associated with actin cables throughout mitosis and the accumulation of both proteins at the cell equator requires filamentous actin. These results indicate that anillin is a conserved cleavage furrow component important for cytokinesis. Interactions with at least two other furrow proteins, actin and the septins, likely contribute to anillin function.

Key words: H cdc10 • pleckstrin homology domain • septin • cleavage furrow • cortex

Introduction

Cytokinesis is the process that partitions the cell surface and cytoplasm of one cell to form two cells (for reviews see Glotzer, 1997; Gould and Simanis, 1997; Field et al., 1999; Hales et al., 1999). To avoid aneuploidy, the cell must successfully coordinate cytokinesis with chromosome segregation. In animal cells, this coordination is achieved, in part, by assembling the cleavage furrow in response to signals from the late anaphase spindle. The exact nature of this signaling remains poorly understood.

In animal cells, the cytoplasm is partitioned by ingress of the cleavage furrow. Cleavage furrow ingress requires a contractile cortical ring of actin and myosin II (for review see Satterwhite and Pollard, 1992; for some more recent examples of the role of myosin II during cytokinesis see Bi et al., 1998, and Lippincott and Li, 1998 [Saccharomyces cerevisiae]; Shelton et al., 1999 [Caenorhabditis elegans]; and Benzanilla et al., 2000 [Schizosaccharomyces pombe]). Based on EM studies, filamentous actin and myosin II within the cleavage furrow have been proposed to have a purse string-like organization (for review see Satterwhite and Pollard, 1992). However, more recent work in tissue culture cells and Dictyostelium have called this organization into question (Fukui and Inoue, 1993; Fishkind and Wang, 1993; Fishkind et al., 1996; for review see Fishkind and Wang, 1995). Further work is necessary to clarify the organization of actin and myosin II in different systems, and to relate this organization to the contractile forces generated during cytokinesis. The mechanisms that lead to furrow assembly and that allow the contractile ring to remain associated with the plasma membrane during contraction are also topics of current investigation.

In addition to cortical contraction, cleavage furrow ingress requires new membrane insertion. A role for syntaxin-mediated membrane fusion has been demonstrated during cytokinesis in Arabidopsis (Lauber et al., 1997) and C. elegans (Jantsch-Plunger and Glotzer, 1999), and, during cellularization, a cytokinesis-related furrowing process in Drosophila embryos (Burgess et al., 1997). Nevertheless, for furrowing events in most animal sys-
tens, it is not known exactly where and when this membrane insertion occurs and to what extent membrane insertion is coupled temporally and spatially to furrow ingression. In principle, insertion of a new plasma membrane could occur anywhere on the surface of the cell. Membrane insertion could also occur before contraction and be stored in structures such as microvilli until additional surface area is required (Fullilove and Jacobson, 1971; Turner and M ahowald, 1977). The process of membrane insertion during cleavage has been studied most extensively in Xenopus embryos (Bluemink and de Laat, 1973; D anilchik et al., 1998). Here, membrane insertion is coupled to furrow ingression and occurs immediately behind the furrow tip. Membrane insertion in this system requires a specialized MT array, disruption of which can prevent progression. In principle, insertion of a new plasma membrane could occur anywhere on the surface of the cell, causing the embryo to rupture (D anilchik et al., 1998). However, the Xenopus embryo is an extremely large and specialized cell, so it is not clear to what extent mechanisms of membrane deposition will be similar in other systems such as vertebrate somatic cells.

One approach to understanding furrow assembly and ingression is to identify the protein components of the cleavage furrow and the interactions between them, and then study the regulation of these interactions in space and time. A number of cleavage furrow components have now been discovered, and some are evolutionarily conserved. Conserved cleavage furrow components include the following: actin, myosin II, formin homology proteins and IQGAP family members (thought to regulate actin dynamics), and the septin family of small GTPases (for review see Field et al., 1999). The septins are found in large protein complexes containing multiple septin family members that can polymerize to form filaments in vitro (for review see Field and Kellogg, 1999).

Another cleavage furrow component that is likely to function during cytokinesis is anillin. Anillin is a Drosophila actin binding protein identified by actin affinity chromatography of embryo extracts. Unlike the septins, actin, and myosin II, anillin specifically localizes to the contractile ring during cytokinesis, and is not found in other contractile actin-rich assemblies such as the stress fibers of cultured cells (Cramer et al., 1997; Kinoshita et al., 1997) or the apical portions of constricting epithelial fibers of cultured cells (Cramer et al., 1997; Kinoshita et al., 1998). The process of mem-}

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### Materials and Methods

#### Buffers

| Buffer     | Composition                        |
|------------|------------------------------------|
| CBS        | 10 mM MES, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, and 320 mM sucrose. |
| TBS        | 0.15 M NaCl, and 20 mM Tris-CI, pH 7.4. |
| TBST       | TBS + 0.1% Triton X-100. A AbDil: TBST, 2% BSA, and 0.1% sodium azide. Mouting media: 0.5% p-phenylenediamine, 20 mM Tris-CI, pH 8.8, and 90 or 10% glycerol as indicated. Sample buffer: 0.25 M Tris-CI, pH 6.8, 3% SDS w/vol, 5% β-ME vol/vol, and 10% glycerol vol/vol. Transfer buffer: 20 mM Tris base, 236 mM glycine, and 20% methanol. |

#### Cloning and Sequencing of a Human Anillin cDNA

A BLAST search of the GenBank database of expressed sequence tags (dbEST) revealed a human cDNA (accession No. R12261) with homology to the COOH terminus of Drosophila anillin. PCR primers based on this sequence (AGCGAAGATACCCATAGG and GACACGCGATTCTCTGGTAAAC) were used to amplify a 193-bp region, using human Hela cell quick-clone cDNA (CLONTECH Laboratories, Inc.) as a template. Using this probe, screening of a human Zap X Library (Hela cDNA; Stratagene) yielded three related clones, the longest of which (4,016 bp) was sequenced. A number of additional 595 bp of coding sequence and an upstream, in-frame stop codon were identified using a 5′/3′ RACE kit (Boehringer) and mRNA isolated from Hela cells using a Quickprep mRNA purification kit (Amersham Pharmacia Biotech) to amplify the 5′ end of human anillin.

#### GFP Constructs

To generate a fusion of green fluorescent protein (GFP) with amino acids 231-1,125 of human anillin (hsanillin), the KpnI-Apal fragment from the hsanillin cDNA was ligated into pEGFP-C1 (CLONTECH Laboratories, Inc.). To make (GFP: 231-982), this construct was digested with EcoR1, and the resulting fragment was ligated into pEGFP-C1 cut with EcoR1. To make the full-length construct, GFP: 3-1,125, nested PCR using the Pfu polymerase (Stratagene) and a human cDNA library (CLONTECH Laboratories, Inc.) as a template was used to amplify the NH₂ terminus of hsanillin. The internal primers were 5′-CGCAAGAAGCTTTGAGTAGATCCTGTTACGG-3′ and 5′-AGACGCTCATCAAGCACCCTC-3′. This product was digested with HindII-BstXI and cloned into the GFP: 231-1,125-containing vector. To make GFP: 1-982, the HindII-BstXI PCR fragment was ligated into GFP: 231-982 cut with the same enzymes. To make all other GFP fusions, PCR with Vent polymerase (New England Biolabs) was used to amplify the appropriate regions. All fragments were digested with HindII-BamHI and ligated into pEGFP-C1.

#### Cell Culture and Transfection

BHK-21 cells were cultured and transfected as described (Heald et al., 1993). BS-C-1 (African green monkey kidney) cells were cultured in DMEM with high glucose. For the blot in Fig. 4, BHK cells were transfected in 6-well plates. The cells were washed twice with PBS and resuspended in 150 μl of sample buffer. 25 μl of each sample was fractionated on a 12% SDS-PAGE gel and Western blotted using an anti-GFP antibody (gift from Aaron Straight, Harvard Medical School, Boston, MA.).

#### Antibody Purification and Labeling

Rabbit antibodies to human anillin were raised against a fusion of GST with amino acids 454-724 of human anillin. To purify the antibodies, the fusion protein was cleaved with Precision protease (A mersham Pharmacia Biotech) to remove the GST and immobilized on a figel-10 (Bio-Rad Laboratories). The antibodies were purified using standard procedures (Harlow and Lane, 1988). A synthetic peptide corresponding to the COOH-terminal 14 amino acids (SRKLEKNKKKGKIF) of Hdc10 (Nakatsuru et al., 1994) was used to raise and affinity purify rabbit polyclonal antibodies as previously described (Field et al., 1998). Labeling of antibodies to Hdc10 and hsaanillin with Oregon green (Molecular Probes) and Cy5 (A mersham Pharmacia Biotech) was performed as previously described (Francis-Lang et al., 1999).

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Abbreviations used in this paper: GFP, green fluorescent protein; Hsaanillin, human anillin; PH, pleckstrin homology.
Fixation, Immunofluorescence, and Microscopy

For the anillin immunofluorescence in Fig. 1, BHK cells were fixed for 5 min with 20 methanol and processed using standard procedures (http://iccbweb.med.harvard.edu/mitchisonlab/). A n-talin anillin antibodies were used at a concentration of 1 μg/ml. For the visualization of Hcdc10, actin and anillin in Fig. 7, BHK cells, grown on coverslips, were fixed for 15 min in 2% formaldehyde in CBS, washed three times with TBS, permeabilized in TBS + 0.5% Triton X-100 for 10 min, rinsed twice with TBS, and blocked in AdbII for 10 min. The coverslips were incubated with directly labeled Cy-5 antianillin and Oregon green anti-Hcdc10 (both at 2 μg/ml) for 40 min, washed three times with TBS, incubated with 0.05 mg/ml TRITC phalloidin (Sigma Chemical Co.) diluted in AdbII for 15 min, washed twice with TBS, and incubated with 1 μg/ml Hoechst in TBS for 5 min. The coverslips were washed twice in TBS and mounted in mounting media (see Buffers) containing 70% glycerol. BHK cells transfected with GFP constructs (see Figs. 5 and 6) were fixed for 10 min with 2% formaldehyde in CBS. The coverslips were washed, permeabilized, and blocked as described above. To stain for Hcdc10, the cells were incubated with 2 μg/ml of directly labeled Cy5 anti-Hcdc10. Three-dimensional images were acquired on a widefield microscope (DeLight; Apllied Precision Inc.). Optical sections were taken at 0.2-μm intervals using an Olympus 100×, 1.4 NA objective and deconvolved using the DeLight software. Either single optical sections or projections of the entire cell are shown, as indicated.

Antibody Injection Experiments

The day before injection, BS-C-1 cells were plated onto 25-mm coverslips. For injection and viewing, coverslips were mounted in a water-jacketed coverslip chamber to maintain a temperature of 34°C. The chamber was placed on the stage of an inverted microscope and injected and viewed by phase optics. The estimated injection volume is 5% of cell volume. Two different concentrations of affinity-purified anti-anillin antibody were injected with similar results (3.4 and 11 mg/ml). Random rabbit IgG (Jackson ImmunoResearch Laboratories) at a concentration of 11 mg/ml was injected as a control. For the overnight experiments, interphase cells were injected, and the coverslip was returned to the incubator for 18–22 h. Cells were fixed and mounted for microscopy as described above. Anti-Hcdc10 antibody was also injected and viewed by phase-contrast microscopy. For time-lapse filming, single cells were injected at prophase or metaphase and filming began immediately. Images were collected at 20-s intervals. A cell was deemed to have completed cytokinesis when the cell was no longer possible to make meaningful measurements that anillin is conserved among metazoans and that at least one additional conserved globular domain. The NH2 termini of the six proteins are less conserved. Pairwise alignments (Tatusova and Madden, 1999) indicate that the human and Drosophila sequences are homologous among their entire lengths, although the level of conservation is significantly higher in the COOH-terminal third of the protein (Fig. 1 B). The COOH-terminal PH domain is the most conserved part of the protein (54% identity between human and Drosophila). A isoconserved between the human and Drosophila sequences are several consensus nuclear localization sequences (NLSSs; Robbins et al., 1991; Hicks and Raikhel, 1995) and one consensus SH3 binding motif (Ren et al., 1993; Lim et al., 1994). In contrast, pairwise alignments reveal no significant homology between the NH2 termini of the predicted Drosophila protein CG4530 or the C. elegans protein Y43F8C.14 and the human and Drosophila NH2-terminal sequences. An intermediate level of NH2-terminal homology is detected between the C. elegans proteins, Y 49F 10.19 and K 108 2.5, and the human and Drosophila proteins. These results indicate that anillin is conserved among metazoans and that at least some organisms may have more than one protein with homology to anillin. Whether these homologous proteins are functionally redundant or involved in disparate processes remains to be determined.

To further characterize the human anillin homologue, we raised and affinity-purified an antibody to amino acids 454–724 of the human protein. Using this antibody, Western blots of extracts from multiple mammalian cell lines revealed a single band migrating at ~180 kD (Fig. 1 C), which is a molecular mass significantly larger than the predicted molecular mass of 124 kD. A similar difference between the apparent molecular mass and predicted molecular mass was observed for Drosophila anillin (see Discussion in Field and Alberts, 1995). The localization of the human protein is essentially the same as that of Drosophila anillin (Field and Alberts, 1995). In mammalian tissue culture cells (Fig. 1 D), anillin concentrates in the nucleus during interphase, although some anillin also appears punctate in the cytoplasm and at the cortex. During mitosis, anillin is enriched at the cortex. Anillin accumulates in the cleavage furrow during cytokinesis and prominently stains the cortex surrounding the midbody late in telophase.

The only known molecular interaction of Drosophila anillin is to bind to actin filaments in vitro. In the Drosophila protein, the actin binding domain has been mapped to the NH2 terminus (Fig. 1 B). To test if the human protein could also bind actin, fragments of human anillin were expressed and purified from bacteria, and an actin cosedimentation experiment was performed. We found that fragments corresponding to amino acids 1–677 and 658–1223 of human anillin bound to actin filaments. More COOH-terminal fragments corresponding to amino acids 454–724 and 983–1,125 of human anillin did not bind actin (data not shown). These results suggest that the minimal actin binding domain for the human protein likely lies between amino acids 231 and 454, similar to its location in the Dro-
The actin binding of fragment 231–677 was less efficient than that of 1–677, suggesting that, as is the case in Drosophila, additional NH$_2$-terminal sequences may influence actin binding.

Injection of Antibodies to Anillin Slows Furrow Ingression and Leads to Failure of Cytokinesis

To determine if anillin is important for cytokinesis, we injected affinity-purified antibody against human anillin into BHK cells that were stained for hsanillin. Three-dimensional widefield data was collected and deconvolved. The interphase cell is a projection of the entire stack; the other images are selected sections. In interphase cells, hsanillin is found primarily in the nucleus, upon nuclear envelope breakdown, hsanillin becomes enriched at the cortex. During cytokinesis, the protein localizes to the cleavage furrow. In late telophase, anillin is concentrated in the cortex surrounding the midbody.

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monkey cells (BS-C-1). Random rabbit IgG at the same concentration was injected as a control. After injection, the cells were cultured for 18–22 h, fixed, and processed for immunofluorescence (see Materials and Methods). The number of nuclei per cell was counted. 89% (54/61) of the cells injected with the antianillin antibody had two or more nuclei compared with 7% (3/46) of injected control cells. The multinucleate cells resulting from the antianillin injection were well spread and contained actin stress fibers (data not shown). These data suggest a role for anillin in cytokinesis but not cell spreading or cell cycle progression.

A failure of cytokinesis can arise from a failure in furrow assembly, ingression (contraction or perhaps membrane insertion), or from a failure of completion, which is the final step that resolves the contracted cortex to generate two topologically distinct cells. To quantitatively analyze cytokinesis in the antibody-injected cells, cells were injected with antianillin antibody in prophase or metaphase (before formation of the cleavage furrow) and time-lapse–imaged using phase-contrast optics. The width of the furrow near the cell equator was measured and plotted versus time. In all cells injected with anillin antibody, the cleavage furrow formed and began to contract. The timing of furrow initiation was not significantly different between experimental (average 204 ± 31.7 s) and uninjected control cells (average 225 ± 80 s). However, the rate of furrow contraction of the antibody-injected cells was significantly slower (P < 0.001; Fig. 2 C) than that of control cells. The rate of furrow contraction of the antibody-injected cells (0.94 ± 0.28 μm/min) was about half that of the control cells (2.09 ± 0.63 μm/min). In 6/9 cells injected with the antianillin antibody, furrow ingression ultimately halted and reversed (Fig. 2). In the three cells where the furrow persisted, ingression eventually stalled and did not reinitiate in the time frame of the experiment (36, 48, and 54 min). Control cells took an average of 9 min to complete cytokinesis. Cumulatively, these results suggest that anillin is important for cytokinesis and may have a role in the assembly, stability, or contractile activity of the cleavage furrow.

**Anillin Localizes to Ectopic Rappaport Furrows in Fused PTK1 Cells**

Recently, a system using fused PTK1 cells was developed to help assess the minimum protein complement necessary to accomplish cytokinesis (Savoian et al., 1999). In fused cells, two spindles exist within one cytoplasm. During cyto-
tokinesis, three cleavage furrows form: two normal furrows that bisect the spindles, and an ectopic furrow which forms between centrosome pairs not previously connected by a spindle. This type of ectopic furrow was previously characterized in echinoderms by Rappaport (1961). Since both types of furrows are functional, components essential for cleavage should be present in both. In contrast, components that fortuitously accumulate in the cleavage furrow may be found only in spindle-bisecting furrows. We examined the localization of anillin in this system and found that anillin localized to all functional furrows, both spindle bisecting and ectopic, which is consistent with the idea that anillin is a ubiquitous component of cleavage furrows (Fig. 3).

**Robust Recruitment of Anillin to the Cleavage Furrow Requires both the PH and Actin Binding Domains**

To examine the domains of anillin required for its localization, we transfected BHK cells with a series of constructs that fuse GFP to regions of anillin (summarized in Fig. 4). Fusions of GFP with full-length anillin and anillin lacking the NH$_2$-terminal 230 amino acids were recruited to the cortex normally during metaphase (Fig. 5, top panel, amino acids 1–1,125 and 231–1,125), and localized robustly to cleavage furrows during cytokinesis (Fig. 5, lower panel, amino acids 1–1,125 and 231–1,125). The localization of these two constructs was identical, except that the fusion with amino acids 231–1,125 failed to localize to nuclei during interphase (see summary in Fig. 4). This suggests that the functionally important NLS may be one of the consensus sequences found in the first 230 amino acids (Fig. 1 B). Fusions with amino acids 1–982 or 231–982 that contain the actin binding domain but delete the PH domain were not recruited to the cortex during metaphase (Fig. 5, top panel, amino acids 1–982) and were only very weakly enriched in the cleavage furrow during cytokinesis (Fig. 5, bottom panel amino acids 231–982). Both of these fusions localized to the midbody late in cytokinesis (data not shown). Interestingly, a fusion of GFP with the COOH-terminal 395 amino acids of anillin, which lacks the actin binding domain, showed no cortical enrichment during metaphase (Fig. 5, top panel, amino acids 1–982) and were only very weakly enriched in the cleavage furrow during cytokinesis (Fig. 5, bottom panel amino acids 231–982). Both of these fusions localized to the midbody late in cytokinesis (data not shown). Interestingly, a fusion of GFP with the COOH-terminal 395 amino acids of anillin, which lacks the actin binding domain, showed no cortical enrichment during metaphase (Fig. 5, top panel, amino acids 1–982) and were only very weakly enriched in the cleavage furrow during cytokinesis (Fig. 5, bottom panel amino acids 231–982). Both of these fusions localized to the midbody late in cytokinesis (data not shown). Interestingly, a fusion of GFP with the COOH-terminal 395 amino acids of anillin, which lacks the actin binding domain, showed no cortical enrichment during metaphase (Fig. 5, top panel, amino acids 1–982) and were only very weakly enriched in the cleavage furrow during cytokinesis (Fig. 5, bottom panel amino acids 231–982). Both of these fusions localized to the midbody late in cytokinesis (data not shown). Interestingly, a fusion of GFP with the COOH-terminal 395 amino acids of anillin, which lacks the actin binding domain, showed no cortical enrichment during metaphase (Fig. 5, top panel, amino acids 1–982) and were only very weakly enriched in the cleavage furrow during cytokinesis.
localization can occur independently of the actin binding domain.

**GFP Fusions Containing the Extended PH Domain Form Ectopic Foci that Contain Hcdc10**

During interphase, anillin is primarily a nuclear protein. However, we found that all constructs that contained the COOH-terminal 197 amino acids of anillin (Fig. 4, amino acids 929–1,125) could form ectopic cortical foci when overexpressed during interphase (Fig. 6). This region of anillin, which we refer to as the extended PH domain, corresponds to the PH domain plus an additional 56 amino acids more NH2-terminal. Deletion of the PH domain abolished the ability to form foci, however, the PH domain itself is not sufficient to confer the ability to form these structures (data not shown). We examined the ectopic foci using probes for actin, myosin II, endogenous anillin (us-
Anillin and Hcdc10 Colocalize to Foci Associated with Actin Cables and Are Simultaneously Recruited to the Cleavage Furrow

The GFP fusion data suggested a possible association between anillin and the septins at the cortex. To test if this association could be observed in untransfected cells, we stained BHK cells for actin, Hcdc10, and anillin (Fig. 7) and performed three-dimensional widefield deconvolution microscopy. We found that Hcdc10 and anillin colocalized to punctate cortical structures throughout mitosis. These puncta were smaller than the ectopic cortical foci formed in cells overexpressing GFP-anillin fusions. Nearly all punctate structures that contained anillin also contained Hcdc10. However, some Hcdc10-containing puncta, particularly those outside the cleavage furrow and near the periphery of the cells did not contain anillin (Fig 7; Late anaphase and Telophase). Interestingly, we found that the majority of septin and anillin puncta were aligned along actin cables throughout mitosis. We also observed punctate foci of myosin II aligned along actin cables during furrow assembly (data not shown); but, at our current resolution, myosin II appeared to be less coincident with anillin than Hcdc10. During metaphase and early anaphase, the actin cables appeared to encircle the cells (Fig. 7, Metaphase and Early anaphase). In late anaphase, as the sides of the cells began to flatten to give the cell a capsule-like shape, the cortical actin cables in the cleavage furrow were pri-
Figure 7. Anillin and Hcdc10 are found in punctate foci that localize along actin cables during mitosis in BHK cells. BHK cells were fixed and stained for actin, Hcdc10, anillin, and DNA. Three-dimensional widefield data sets were collected and deconvolved. Shown are projections of entire cells. During metaphase and early anaphase, actin is found in cables that often appear to encircle the cell. During late anaphase, as the cell initiates contraction, the actin cables appear to align parallel to the spindle axis. As contraction proceeds, more actin cables within the cleavage furrow are observed perpendicular to the spindle axis but, within the actively contracting region near the base of the cell, the actin cables are still parallel to the spindle axis. During all phases of mitosis, anillin and Hcdc10 colocalize to foci that align along actin cables.
marily aligned along the spindle axis. These axially aligned actin filaments appeared to be enriched on the sides of the cell (Fig. 7, Late anaphase), although we also observed them on the top surfaces of some cleavage furrows. Later in telophase, actin cables perpendicular to the spindle axis were also observed. However, these cables were generally enriched to either side of the center of the cleavage furrow and often appeared to encircle the daughter cells rather than to form a circumferential ring at the equator (Fig. 7, Telophase).

We also examined the relative timing of recruitment of anillin and Hcdc10 to the cortex and to the cleavage furrow (a sample field of cells is shown in Fig. 8 A). During interphase, when anillin was primarily nuclear, the septin Hcdc10 was already found in punctate cortical foci. We should note that the nuclear localization of anillin is more prominent in methanol-fixed cells (Fig. 1 D) than in cells fixed with formaldehyde (Fig. 8 A). In metaphase cells, anillin and Hcdc10 colocalize to punctate cortical foci. In anaphase and telophase cells, foci containing anillin and Hcdc10 are found in the cleavage furrow. The timing of cleavage furrow recruitment was identical for the two proteins. Whenever we detected a concentration of one component near the equator, the other was always present. The extent of the region of the cortex where the proteins were enriched was identical throughout furrow assembly and ingestion. The timing of furrow recruitment and the extent of the region of enrichment was also identical for myosin II (data not shown).

**Anillin and Septins Localize to the Cortex in the Absence of Actin**

The localization of anillin and the septin Hcdc10 along actin cables between metaphase and telophase suggested that filamentous actin might be important for their concentration in the furrow. To test if actin filaments are required for anillin and septin localization during cytokinesis, we treated cells with the actin depolymerizing drugs latrunculin A or latrunculin B, fixed, stained, and imaged cells in late mitosis. As expected (Spector et al., 1989), no cleavage furrow was formed in the presence of either drug, and filamentous actin was reduced in amount and disorganized in location (Fig. 8 B). In the absence of a furrow, it is difficult to distinguish anaphase and telophase, but anillin was not localized to the equator in any postanaphase cells we observed after latrunculin A treatment. However, both proteins were still colocalized in foci and largely restricted to the cortex. We conclude that anillin-septin colocalization and maintenance of their association with the cortex do not depend on organized, filamentous actin, whereas their localization to the presumptive cleavage furrow does.

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**Figure 8.** A nillin and the septin Hcdc10 colocalize on the cortex of latrunculin-treated cells but do not accumulate in the cleavage furrow. (A) A typical field of BHK cells fixed and stained for DNA (top), anillin (bottom, red), and Hcdc10 (bottom, green). Three-dimensional widefield data sets were collected and deconvolved. A projection of the field is shown. During interphase (I) Hcdc10 is present in punctate cortical structures on the cortex. Anillin is in the nuclei of most, but not all, interphase cells. Note that the nuclear staining is somewhat weaker in formaldehyde-fixed cells compared with the methanol-fixed cells shown in Fig. 1. At metaphase (M), anillin concentrates at the cortex where it colocalizes with septin foci. At telophase (T), foci containing anillin and Hcdc10 are concentrated in the cleavage furrow. (B) BHK cells grown on coverslips were treated with 2 μg/ml latrunculin A (a gift from Miranda Sanders and Phil Crews, University of California, Santa Cruz) or latrunculin B (Calbiochem) in normal culture media for 60 min before processing for immunofluorescence. In the left panel, a merge of DNA (red) and actin (green) is shown. In the right panels, a merge of anillin (red) and the septin Hcdc10 (green) is shown. Images were collected as described for Fig. 7 and are projections of entire cells. When the actin is depolymerized by latrunculin treatment, neither Hcdc10 nor anillin are recruited to the cell equator during mitosis; instead, they colocalize to large cortical spots.
Discussion

Anillin Is Conserved among Metazoans

Human and Drosophila anillin share several conserved features including the presence of consensus nuclear localization and SH3 binding sequences, and a COOH-terminal PH domain. The existence of an anillin homologue in Xenopus laevis (Straight, A., personal communication) and of multiple anillin homologues in C. elegans (this report) indicates conservation of anillin among metazoans. Although S. cerevisiae does not appear to have an anillin homologue, the Mid-1 protein, which is required for septation in the fission yeast S. pombe, is similar to anillin in localization (nucleus and cleavage furrow) and also contains a COOH-terminal PH domain. However, there is no other obvious sequence homology (Bähler et al., 1998).

Anillin Function Is Important for Cytokinesis

Injection of antibodies to anillin into mammalian tissue culture cells slows the rate of furrow insertion and prevents the completion of cytokinesis. To more precisely define the pathway in which it acts, it is useful to consider the subprocesses that together comprise cytokinesis (for reviews see Saunders and Field, 1994; Glotzer, 1997; Field et al., 1999). Initiation, a signaling process that determines where and when the furrow assembles (for reviews see Rappaport, 1996; Oegema and Mitchison, 1997), is thought to depend on components positioned by the late anaphase spindle microtubules, such as kinesins, kinesins, and perhaps chromosomal passenger proteins. Furrow assembly depends on cortical components and the actin cytoskeleton. Furrow formation can be subdivided into two subprocesses, furrow contraction, which depends on actin and myosin II (for review see Satterwhite and Pollard, 1992) and membrane insertion, which has been shown in a number of systems to require vesicle targeting and exocytic mechanisms (Bluemink and de Laat, 1973; Burgess et al., 1997; Lauber et al., 1997; Danilchik et al., 1998; Jantsch-Plunger and Glotzer, 1999). The relative importance of contraction and membrane insertion during furrow progression is likely to depend on the system, and has not yet been assessed for vertebrate somatic cells. Finally completion, the process by which the surface area of the cell is finally partitioned, is thought to require midbody microtubules and membrane fusion events (for review see Field et al., 1999). Our localization and antibody injection data suggest that anillin functions during furrow assembly or ingestion, and possibly in completion, but not in initiation. Within ingestion, we cannot yet determine whether anillin is required primarily for furrow contraction or membrane insertion. The close association of anillin with actin cables in the furrow suggests a role in furrow assembly or contraction. However, the close association of anillin with septins, which have in turn been implicated in exocytotic regulation (Hsu et al., 1998; Beites et al., 1999), suggests that a role in membrane insertion cannot be ruled out.

Functional Domains of Anillin

The Conserved COOH Terminus. Both sequence conservation and localization of GFP fusions suggest a critical role for the COOH-terminal third of anillin (amino acids 731-1,125) that contains the PH domain. When fused to GFP, this domain is sufficient to confer localization to ectopic cortical foci containing the septin Hcdc10 in interphase cells and clear, although relatively weak, localization to the cleavage furrow during cytokinesis. The COOH-terminal PH domain and an additional 56 amino acids NH2 terminal (amino acids 929-1,125, the extended PH domain) is sufficient to mediate a tight colocalization between anillin and the septins at the cortex during interphase. The full-length endogenous anillin also colocalizes with septins in the cortical foci during mitosis. This colocalization could be explained by a direct physical interaction between the extended PH domain and the septin complex or by contacting to some cortical structure without a direct interaction. So far, we have not seen a direct anillin–septin interaction in coimunoprecipitation experiments. However, these experiments were performed on soluble, as opposed to membrane-bound, protein pools, and the GTP binding status of the septin complex was not manipulated (Oegema, K., and C. Field, unpublished results).

The PH Domain. The COOH-terminal anillin PH domain is necessary for all targeting events, including the formation of ectopic septin-containing foci in cells overexpressing anillin GFP fusions, but is not on its own sufficient to confer any specific localization. PH domains are protein modules of ~120 amino acids thought to mediate recruitment of proteins to the plasma membrane through interactions with lipids (Blomberg et al., 1999). Structural studies indicate that PH domains are electrostatically polarized with a positively charged face that has been postulated to confer the affinity and specificity for different lipids (Leonard et al., 1996; Rameh et al., 1997). The anillin PH domain is closest in charge to the PH domains of spectrin and dynamin that bind phosphoinositides relatively weakly and nonspecifically. These low affinity PH domains are thought to confer membrane targeting upon oligomerization (Klein et al., 1998) or in conjunction with other domains of the protein (Leonard et al., 1996). In addition to lipid binding by the anillin PH domain, an interaction with the septins may also be required to target anillin to the cortex. During interphase in BHK cells, the septin Hcdc10 is present on the cortex in the absence of anillin (Fig. 8 A). A nillin may localize to the cortex by targeting to septin-containing cortical structures when released from the nucleus upon entry into mitosis. Cortical targeting of septins may occur by direct association with membranes, as suggested by recent data that the mammalian septin H5 binds specifically to phosphatidylinositol polyphosphate (Zhang et al., 1999).

The NH2-terminal Actin-binding Domain. A nillin also has a conserved NH2-terminal actin-binding domain required for both robust cleavage furrow localization and cortical localization at metaphase. Careful colocalization shows that endogenous anillin/septin foci colocalize with actin cables throughout mitosis. During cytokinesis, actin cables tend to align along the spindle axis, rather than circumferentially as a simple purse string model might predict. The limitations of the purse string model, and possible implications of noncircumferential actin cables have been discussed elsewhere (Fukui and Inoue, 1991; Fishkind and Wang, 1995). The fact that anillin can bind directly to actin
in vitro makes it an attractive candidate to mediate the alignment of anillin/septin-containing foci along actin cables. However, the possibility that the colocalization of anillin/septin foci along actin cables is mediated by interactions between actin and the septins cannot be ruled out (K Inoshita et al., 1997; Glotzer, 1997). Treatment of cells with actin-depolymerizing drugs abolished the accumulation of anillin and H cdc10 in the cleavage furrow. However, anillin and the septins remain associated with the cortex in the latrunculin-treated cells, suggesting that maintenance of their cortical localization does not require actin. Experiments examining the kinetics of recruitment will be required to determine if targeting of anillin and H cdc10 to the cortex can occur normally in the absence of actin.

Cumulatively, our results suggest that anillin may function together with the septins, actin, and myosin II during the assembly and ingestion of the cleavage furrow. What might anillin be doing during cytokinesis? An anillin is an actin-binding protein, and fragments of Drosophila anillin also bundle actin. Therefore, anillin could have a role in the formation or organization of actin cables in the cleavage furrow or in the dynamics of actin organization during cleavage. A natterative model is that anillin might mediate interactions between actin and the septin/membrane cytoskeleton, perhaps linking the cleavage furrow to the plasma membrane. These interactions could be important for cleavage furrow assembly or for maintaining structural integrity and membrane attachment of the cleavage furrow during contraction.

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