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cyk-1: a *C. elegans* FH gene required for a late step in embryonic cytokinesis

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SUMMARY

A maternally expressed *Caenorhabditis elegans* gene called *cyk-1* is required for polar body extrusion during meiosis and for a late step in cytokinesis during embryonic mitosis. Other microfilament- and microtubule-dependent processes appear normal in *cyk-1* mutant embryos, indicating that *cyk-1* regulates a specific subset of cytoskeletal functions. Because cytokinesis initiates normally and cleavage furrows ingress extensively in *cyk-1* mutant embryos, we propose that the wild-type *cyk-1* gene is required for a late step in cytokinesis. Cleavage furrows regress after completion of mitosis in *cyk-1* mutants, leaving multiple nuclei in a single cell. Positional cloning and sequence analysis of the *cyk-1* gene reveal that it encodes an FH protein, a newly defined family of proteins that appear to interact with the cytoskeleton during cytokinesis and in the regulation of cell polarity. Consistent with *cyk-1* function being required for a late step in embryonic cytokinesis, we show that the CYK-1 protein co-localizes with actin microfilaments as a ring at the leading edge of the cleavage furrow, but only after extensive furrow ingression. We discuss our findings in the context of other studies suggesting that FH genes in yeast and insects function early in cytokinesis to assemble a cleavage furrow.

Key words: Cytokinesis, FH gene, Microfilaments, Microtubules, Embryogenesis, *Caenorhabditis elegans*

INTRODUCTION

Cytokinesis in animal cells involves a circumferential activation of the cortical actin-myosin cytoskeleton to form an ingressing furrow in the plasma membrane. The cleavage furrow ultimately bisects the mitotic spindle and resolves to yield two separate daughter cells, in a process that appears to involve a sequence of discrete steps (for a review see Glotzer, 1997). These steps include positioning of a cleavage furrow, assembly of an actin and myosin contractile ring, invagination of the plasma membrane, and finally a termination step that partitions the two daughter nuclei into separate cells. Although several genes required for cytokinesis have been identified (see below), evidence that their corresponding gene products mediate a sequence of discrete steps remains minimal.

Genetic studies in several organisms show that members of the recently defined FH family of genes are required for cytokinesis, the regulation of cell polarity, or both (Frazier and Field, 1997; Wasserman, 1998). The FH family is named for two 'formin homology' regions, FH1 and FH2, which are conserved in sequence and, to some extent position, in all family members (see below). FH genes include *fus1* and *cdc12* in *Schizosaccharomyces pombe* (Petersen et al., 1995; Chang et al., 1997), *BNI1* and *BNR1* in *Saccharomyces cerevisiae* (Evangelista et al., 1997; Imamura et al., 1997), *sepA* in *Aspergillus* (Harris et al., 1997), *diaphanous* and *cappuccino* in *Drosophila melanogaster* (Castrillon et al., 1994; Emmons et al., 1995), the *limb deformity* (*ld*) locus and *mDia* in *Mus musculus* (Woychik et al., 1990; Watanabe et al., 1997) and the human deafness gene *DFNA1* (Lynch et al., 1997).

FH proteins are predicted to range in size from about 100 to 200 kDa. In addition to the shared FH1 and FH2 regions, FH proteins exhibit lower levels of sequence similarity throughout their length, with the C-terminal region showing the highest sequence conservation (Frazier and Field, 1997; Wasserman, 1998). The FH1 and FH2 sequences are separated by about 100 amino acids and are roughly centered in the polypeptide or displaced towards the C terminus. In most FH proteins, the FH1 and FH2 regions are flanked by putative coiled-coil domains.

Yeast two-hybrid, GST pull-down and co-immunoprecipitation studies indicate that Bni1p and Bnr1p in *S. cerevisiae*, Cappuccino in *D. melanogaster*, Cdc12p in *S. pombe* and mDia protein in *M. musculus* all interact with profilins through their FH1 domains (Evangelista et al., 1997; Imamura et al., 1997; Manseau et al., 1996; Chang et al., 1997; Watanabe et al., 1997). Moreover, the FH1 domain of the mouse *ld* protein interacts with some SH3 and WW domains in vitro (Chan et al., 1996). Profilin appears to regulate actin dynamics and function (Theriot and Mitchison, 1993), suggesting that FH proteins may influence the actin

cytoskeleton through their FH1 domains. No proteins have been found that bind only the FH2 region. Cdc42p in *S. cerevisiae*, however, interacts with the N-terminal region of Bni1p, while Bnr1p binds a different Rho family member, Rho4p (Evangelista et al., 1997; Imamura et al., 1997). Thus, FH proteins interact with the actin cytoskeleton and may be effectors of small G-proteins.

Because actin microfilaments are required for the formation and ingression of a cleavage furrow during cytokinesis, and because FH1 domains interact with proteins that regulate or associate with actin microfilaments, FH proteins may function in cytokinesis by a localized modification of the membrane-associated actin cytoskeleton (Frazier and Field, 1997; Wasserman, 1998). For example, Cdc12p in *S. pombe* is found in the contractile ring during cytokinesis and appears to be required for actin ring assembly (Chang et al., 1997). In contrast, the mouse FH proteins encoded by the *ld* locus are found in the cytoplasm and nucleus, and the severity of mutations in the *ld* locus correlates with the loss of nuclear localization (Chan and Leder, 1996). Mutational inactivation of the mouse *ld* locus perturbs limb and kidney development, but the cellular functions of the encoded formin isoforms remain unknown (Woychik et al., 1990; Chan and Leder, 1996). The different phenotypes that result from mutational inactivation of different FH genes, and the varied protein localization patterns of the FH proteins, indicate that they participate in multiple processes, perhaps united only by a conserved interaction with the cytoskeleton.

To investigate the mechanism of FH function, we have examined the requirements for a maternally expressed FH gene in *C. elegans* called *cyk-1*, for cytokinesis-defective. Our genetic studies suggest that *cyk-1* is required for a late step in embryonic cytokinesis. Consistent with this hypothesis, we show that the CYK-1 protein localizes to the leading edge of the cleavage furrow near the end of cytokinesis.

MATERIALS AND METHODS

Strains and alleles

Standard procedures for nematode culture and genetics were followed (Brenner, 1974). A genetic screen for non-conditional, recessive, maternal-effect, embryonic-lethal mutants was performed as described previously (Kemphues et al., 1988). Long or Unc animals from the following strains were used to obtain *cyk-1* mutant embryos from homozygous mutant mothers for phenotypic analysis: *lon-1(e185) cyk-1(or36)/ qC1 III*; *cyk-1(or36) unc-36(e251)/qC1 III; cyk-1(or36) unc-32(e189)/qC1 III*; *cyk-1(t1611) unc-32 (e189)/qC1 III:him-3 (e1147) IV* and *cyk-1(t1568) unc-32(e189)/qC1 III; him-3 (e1147) IV*. We saw no variation in phenotype when homozygous mutant mothers were grown at 15°C or at 25°C, indicating that the mutation is not temperature-sensitive. Subsequently, all strains were grown at room temperature (approx. 22°C).

Microscopy and immunofluorescence

Living embryos were mounted on agar pads under coverslips for viewing with Nomarski optics on a Zeiss Axioskop, as described previously (Sulston et al., 1983). Photographs were taken with a 35 mm camera using Kodak Technical Pan film processed with HC110 developer. Digitized images from a Zeiss LSM3 confocal microscope were also used. To analyze the timing of the cell cycle (Fig. 1B) we observed 7 wild-type and 7 *cyk-1(or36)* embryos. 0 minutes was defined as the time at which the maternal and paternal pronuclei touched. Images on 35 mm film were scanned into a PowerMac computer using a Polaroid Sprintscan. Figures were made using Adobe Photoshop 4.0 and Canvas 5.0.

Procedures were as described for P-granule staining using mAb OIC_1D_4 (Strome and Wood, 1983) and for NMY-2 staining (Guo and Kemphues, 1996). Embryo stage was determined by DAPI-staining of embryos. Rhodamine- or FITC-conjugated phalloidin (Molecular Probes, Eugene OR) was used for detecting actin microfilaments. The manufacturers' protocol was followed and slides were stained for 20-30 minutes, then washed as in Guo and Kemphues (1995).

Molecular cloning of *cyk-1*

Positional mapping using the strain *sma-3(e491) unc-36/cyk-1(or36) III* placed *cyk-1* between 0.794 and 0.833 map units to the left of *unc-32* on chromosome III. In this region the cosmid F11H8 contains a predicted gene, F11H8.4, closely related to *D. melanogaster diaphanous* (S. Wasserman, personal communication). Cosmid F11H8 (obtained from Alan Coulson, Sanger Center, Cambridge, England) was used for germline transformation as described in Mello et al. (1991). Transgenic *lon-1(e185) cyk-1(or36)/qC1* animals carrying F11H8 and a dominant allele of *rol-6* segregated Lon Rol animals that were rescued in the first generation. Rescue did not persist in subsequent generations.

We sequenced the genomic DNA spanning the predicted ORF, F11H8.4, in our mutant alleles by PCR amplification. Comparison to N2 genomic sequence (or *lin-2(e1309)*, the parental strain used for mutagenesis; see Kemphues et al., 1988), shows a C→T mutation at codon position 1314 (CGA→TGA) in allele *or36*. This mutation introduces an opal stop codon following the second coiled-coil region, predicted using an algorithm described in Lupas et al. (1991; Fig. 4). In the alleles *t1568*, codon 1212 (CAA) is mutated to TAA; in *t1611* codon 1238 (CGA) is mutated to TGA. The *t1568* and *t1611* sequences were compared to each other, as the strain used for mutagenesis was not sequenced. All three alleles of the mutant genes were cloned into plasmids as three overlapping fragments from genomic DNA using PCR. Two independent clones from separate PCR reactions were obtained for each fragment, and one strand sequenced from each clone. No other differences in sequence were found throughout the remaining coding sequences for all three alleles.

Using a mixed staged embryonic cDNA library (gift from Pete Okkema), we cloned several cDNAs that together encode the entire predicted ORF. The radiolabeled probe was produced using a PCR-amplified template containing exon 10 from the F11H8 cosmid, using primers Ex10A and Ex10BXh (Genosis), followed by random primed DNA synthesis using [^{32}P]dCTP (Amersham) (primers, Ex10A: TAA TAC GAC TCA CTA TAG GGT CGT CAT GAA TGA CTT GTG CTG TC; Ex10BXh: CCA GAG CTC TCC CAA AAA TAC CTC TTC CTC CGC C). Both strands of one cDNA containing exons 7-14 (accession number AF062008), and another containing exons 1-3 (accession number AF062007), were sequenced. The intron/exon structure of exons 3-6 shown in Fig. 4 is that predicted by Genefinder.

cyk-1 RNA injection

Single-stranded *cyk-1* RNA for microinjection was made by in vitro transcription as follows. The plasmid p1.6A3, containing 1.6 kb of *cyk-1* cDNA sequence encoding exons 7-11 inserted into the cloning site within pGEM-T (Promega), was linearized with *Xba*I (New England Biolabs). RNA was synthesized from this template using SP6 RNA polymerase (Boehringer-Mannheim). Although *cyk-1* RNA microinjection led to embryonic lethality due to cytokinesis defects, we observed no sterility or defects in oocyte production in the microinjected animals.

cyk-1/Deficiency

sma-3 (e491) cyk-1(or36)/+ males were crossed into *nDf16/qC1* hermaphrodites and the outcrosses were scored for the presence of small (Sma) worms as *nDf16* uncovers both *sma-3* and *cyk-1*. These worms were viewed under Nomarski optics as described above. As a

control, *sma-3 unc-36(e251)/+* males were crossed into *nDf16/qC1* hermaphrodites. Small, Unc animals were identified (*nDf16* uncovers *unc-36*) and were compared with *sma-3 cyk-1/nDf16* animals. Oocyte production defects and the resulting sterility were not seen in the *sma-3 unc-36/nDf16* animals. Animals homozygous for the deficiency, produced by heterozygous mothers, die during embryogenesis and fail to hatch.

cyk-1 mRNA and protein expression

Synthesis of digoxigenin-labeled antisense RNA probes (Boehringer-Mannheim) and in situ hybridizations were done as described (Seydoux et al., 1994), except that no Proteinase K step was included. For SP6 (antisense) and T7 (sense) polymerase templates, we used *Xba*I- or *Nde*I-linearized p1.6A3, respectively. We compared antisense with sense staining as a negative control, and we used staining with *mex-3* antisense RNA made from the pJP621 plasmid as a positive control (Draper et al., 1996).

We generated two rabbit polyclonal antisera to CYK-1 reactive against an N-terminal fragment (amino acid residues 2-355 fused to GST), and a C-terminal fragment (amino acids 834-1159, with a 6X-His tag at the N terminus). Two fixation methods were used with these antisera. (1) Hermaphrodites were cut open on a polylysine-coated slide, and embryos permeabilized by the freeze-crack technique (Bowerman et al., 1992), fixed by immersion in methanol (room temperature, 15 minutes), washed (3×, 4 minutes each) in Tris-Tween (Bowerman et al., 1992), then incubated in a blocking solution (see below). (2) Embryos were permeabilized by alkaline hypochlorite treatment, then fixed in 3% formaldehyde as described in Aroian (1997), except that the procedure was done in baskets made from Eppendorf tubes with a hole punched in the lid and covered with a porous membrane (8 μm pore size, Osmonics, Livermore, CA). Alternatively, worms were cut open on a slide in a small drop of water, which was replaced with the formaldehyde fixative. Embryos were permeabilized by squashing. After fixing for 10 minutes, slides were frozen in liquid nitrogen for 10 minutes, the coverslips were removed, and the slides were treated as in Aroian (1997).

Pre-immune sera showed cytoplasmic background staining similar to that of the immune sera. P-granule staining was observed in both preimmune and immune sera. No cleavage furrow staining was detectable in *cyk-1(t1568)* embryos, confirming the specificity of the observed staining pattern. Only background staining was detectable when the N-terminal immune serum was pre-incubated with the corresponding fragment of CYK-1 protein used for immunization. When embryos are fixed with formaldehyde, the immune serum raised against the N-terminal fragment detects nuclear staining at a low frequency (about 20-30%). Both rabbit polyclonal immune sera showed the same CYK-1 cleavage furrow staining and meiotic staining pattern in WT embryos. The images in Fig. 6 were obtained using the N-terminal fragment-recognizing antiserum, and embryos were fixed using Method 2.

Embryos from all mutant alleles of *cyk-1* were stained with the N-terminal fragment antiserum. *or36* embryos show detectable protein. No staining was observed in most *cyk-1(t1611*) embryos, although in about 5% of *t1611* embryos foci of staining were seen associated with the plasma membrane.

RESULTS

cyk-1 mutants are defective in embryonic cytokinesis

We isolated three alleles of *cyk-1* in screens for recessive, maternal-effect, embryonic-lethal mutations (see Materials and methods). The three alleles result in similar mutant phenotypes; unless otherwise indicated, we report results from an analysis of mutant embryos produced by self-fertilization in homozygous *cyk-1(or36)* mutant hermaphrodites.

In developing *cyk-1(or36)* mutant embryos, observed in utero using Nomarski optics (Materials and methods), early embryonic cells initiate cytokinesis normally but almost invariably fail to complete it (Fig. 1A). During the first round of mitosis, a cleavage furrow forms at the correct position, bisecting the posteriorly displaced mitotic spindle. The cleavage furrow ingresses extensively and stably maintains a deep position, regressing only after daughter nuclei are visible. In the next round of mitosis, the mitotic spindle is tetrapolar due to the presence of four centrosomes in a single cell. The resulting cleavage furrows bisect each arm of the spindle in a corresponding tetrapolar fashion, again regressing only after nuclei form.

Although their phenotypes are otherwise nearly indistinguishable, the cleavage furrows in *t1568* and *t1611* embryos are less stable than in *or36* embryos. When mounted on agarose pads and viewed under the pressure of a coverslip, the cleavage furrows in *t1568* and *t1611* embryos frequently fail to form. But when observed in utero in young adult mutant mothers mounted on agar pads, most *or36*, *t1568* and *t1611* mutant embryos show a late defect in cytokinesis. All *or36* mutant embryos observed in utero exhibited a late defect in cytokinesis at the first cleavage (n=18) and second cleavage (n=28) attempts. However, during the first cleavage attempt, some *t1568* (1/11) and some *t1611* (4/12) mutant embryos exhibited an earlier defect in cytokinesis, with cleavage furrows regressing after moderate amounts of pinching in (data not shown); the remainder showed a typical late defect. During all second cleavages scored in utero, furrows ingressed to or near the center of the embryo, regressing only after nuclei formed (n=28, 11 and 9 for *or36*, *t1568* and *t1611*, respectively). The cytokinesis defect is highly penetrant in all three *cyk-1* alleles. The first two cleavages are never successful, and additional rounds of mitosis and failed cytokinesis continue, resulting in the production of unicellular embryos filled with many nuclei of variable size (Fig. 1A). Rarely, we see embryos that appear to have pinched off one or two small membrane-bound compartments containing multiple nuclei (3/586 late stage *or36* embryos scored). Although we observed a slight delay between pronuclear meeting and the initiation of cytokinesis (Fig. 1B), *cyk-1* mutant embryos undergo many rounds of mitosis and eventually express markers characteristic of post-mitotic, differentiated cell types (data not shown).

1-cell stage *cyk-1* mutant embryos fail to pinch off polar bodies during meiosis and are partially defective in pseudocleavage

Another prominent defect in *cyk-1* mutant embryos is their inability to extrude polar bodies during meiosis (Fig. 2A). After fertilization, the maternal pronucleus undergoes meiosis I and II and the embryo partitions discarded chromosomes into two vesicles called polar bodies that pinch off from the plasma membrane (Albertson and Thomson, 1993; Albertson et al., 1997). In *cyk-1* mutant embryos the meiotic segregation of chromosomes appears normal, but no chromosomes are extruded and polar bodies do not form (Fig. 2A). As a result, extra nuclei appear in *cyk-1* mutant embryos near the anterior end of the embryo, which often later associate with nuclei that form after the first embryonic mitosis. The only other defect we detected in *cyk-1* mutant embryos is a nearly complete failure to form pseudocleavage furrows (Fig. 2B). In wild-type

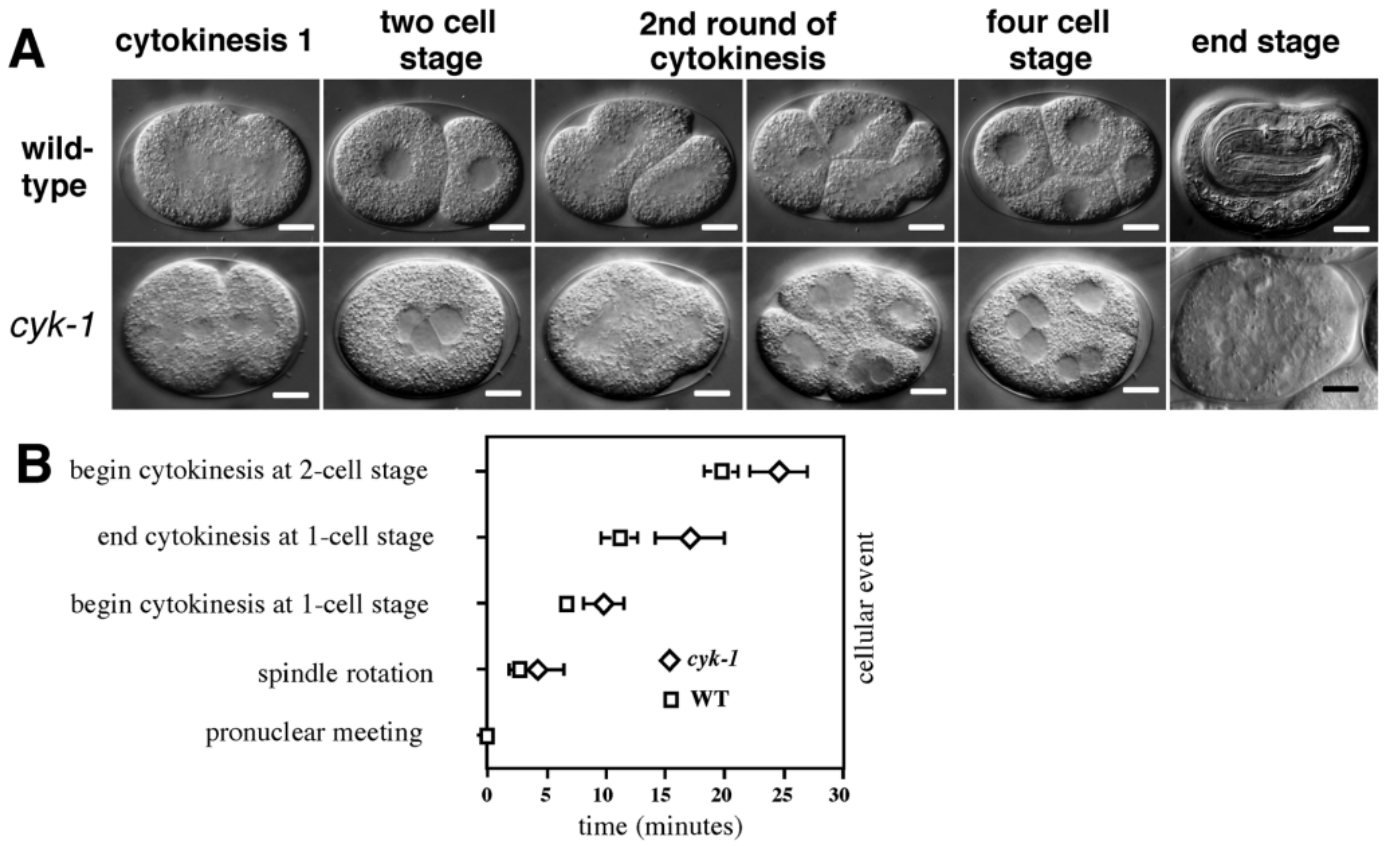


Fig. 1. *cyk-1* embryos are unable to complete cytokinesis. (A) Nomarski micrographs showing cytokinesis occurring at the 1- and 2-cell stages in wild type and at the equivalent stages in *cyk-1* mutant embryos. Note that the cleavage furrows in *cyk-1* mutant embryos persist even as nuclei reform. A tetrapolar spindle forms (observable in Nomarski images as an area cleared of yolk granules) at the second cleavage in *cyk-1* embryos, with bisecting tetrapolar cleavage furrows. A late stage wild-type embryo is shown just prior to hatching; a late stage *cyk-1* mutant embryo is shown with hypodermal-like nuclei visible in a multinucleated single cell. We also have observed octapolar spindles during the third round of embryonic mitosis, with pronounced cleavage furrows bisecting each spindle arm. In most embryos after the first two rounds of mitosis, though, large numbers of interconnected spindles appear to trigger widespread contractions of the plasma membrane without extensive furrow formation (K.A.S. and B.B., data not shown). In this and subsequent figures, anterior is to the left and ventral at the bottom; bar, approximately 10 μm. *C. elegans* embryos are about 50 μm in length. (B) Cell cycle timing during the first and second cell cycles in *cyk-1* mutant embryos compared to wild-type embryos (±1 s.d.).

embryos, the pseudocleavage furrow is a prominent but transient ingression in the plasma membrane that forms early in the first zygotic cell cycle and then regresses as the maternal and paternal pronuclei meet near the center of the embryo. As *nop-1* mutant embryos do not undergo pseudocleavage but otherwise develop normally (Rose et al., 1995), it seems unlikely that the near absence of pseudocleavage furrows in *cyk-1* embryos significantly affects subsequent steps in development.

Most cytoskeletal functions appear normal in *cyk-1* mutant embryos

Many events in the early embryo that require a functional cytoskeleton occur normally in *cyk-1* mutant embryos. These events include pronuclear migration, spindle assembly and positioning, and anaphase chromosome segregation (Figs 1A, 2A,B and data not shown). Thus, mutational inactivation of *cyk-1* appears not to affect most cytoskeletal functions. We also examined P-granule segregation, a process that, like cytokinesis, requires microfilaments (Hird et al., 1996). P-granules are cytoplasmic structures initially present throughout the early 1-cell stage zygote that are segregated to the posterior cortex before the first embryonic mitosis. In contrast to the cytokinesis defect, P-granule localization appears normal in *cyk-1* mutant embryos (Fig. 3A), consistent with other functions of the actin cytoskeleton being unaffected.

The cleavage defect in *cyk-1* mutant embryos occurs late in cytokinesis

Observation of dividing embryonic cells suggests that cytokinesis initiates normally in *cyk-1* mutant embryos and that extensive ingression of the cleavage furrow occurs (Fig. 1A). To more accurately assess cleavage furrow ingression, we used phalloidin staining of fixed *cyk-1* mutant embryos to visualize filamentous actin associated with the cytoplasmic cortex (Strome, 1986; Aroian et al., 1997). The cleavage furrows that form during the second attempt at cytokinesis in *cyk-1* mutant embryos extend far into the cell and often meet in the center (Fig. 3B). Although in living *cyk-1* embryos, the first attempt at cytokinesis results in extensive migration of the cleavage

furrow into the cell (Fig. 1A), the first cleavage furrow is difficult to preserve during fixation of these mutant embryos. A cleavage furrow is detectable but does not extend deeply into the interior (Fig. 3B).

To further assess cleavage furrow formation and function in *cyk-1* mutant embryos, we also examined the distribution of NMY-2, a non-muscle myosin II required for both polarity and embryonic cytokinesis (Guo and Kemphues, 1996). In early stage wild-type embryos, NMY-2 localizes to the cortex of blastomeres (Guo and Kemphues, 1996), and is enriched in the cleavage furrow during cytokinesis (Fig. 3C). We found that NMY-2 is present in the cleavage furrows of *cyk-1* mutant embryos (Fig. 3C). In summary, actin and myosin are present in the cleavage furrow and mediate extensive ingression in most *cyk-1* mutants.

The *cyk-1* sequence predicts an FH protein

By positional cloning and a gene candidate approach, we determined that *cyk-1* corresponds to the predicted gene F11H8.4, identified by the *C. elegans* genome consortium (Fig. 4). The sequence of the wild-type *cyk-1* gene indicates that it is a new member of the FH gene family (Castrillon and Wasserman, 1994; Frazier and Field, 1997; Wasserman, 1998). The predicted CYK-1 protein is most similar to *mDia* in *M. musculus* and to *diaphanous* in *D. melanogaster*, respectively sharing 32% and 31% overall amino acid identity, and 45% identity in their FH2 domains (Fig. 4D). The *cyk-1* FH1 domain contains multiple stretches of polyproline and glycine, positioned a conserved distance N-terminal to the FH2 domain.

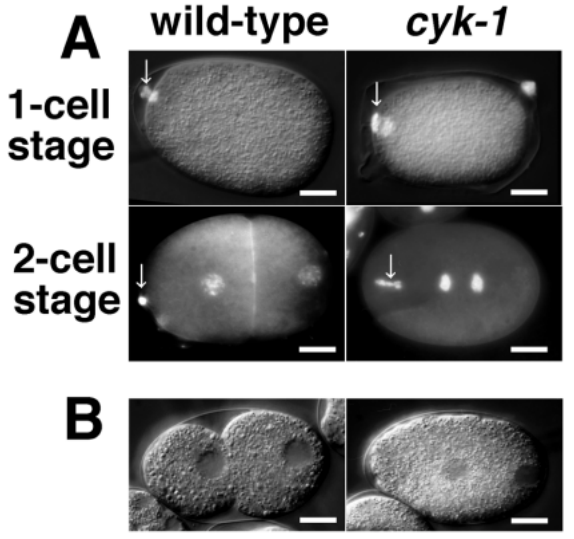


Fig. 2. Polar body extrusion and pseudocleavage do not occur in *cyk-1* mutant embryos. (A) DAPI staining of chromosomes during meiosis at the 1-cell stage, or during prophase at the 2-cell stage in wild type and telophase of the first cell cycle in *cyk-1* mutant embryos. Nomarski images of the fixed embryos are overlaid on the DAPI immunofluorescence micrographs of the 1-cell stage embryos to show cell boundaries. Arrows point to the meiotic chromosomes; DAPI-stained mitotic nuclei are also visible at the 2-cell stage. (B Nomarski images of a wild-type *C. elegans* embryo undergoing psuedocleavage during pronuclear migration and of the same stage in a *cyk-1* mutant embryo. Note that membrane ruffling is still seen although the pseudocleavage furrow is absent in the mutant embryo.

To verify the gene identity and determine the nature of the mutations, we sequenced F11H8.4 cloned from the genomic DNA of homozygous *cyk-1* mutant animals (Materials and methods). All three alleles, *or36, t1611* and *t1568,* have premature stop codons near the 3′ end of the gene: *or36* in the 13th exon, and *t1568* and *t1611* in the 12th exon, each after roughly 90% of the coding sequences, following the FH1 and

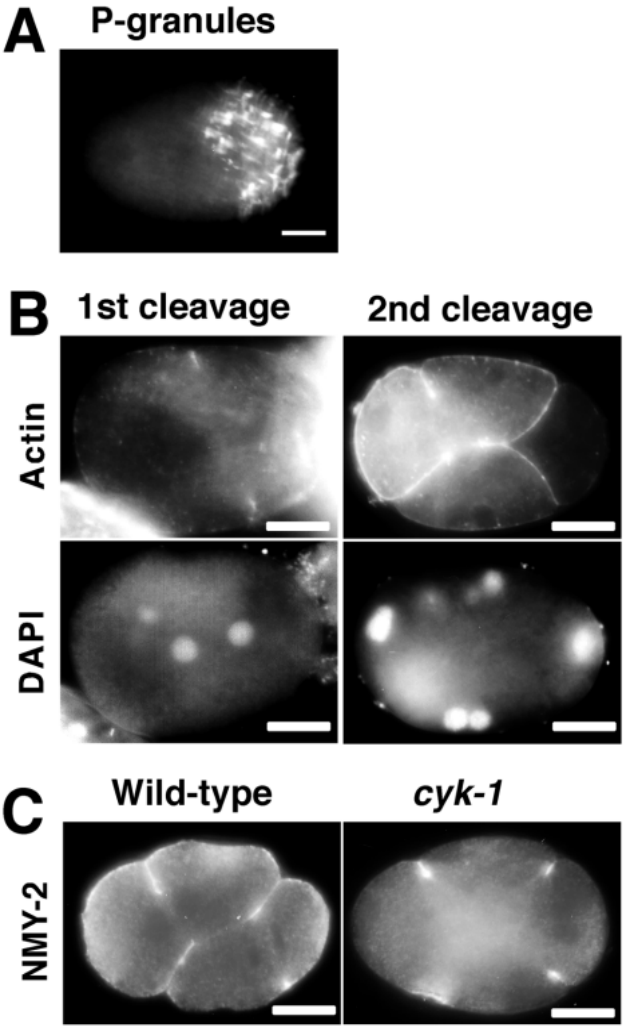


Fig. 3. Asymmetric localization of P-granules and the early stages of cytokinesis occur normally in *cyk-1* mutant embryos. (A) An immunofluorescence micrograph showing P-granule localization in a *cyk-1* mutant embryo near the end of the first cell cycle. (B) Rhodamine-conjugated phalloidin was used to visualize actin microfilaments during the first and second attempts at cytokinesis in *cyk-1* mutant embryos (Materials and methods). The formation and ingression of a cleavage furrow is indicated by the presence of actin microfilaments associated with the cell cortex of the cleavage furrows. Photomicrographs of DAPI-stained embryos indicate the positions of nuclei. Variable numbers of nuclei reform in *cyk-1* mutant embryos after completion of the second round of mitosis, presumably due to aneuploidy and aberrant chromosomal segregation with a tetrapolar spindle (see Fig. 2A). (C) Embryos undergoing the second round of embryonic mitosis, stained with an affinity-purified polyclonal rabbit antiserum that recognizes the non-muscle myosin II, NMY-2. NMY-2 is present in the cleavage furrows of both wild-type and *cyk-1* mutant embryos during cytokinesis.

FH2 coding sequences (Fig. 4C). Intriguingly, the position of these nonsense mutations in *cyk-1* is similar to the position of a truncating mutation in the human FH gene, *DFNA1*, that causes progressive deafness (Lynch et al., 1997).

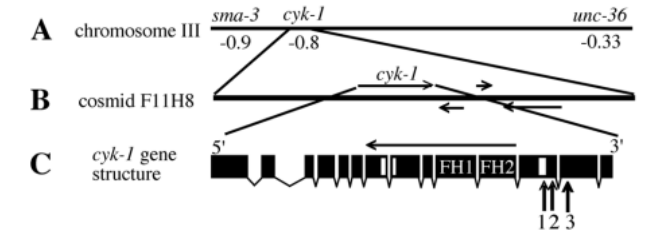
cyk-1 may have an additional role during oogenesis

Because our three mutant alleles of *cyk-1* may not be null, we used a genetic test to determine if they retain any function. We generated *cyk-1* hemizygous worms (*sma-3 cyk-1(or36)/nDf16*) to determine the phenotype associated with a single copy of the mutant allele (see Materials and methods). Although homozygous *cyk-1(or36)* hermaphrodites are fertile, hemizygous mutant animals are sterile. The sterility appears to involve a failure of plasma membranes to enclose mature oocyte nuclei during oogenesis (Fig. 5A-C). The same phenotype is seen with the *t1568* and *t1611* alleles (H.S. and R.S., data not shown). These results suggest that our alleles of *cyk-1* may retain some function and that *cyk-1* may have an additional role during the cellularization of oocytes. Intriguingly, mutations in a human homologue of *diaphanous* have recently been reported to be associated with premature ovarian failure and sterility (Bione et al., 1998).

As an alternative approach to reducing or eliminating *cyk-1* function, we microinjected *cyk-1* RNA into wild-type nematode ovaries. Microinjection of RNA into the syncytial ovary of wild-type *C. elegans* hermaphrodites eliminates gene function for most maternal genes tested in *C. elegans*, frequently phenocopying known null mutants and causing a loss of detectable protein (Fire et al., 1998). Young adult wild-type hermaphrodites were microinjected with *cyk-1* RNA (Materials and methods). In 12/12 animals, all embryos produced starting approximately 15 hours after microinjection were multinucleate and indistinguishable from *or36*, *t1568* and *t1611* mutant embryos. Embryos from microinjected mothers were observed in utero during the first and second attempts at embryonic mitosis using Nomarski optics. In all cases, the cleavage furrows invaginated extensively and then regressed: *n*=11 for the first cleavage, *n*=12 for the second cleavage (Fig. 5D-F). Thus the late defect in embryonic cytokinesis is not unique to our mutant alleles and may represent the embryonic loss-of-function phenotype for this locus (see Discussion).

CYK-1 protein localizes to the leading edge of the cleavage furrow late in cytokinesis.

To determine when and where in cytokinesis *cyk-1* might act, we generated rabbit polyclonal antisera that specifically recognize the CYK-1 protein. Consistent with it acting at a late step in cytokinesis, CYK-1 protein is detectable only well after the initiation of furrow ingression, when it forms a ring at the leading edge of the cleavage furrow (Fig. 6A). In five different wild-type embryos fixed during the first embryonic cytokinesis, actin but not CYK-1 was detectable when the contractile rings were 85%, 76%, 66%, 65% and 50% of the cell diameter. We detected CYK-1 at the leading edge of the first cleavage furrow in five wild-type



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organism	gene name	entire gene identity	entire gene similarity	FH2 domain identity	FH2 domain similarity
S. cerevisiae	*BNI1*	23%	46%	32%	52%
	BNR1	20	44	21	48
S. pombe	*fus1*	19	40	32	52
	cdc12	19	42	33	51
A. nidulans	*sepA*	23	46	32	52
D. melanogaster	*cappuccino*	24	46	26	60
	diaphanous	**31**	**52**	**45**	**68**
M. musculus	*formin*	23	46	26	53
	mDia	**32**	**54**	**45**	**68**
C. elegans	C46H11.11	25	45	28	52

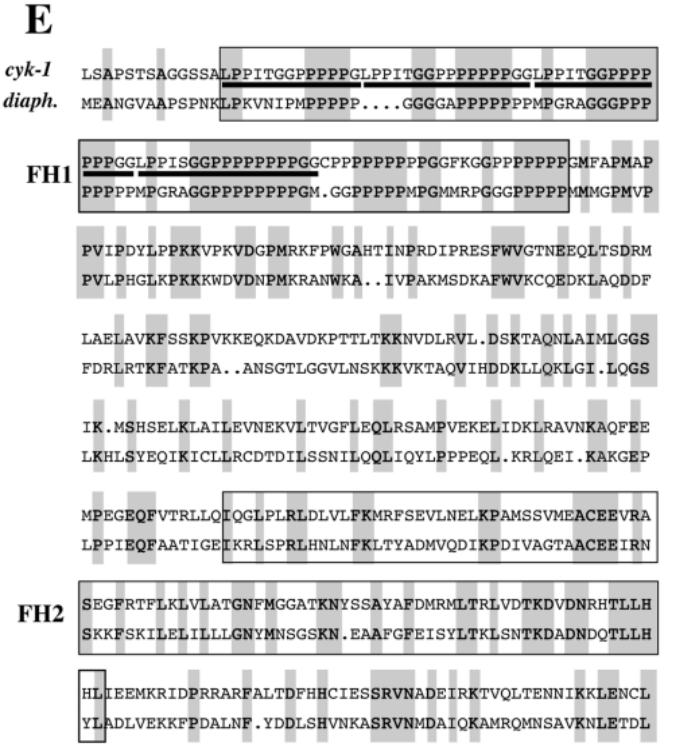

Fig. 4. Cloning and characterization of the *cyk-1* gene. (A) The chromosomal localization of *cyk-1*. (B) The cosmid F11H8 contains the *cyk-1* gene and rescues the *cyk-1* defect. Arrows represent other predicted genes. (C) The predicted and partially confirmed intron/exon structure of the *cyk-1* gene is shown (see Materials and methods). Vertical arrows point to the positions of premature stop codons present in the *cyk-1* mutant alleles *t1568* (1), *t1611* (2) and *or36* (3). See Materials and methods for the sequences of these mutant alleles. The horizontal arrow indicates the region of the corresponding cDNA from which RNA was made for RNAi. The open boxes represent predicted coiled-coil domains. (D) A comparison of *cyk-1* to several other FH genes reveals that *cyk-1* is a member of the FH gene family and that its closest identified relatives are *mDia* in *M. musculus* and *diaphanous* in *D. melanogaster*. (E) A portion of the predicted protein sequence of the *cyk-1* gene (amino acids 693-1,112) compared to that of the closely related FH gene, *diaphanous* (amino acids 493-904). Sequence identities are shaded in gray. The FH1 and FH2 domains are boxed and the proline repeats are underlined.

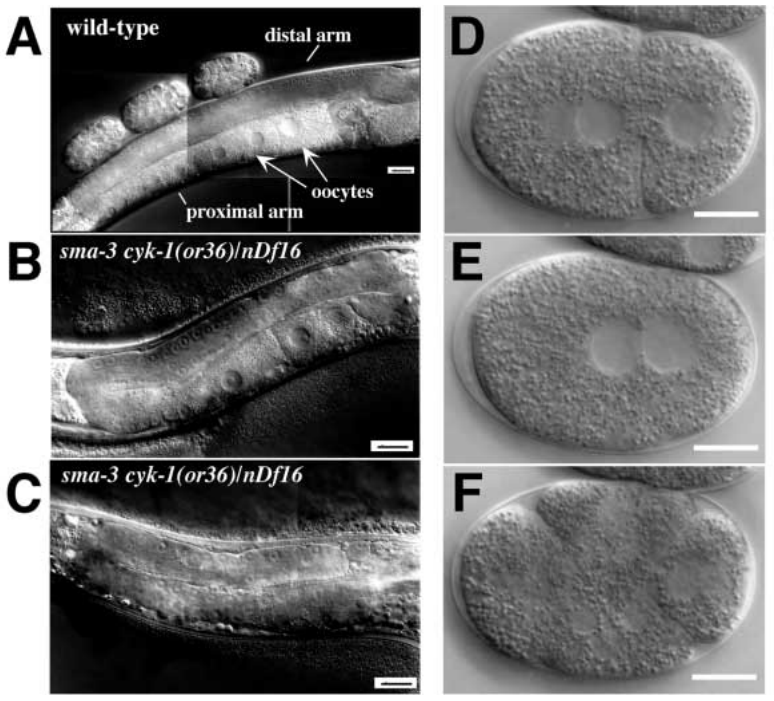

Fig. 5. (A-C) Oocytes fail to form correctly in *sma-3 cyk-1*/*nDf16* animals. Nomarski micrograph of a gonad from a wild-type worm. In the distal arm of the gonad, germ nuclei are part of a syncitium that shares a common cytoplasmic core. As they round the bend of the gonad and enter the proximal arm, nuclei begin to differentiate and become completely cellularized to form a mature oocyte (Albertson et al., 1997). The bottom two panels are Nomarski micrographs of gonad arms in *sma-3 cyk-1/Df* animals. In these hemizygous *cyk-1(or36)* mutants, the cellularization of oocytes in the distal arm fails to variable degrees. A less severe example is shown in B, a more severe example in C. (D-F) RNAi phenocopies mutations in *cyk-1*. RNA inhibition of *cyk-1* function yields the same phenotype as *cyk-1*(*or36*). (D) A well-defined cleavage furrow is established. (E) This cleavage furrow does not go to completion but regresses when nuclei form. (F) A second attempt at cytokinesis shows the tetrapolar cleavage characteristic of mutations in *cyk-1*.

embryos when the contractile rings were 49%, 29%, 22%, 22% and 18% of the cell diameter. Thus only after the cleavage furrows close down substantially around the midzone of the mitotic spindle do detectable amounts of CYK-1 co-localize with the actin contractile ring. We see a similar pattern of CYK-1 localization to the leading edge of cleavage furrows late in cytokinesis during subsequent embryonic cell divisions (data not shown). Earlier in development we also detect CYK-1 near the site of polar body extrusion during meiosis (Fig. 6B), consistent with a meiotic requirement for *cyk-1* function (Fig. 2).

While we do not detect CYK-1 antibody staining in *t1568* and most *t1611* mutant embryos (Materials and methods), we do see localization of CYK-1 to the leading edge of abortive cleavage furrows in *or36* embryos (Fig. 6C). Thus truncation of CYK-1 after the FH2 sequence and the putative coiled-coil region near the C terminus (Fig. 4C) does not appear to preclude CYK-1 localization to the leading edge of the cleavage furrow, and sequences in the C-terminal 10% of the predicted CYK-1 protein may be important for the termination of cytokinesis.

Maternal expression of *cyk-1* mRNA during oogenesis

Using in situ hybridization (Materials and methods), we found that *cyk-1* mRNA is detectable in the germline and throughout all blastomeres in early stage embryos, fading rapidly to background levels after the 28-cell stage in embryogenesis (Fig. 7), with persistence of mRNA seen in the germline lineage (Fig. 7D). This pattern of accumulation is typical of many maternally expressed genes in *C. elegans* (Seydoux and Fire, 1994), indicating that both by genetic criteria and by in situ hybridization, *cyk-1* appears to be maternally expressed. Although our data do not rule it out, we see no evidence for zygotic expression: *cyk-1* mRNA levels fade throughout the embryo early in development, and we see no zygotic rescue of the *cyk-1* mutant phenotype when wild-type males are mated to homozygous mutant hermaphrodites (data not shown). Thus, it is possible that *cyk-1* is required specifically for embryonic cytokinesis, and perhaps for earlier events during oogenesis, with maternal supplies sufficing for these requirements.

DISCUSSION

Our analysis suggests that the maternally expressed *C. elegans* FH gene *cyk-1* is required for a late step in embryonic cytokinesis. During the first two mitotic cycles in most *cyk-1* mutant embryos, cleavage furrows form and extend far into the interior of the embryo, but invariably regress after the completion of mitosis. Consistent with it acting at a late step in cytokinesis, we detect CYK-1 protein at the leading edge of the cleavage furrow only after substantial ingression has occurred. Although our alleles may not be null and *cyk-1* may have an additional role during oogenesis, reducing *cyk-1* function by mutation, or by microinjecting *cyk-1* RNA into the ovaries of wild-type nematodes, results in the production of mutant embryos with a late defect in cytokinesis. Moreover, in *cyk-1(t1568)* and *cyk-1(t1611)* mutant embryos, no CYK-1 protein is detectable in the cleavage furrow during mitosis. Thus reduction or elimination of *cyk-1* function in the early embryo results in a late defect during cytokinesis, suggesting a late requirement for FH gene function during cytokinesis. *cyk-1* mutant embryos also are defective in the extrusion of polar bodies during meiosis, and in the formation of membrane invaginations called pseudocleavage furrows that transiently form shortly after fertilization. Thus these latter two processes are mechanistically related to cytokinesis.

Genetic evidence that cytokinesis proceeds through a sequence of discrete steps

Although several genes are now known to be required for cytokinesis, most appear to encode products involved in the assembly or early function of the contractile ring. The late defect in *cyk-1* mutant embryos argues that the initial steps of

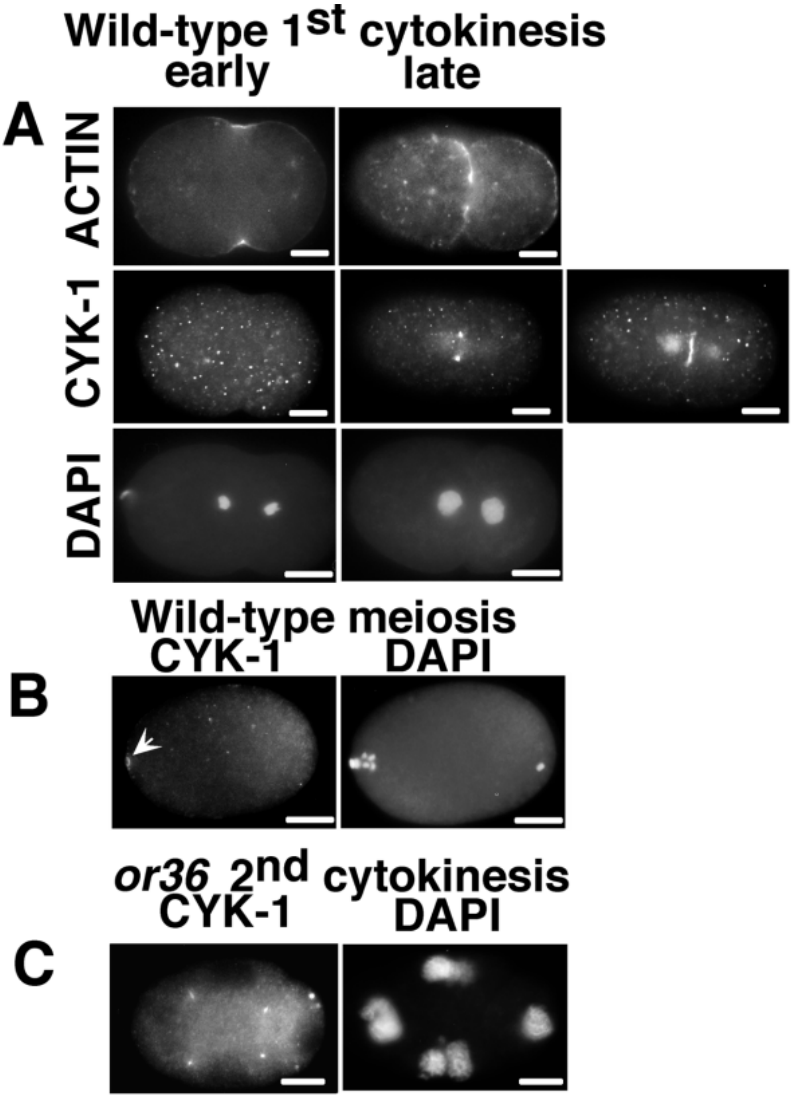

Fig. 6. CYK-1 protein localizes to the leading edge of the cleavage furrow at a late stage in furrow ingression. Rhodamine-conjugated phalloidin applied to formaldehyde-fixed embryos was used to detect actin microfilaments, rabbit polyclonal antibodies were used for detecting CYK-1 protein, and DAPI was used for staining chromosomes in embryos undergoing cytokinesis. (A) Early in cytokinesis (left column) in wild-type embryos, actin microfilaments appear enriched in the cleavage furrow, but no CYK-1 is detectable. CYK-1 is detectable only later in cytokinesis (right column), as a ring at the leading edge of the cleavage furrow. The ring is visible as two points of staining in a confocal cross section through the center of the ring (middle panel of the second row from the top), and as a line in a section through the same embryo that passes along the top of the CYK-1 ring (rightmost panel of the second row from the top). The cytoplasmic 'dots' and the faint nuclear staining are not reproducible and are not specific for CYK-1 (see Materials and methods). (B) CYK-1 protein is associated with the plasma membrane at the site of polar body extrusion during meiosis (arrow), with DAPI staining showing the maternal chromosomes to the left (paternal pronucleus is to right). (C) CYK-1 is present as four foci of staining in the center of a *cyk-1(or36)* mutant embryo undergoing a second, tetrapolar attempt at cytokinesis, with DAPI staining showing the position of reforming nuclei.

cytokinesis, i.e. positioning and assembly of functional cleavage furrows and contraction of the actin-myosin ring, occur normally or nearly normally. CYK-1 appears to be required at a later step, either during a late phase of furrow ingression or subsequently during the process of termination. Consistent with the late defect in cytokinesis in *cyk-1* mutant embryos, CYK-1 protein localizes to the leading edge of the cleavage furrow late in cytokinesis. Intriguingly, inactivation of Rho family member G-proteins in *Xenopus* embryos, or of a Ras-GAP in *Dictyostelium*, blocks cytokinesis without disturbing the assembly and initial ingression of cleavage furrows (Drechsel et al., 1997; Adachi et al., 1997; for a review see Chisolm, 1997). The involvement of G-proteins at a late step in cytokinesis is interesting in light of the genetic and biochemical evidence that the FH proteins Bni1p and Bnr1p in *S. cerevisiae* interact with Rho family G-proteins (see Introduction). Perhaps interactions such as these promote the final steps that terminate cytokinesis.

Do FH proteins function at an early or at a late step in cytokinesis?

In contrast to our evidence suggesting CYK-1 acts late in cytokinesis, studies of FH genes in *S. pombe*, *S. cerevisiae* and *D. melanogaster* have led investigators to suggest a role early in cytokinesis, regulating the assembly of actin microfilaments to form a contractile ring (Chang et al., 1997; Imamura et al., 1997; Giansanti et al., 1998). In contrast to *S. cerevisiae* and *S. pombe*, actin microfilaments in early *C. elegans* blastomeres are present throughout the cortex both during interphase and during mitosis (Aroian et al., 1997), with local actin-myosin generated force producing the cleavage furrow, as in other animal cells (Frazier and Field, 1997). Because of this difference in the behavior of the actin cytoskeleton, perhaps FH genes have evolved to perform somewhat different functions during cytokinesis in animal and in yeast cells.

Alternatively, it may be difficult to detect a late defect in cytokinesis based on analyses of fixed specimens. Only by viewing cytokinesis in living embryos could we detect a late defect in cytokinesis in two of our three mutant *cyk-1* alleles (see above). The finding that a contractile ring is absent in fixed tissues in *diaphanous* mutants in *D. melanogaster* and in *cdc12* mutants in *S. pombe* could be due to an early defect in cytokinesis, or perhaps to a similar difficulty in preserving abnormal cleavage furrows in mutant cells during fixation.

It is also possible that *cyk-1* has a role early in cytokinesis

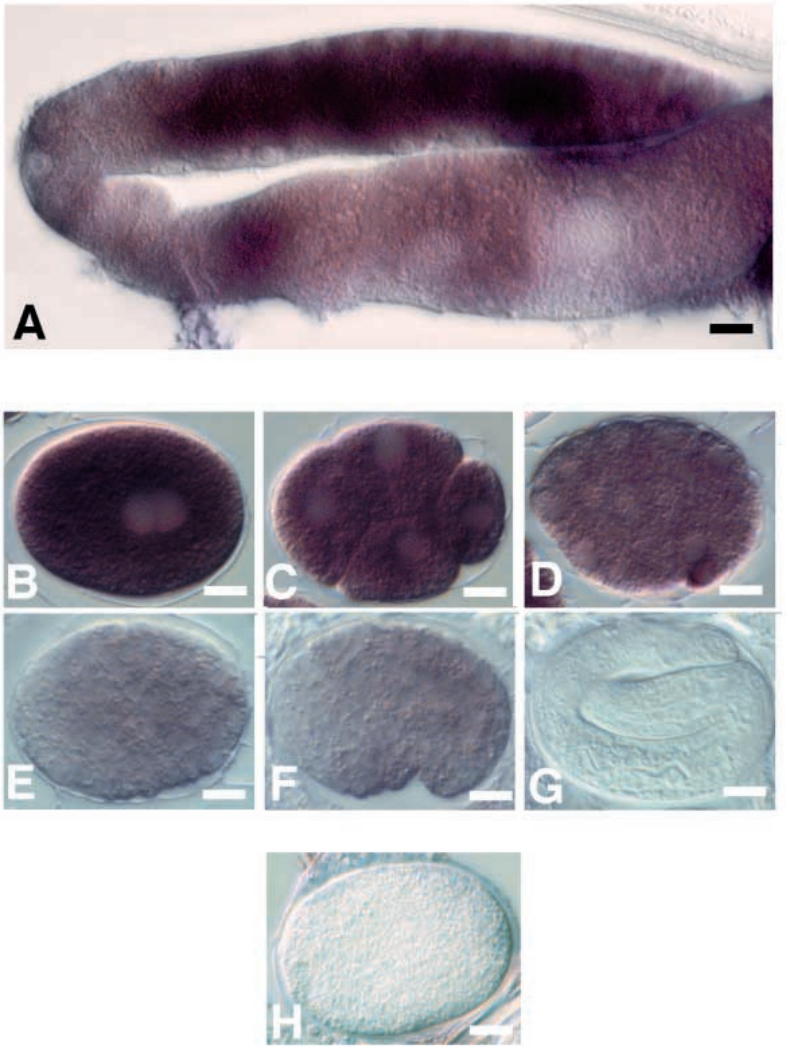

Fig. 7. *cyk-1* mRNA abundance as determined by in situ hybridization with labeled antisense RNA. (A) *cyk-1* RNA expression in the gonad of a wild-type worm. *cyk-1* RNA abundance in 1-cell (B), 4-cell (C), 28-cell (D), ~100-cell (E), ~500-cell (F) and threefold stage (G) wild-type embryos. (H) A 1-cell stage wild-type embryo stained with labeled sense *cyk-1* RNA as a negative control.

that is less apparent in our mutants, and that a late role in *S. pombe* and *D. melanogaster* might be masked by the observed early defects. In support of this view, we note that the initiation of a productive cleavage furrow at the 1-cell stage requires more time in *cyk-1* mutants than in wild-type embryos (Fig. 1B). Furthermore, the cleavage furrows that form during the first mitosis in *t1568* and *t1611* mutant embryos sometimes regress after little progress towards the center of the embryo. Additionally, the finding that cleavage furrows in *cyk-1* mutant embryos are more difficult to preserve during fixation than is true for wild-type embryos could perhaps reflect a defect in their initial assembly. Indeed, CYK-1 might be present diffusely in early cleavage furrows, but concentrate to detectable levels only late in cytokinesis. While our data do not rule out an early role for *cyk-1* function during cytokinesis, we believe they most clearly suggest a requirement for *cyk-1* function at a late step in embryonic cytokinesis, even if *cyk-1* also plays additional early role(s). This intriguing observation has led us to speculate on a mechanism for CYK-1 function.

A model for CYK-1 function: bridging microfilaments and microtubules

We propose that CYK-1 acts late in cytokinesis by bridging microfilaments and microtubules as midzone spindle microtubules become tightly encircled by the cleavage furrow near the end of cytokinesis (Fig. 8). Such a bridging function might stabilize the cleavage furrow prior to its resolution by joining the microfilament-associated plasma membrane to

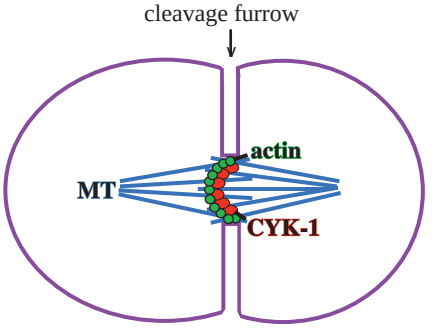

Fig. 8. A model for *cyk-1* function during cytokinesis. A cartoon of a 1-cell *C. elegans* embryo undergoing cytokinesis is shown. *cyk-1*, depicted as red ovals, forms a ring at the leading edge of the cleavage furrow that bridges the actin (green) and microtubule (blue) cytoskeletons.

midzone microtubules. In support of the necessity of such a function in cytokinesis, several groups have recently reported a requirement for midzone microtubules throughout cytokinesis (Giansanti et al., 1998; Wheatley and Wang, 1996; Eckley et al., 1997). In this model of CYK-1 function, the FH1 domain of the predicted CYK-1 protein would bind to plasma membrane-associated microfilaments, perhaps through a nematode profilin or through an SH3 or WW domain protein (see Introduction). We further propose that CYK-1 might bind, directly or indirectly, through a different domain to midzone microtubules. This simple model does not exclude similar bridging functions for other proteins, or interactions of CYK-1 with additional proteins associated with the cleavage furrow.

While speculative, this model for CYK-1 function is consistent with a late defect in cytokinesis, and with the late localization of CYK-1 to the leading edge of the cleavage furrow. Perhaps CYK-1 must associate with both microfilaments and microtubules to concentrate at the leading edge of the cleavage furrow late in cytokinesis, once the actin-myosin contractile ring and the microtubule spindle are tightly apposed to one another. We also found that formation of the pseudocleavage furrow after fertilization is defective in *cyk-1* mutant embryos, another process that requires both microfilaments and microtubules (Hill and Strome, 1990; Hird and White, 1993). Finally, we note that inactivation of the *D. melanogaster* FH gene *cappuccino* results in a disorganization of the microtubule cytoskeleton during oogenesis (Manseau et al., 1996). Perhaps *cappuccino* organizes or polarizes microtubules by mediating an interaction between microfilaments and microtubules during insect oogenesis. It is intriguing to speculate that enabling concerted action between the actin- and microtubule-based cytoskeletons is a general property of FH proteins.

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