CYK-4: A Rho Family GTPase Activating Protein (GAP) Required for Central Spindle Formation and Cytokinesis

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Abstract. During cytokinesis of animal cells, the mitotic spindle plays at least two roles. Initially, the spindle positions the contractile ring. Subsequently, the central spindle, which is composed of microtubule bundles that form during anaphase, promotes a late step in cytokinesis. How the central spindle assembles and functions in cytokinesis is poorly understood. The *cyk-4* gene has been identified by genetic analysis in *Caenorhabditis elegans*. Embryos from *cyk-4(t1689ts)* mutant hermaphrodites initiate, but fail to complete, cytokinesis. These embryos also fail to assemble the central spindle. We show that the *cyk-4* gene encodes a GTPase activating protein (GAP) for Rho family GTPases. CYK-4 activates GTP hydrolysis by RhoA, Rac1, and Cdc42 in vitro. RNA-mediated interference of RhoA, Rac1, and Cdc42 indicates that only RhoA is essential for cytokinesis and, thus, RhoA is the likely target of CYK-4 GAP activity for cytokinesis. CYK-4 and a CYK-4:GFP fusion protein localize to the central spindle and persist at cell division remnants. CYK-4 localization is dependent on the kinesin-like protein ZEN-4/CeMKLP1 and vice versa. These data suggest that CYK-4 and ZEN-4/CeMKLP1 cooperate in central spindle assembly. Central spindle localization of CYK-4 could accelerate GTP hydrolysis by RhoA, thereby allowing contractile ring disassembly and completion of cytokinesis.

Key words: cell division • spindle midzone • *Caenorhabditis elegans* • Rho GTPase • kinesin

Introduction

The process of cytokinesis produces two daughter cells from a single parental cell and permanently segregates the products of the cell division cycle. Cytokinesis is one of the few processes in biology known to require coordination between microtubules and actin filaments. Indeed, in animal cells there are two steps in cytokinesis that rely on interactions between microtubules and the actin-based contractile ring (for reviews see Glotzer, 1997; Field et al., 1999). In the first instance, the microtubule-based mitotic spindle specifies the position of the contractile ring. This allows the division plane to be positioned so that the separated chromosomes are partitioned equally into the two daughter cells. Subsequently, after ingression of the cleavage furrow, there is a second step that depends on both microtubules and the contractile ring. Completion of cytokinesis requires the central spindle, which contains bundled, antiparallel microtubules. The molecular mechanisms underlying these two microtubule-dependent steps in cytokinesis are not known. In this report, we describe a protein that is essential for this second microtubule-dependent step and, thus, defines a functional link between the central spindle and the contractile ring.

The degree to which the progression of cytokinesis depends on the central spindle varies somewhat in different experimental organisms. In invertebrate embryos, a transient interaction between astral microtubules of the mitotic spindle and the cell cortex is sufficient to position the cleavage furrow (Rappaport, 1985). Furrows specified in this manner ingress, but if the spindle is removed, these furrows do not usually complete cytokinesis (Rappaport, 1985). In contrast, in *Drosophila* spermatocytes, contractile ring formation requires the central spindle (Giansanti et al., 1998). Similarly, in cultured mammalian cells, astral microtubules appear to be insufficient to induce furrow ingression, instead the presence or absence of a central spindle determines whether or not a cleavage furrow forms (Cao and Wang, 1996; Wheatley and Wang, 1996b; Eckley et al., 1997; Rieder et al., 1997; Savoian et al., 1999). Moreover, in cultured cells and in sea urchin embryos, the central spindle is also required for completion of cytokinesis

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(Wheatley and Wang, 1996a; Larkin and Danilchik, 1999). In *Caenorhabditis elegans* embryos, as in invertebrate embryos, only the later stages of cytokinesis appear to depend on the central spindle. Embryos depleted of the kinesin-like protein ZEN-4/CeMKLP1 fail to assemble the central spindle, yet cleavage furrows form and ingress, but cytokinesis does not proceed to completion (Powers et al., 1998; Raich et al., 1998). In summary, the initiation of cytokinesis depends on the central spindle in some but not in all organisms, whereas there appears to be a general requirement for the central spindle for the completion of cytokinesis in animal cells. While it is clear that the central spindle plays an important role in cytokinesis, the underlying mechanism remains elusive.

Cleavage furrow ingression is driven by the actin-based contractile ring. Like many actin-based structures, the contractile ring requires the RhoA GTPase for its assembly. Rho family GTPases are thought to act as molecular switches that cycle between inactive GDP-bound forms and active GTP-bound forms; their ability to exchange and hydrolyze GTP is regulated by additional factors, the so-called guanine nucleotide exchange factors (GEFs)[1] and GTPase activating proteins (GAPs). Inactivation of RhoA by the exoenzyme C3 (Aktories and Hall, 1989), inhibits cytokinesis in a wide variety of experimental settings by causing disassembly of cortical actin structures and the contractile ring (Kishi et al., 1993; Mabuchi et al., 1993; Moorman et al., 1996; Drechsel et al., 1997; O'Connell et al., 1999). Further, a Rho GEF is essential for cytokinesis (Prokopenko et al., 1999; Tatsumoto et al., 1999). GTP-bound RhoA interacts with a number of putative effectors including formins, Rho kinase, Citron kinase, and a regulatory subunit of myosin phosphatase (for review see Van Aelst and D'Souza-Schorey, 1997). The requirement for RhoA in cytokinesis may reflect its ability to regulate formins since members of the formin gene family are required for cytokinesis in budding yeast (BNI1/BNR1) (Imamura et al., 1997), fission yeast (Cdc12) (Chang et al., 1997), *Drosophila* (*dia*) (Castrillon and Wasserman, 1994) and *C. elegans* (*cyk-1*) (Swan et al., 1998). Several formins also bind to profilin (Chang et al., 1997; Evangelista et al., 1997; Imamura et al., 1997; Watanabe et al., 1997), a key regulator of actin polymerization. It is conceivable that GTP-bound RhoA promotes contractile ring assembly by activating actin polymerization via the formins and by activating myosin motor activity.

There is compelling evidence that the microtubule-based central spindle and the actin-based cleavage furrow are both essential for cytokinesis. How do these two cytoskeletal polymers interact? There are a few cases in which an interaction between the microtubule and actin cytoskeletal systems have been characterized. Examples include nuclear positioning in budding yeast (Carminati and Stearns, 1997; Fujiwara et al., 1999; Miller et al., 1999), spindle orientation in epithelial cells (Busson et al., 1998) and in certain asymmetrically dividing cells, such as the posterior blastomere of the two cell *C. elegans* embryo (Hyman and White, 1987; Waddle et al., 1994; Skop and White, 1998; Gönczy et al., 1999a). In these examples, there is evidence that the dynein–dynactin microtubule motor complex may mediate the interaction of microtubules with the cell cortex.

In this study, we have characterized the role of the *cyk-4* gene in the early divisions of the *C. elegans* embryo. We show that CYK-4 is required for the late stages of cytokinesis. Interestingly, *cyk-4* mutant embryos fail to assemble the central spindle. Positional cloning and localization studies revealed that the *cyk-4* gene encodes a novel GAP for the Rho family of GTPases that localizes to the central spindle. The missense mutation in the *cyk-4(t1689ts)* allele is found in a domain dispensable for GAP activity, suggesting that CYK-4 may have another function in addition to activating GTP hydrolysis by Rho family proteins. Accordingly, we find that CYK-4 and the kinesin-like protein ZEN-4/CeMKLP1 are interdependent for their proper localization. Based on these data, we propose a model by which CYK-4 acts in concert with ZEN-4/CeMKLP1 to assemble the central spindle. The concentration of CYK-4 to the central spindle would serve to target the GAP domain to the fully ingressed contractile ring, where it could promote GTP hydrolysis by RhoA, thereby facilitating the completion of cytokinesis.

Materials and Methods

Strains and Alleles

The *cyk-4(t1689ts)* allele was identified in a search for maternal effect lethal mutations on chromosome III (Gönczy et al., 1999b). The strains DR104, BW1535, BW1369, PD4792, and RW7000 were obtained from the *Caenorhabditis* Genetics Center. The strain EU716 containing the *zen-4(or153ts)* allele will be described in detail elsewhere (a gift from A. Severson, D. Hamill, and B. Bowerman, University of Oregon). The end points of the deficiency tDf10 are not molecularly defined, but it uncovers *cyk-4*, *lit-1*, and *bli-5* and it does not uncover *unc-64*.

The *cyk-4(t1689ts)* III, *zen-4(or153ts)* IV double mutant embryos were constructed as follows. PD4792 (mIs11 IV) was crossed with MG22 (*unc-32(e189)cyk-4(t1689ts)*/qC1 *dpy-19(e1259) glp-1(q339)* III) and picking an UncGFP F2 progeny that was then crossed to MG22 and a F2 progeny was isolated (strain MG212) that had the genotype *unc-32(e189) cyk-4(t1689ts)/qC1 dpy-19(e1259) glp-1(q339)* III, mIs11 IV. Strain EU716, *zen-4(or153ts)*, was crossed to MG22 and a DpyUncTs F2 progeny was isolated with genotype *unc-32(e189) dpy-18(e364)* III, *zen-4(or153ts)* IV was isolated, MG182. This was crossed to males from MG22 and an F2 progeny of genotype *unc-32(e189) dpy-18(e364)/qC1* III, *zen-4(or153ts)* IV was isolated (strain MG189). Males from MG22 were crossed to MG212 and the GFP+ F1 male progeny of this cross (genotype *unc-32 dpy-18/qC1* mIs11/+ or *unc-32 cyk-4/qC1* mIs11/+) were crossed to MG189, and a strain of genotype *unc-32 cyk-4/qC1* III; mIs11/*zen-4(or153ts)* IV was isolated. From this strain, we isolated a line of genotype *unc-32 cyk-4/qC1* III; *zen-4(or153ts)* IV and found that in thousands of progeny, no Uncs were produced. In parallel, we isolated a line of genotype *unc-32 cyk-4* III; mIs11/*zen-4(or153ts)* IV and found that in thousands of progeny, no non-GFP worms were produced.

Antisera

CYK-4–specific antisera were produced in rabbits using a His_6-CYK-4 fusion protein as an immunogen (containing amino acids 407–613 of cyk-4). A GST-CYK-4 fusion, containing amino acids 407–669 of CYK-4, was coupled to a Hi-Trap NHS resin (Amersham Pharmacia Biotech) and used to affinity purify anti-CYK-4 antibodies that were used at a final concentration of 1:300. The antibodies used for the studies are specific for CYK-4 since the staining can be blocked with antigen, a similar pattern is observed when anti-GFP antibodies are used to detect a CYK-4:GFP fusion construct, and the staining pattern is disrupted in cyk-4 mutant embryos. The rat monoclonal YOL 1/34 antitubulin antibody was used at a

[1]*Abbreviations used in this paper:* GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; GFP, green fluorescent protein; RNAi, RNA-mediated interference; ts, temperature-sensitive.

dilution of 1:200–500. Anti-GFP antibodies (Roche) were used at a dilution of 1:500. Antisera specific for ZEN-4/CeMKLP1 was provided by Bill Saxton and Susan Strome (University of Indiana, Bloomington, IN) and used at a dilution of 1:4,000. Antisera specific for AIR-2 was provided by Andy Golden (National Institutes of Health, Bethesda, MD) and used at a dilution of 1:1,000.

Genetic Mapping of CYK-4

The *cyk-4* locus maps under the deficiency tDf6 which deletes a large fraction of the right arm of LGIII. Embryos derived from mothers with the genotype *cyk-4(t1689ts)*/tDf6 also exhibit extensive furrow ingression. Recombination mapping using *unc-32(e189) cyk-4(t1689ts)/dpy-18(e364) unc-25(e156)* placed *cyk-4* distal to (or very close to) *unc-25* (23/23 Dpy non Unc's carried the *cyk-4* mutation). Recombination between *dpy-18 cyk-4*/RW7000, which carries several Tc1 elements including one on the cosmid F14F7, gave rise to 45 Dpy non-Ts animals, three of which lacked the TC1 insertion on F14F7, indicating that the *cyk-4* gene is distal to this cosmid. Crosses to strains carrying the deficiencies ctDf3, ctDf2, and tDf10 revealed that *cyk-4(t1689ts)* is not uncovered by ctDf3 and is uncovered by both ctDf2 and tDf10. Since tDf10 does not uncover *unc-64, cyk-4(t1689ts)* must be distal to *unc-64*.

Time-lapse Recordings

Time-lapse Nomarski imaging was performed as described previously (Jantsch-Plunger and Glotzer, 1999). Time-lapse imaging of CYK-4:GFP was performed on a Zeiss Axiovert microscope using a 100×/1.3 neofluor objective. The illumination source, an Atto-arc HBO-103, was reduced to 50% intensity. An intensified cooled CCD camera (GenIV pentamax; Princeton Instruments) was used for image acquisition. The camera and other electronics were controlled with MetaMorph software (Universal Imaging). Typical acquisition times were 40–80 ms. Every 10 s, four to five fluorescent images were acquired at different focal planes and a Nomarski image was acquired. The fluorescent images were projected onto a single frame using the maximum intensity from the stack of images. Under these conditions, embryos could be filmed for >1 h without affecting the cell cycle timing or pattern of cell divisions.

Rescue Experiments

To identify the *cyk-4* gene in this region, cosmid DNA (from stocks provided by Alan Coulson, Sanger Center, Hinxton, UK) was coinjected with the *rol-6(su1006)* dominant marker (Mello et al., 1991) into the gonad of *unc-32 cyk-4/qC1* worms. Heterozygous F1 hermaphrodites that carried the rol-6 dominant marker were cloned to individual plates at 25°C and the presence of Unc progeny, indicating zygotic rescue of the *cyk-4* mutation, was assessed. Individual *unc-32 cyk-4* worms carrying the extrachromosomal arrays were cloned to individual plates to assess the extent of germline rescue. The *cyk-4* genomic rescue construct MP17, contains a 4.9-kb genomic XbaI fragment excised from K08E3 and inserted into pBS-KS+.

RNA Interference

Approximately 500 bp of DNA corresponding to the predicted coding regions of Rho (Y51H4A.B), Rac-1 (C09G12.8B), Cdc42 (R07G3.1), F22E12.2, Y32F6B.3, K08D3.9, K08E3.2, K08E3.3, K08E3.4, K08E3.6, K08E3.7, K08E3.8 were amplified by PCR and cloned into pGEM-T (Promega). Double stranded RNA was transcribed (Ambion), annealed, and injected into the gonads of wild-type N2 hermaphrodites as described (Fire et al., 1998).

Production of CYK-4:GFP Transgenes

The GFP cassette from vector pPD119.16 (a gift from A. Fire, Carnegie Institute, Baltimore, MD) was excised with BspLUIII and inserted into the unique NcoI site of MP17 (see above). This construct was linearized with XbaI, and complex arrays containing linearized genomic DNA and linearized rol-6(su1006) DNA were mixed in a ratio of 1:100:1 and injected into *unc-32(e189) cyk-4(t1689ts)/qC1* hermaphrodites. Rolling F1 heterozygotes were singled out at 25°C and rolling Unc F2 animals were picked. A line MG110, *cyk-4 xsEx1[cyk-4:GFP],* was obtained that gave stable rescue of the *cyk-4(t1689ts)* mutation.

The structure of the *cyk-4* gene was established by analysis of a large number of expressed sequence tag sequences available in the sequence databases at the Sanger Center and the National Institute of Genetics and by sequencing the clones yk63D6 and yk104g12 (provided by Yuji Kohara, National Institute of Genetics). The structure of the gene is identical to the structure predicted by the *C. elegans* Genome Consortium.

Immunolocalization

Immunolocalization studies were performed as previously described (Jantsch-Plunger and Glotzer, 1999). In brief, gravid hermaphrodites were placed on aminopropyl-silane–treated slides, a coverslip was added, and sufficient pressure to extrude the embryos was applied. The slide was placed into liquid nitrogen. The coverslip was removed while the sample was still frozen, the preparation was fixed with –20°C methanol, and antibody staining was performed according to standard procedures.

Biochemical Analysis of CYK-4

The coding regions of Rho, Rac, and Cdc42 were PCR-amplified and cloned into pET28b with a COOH-terminal polyhistidine tag. The GTPases were expressed at 25°C and purified using Ni^{2+}-NTA agarose (QIAGEN). Proteins were dialyzed into 50 mM Tris, pH 7.5, 50 mM NaCl, and 5 mM $MgCl_2$, and quick frozen. The GAP domain of CYK-4 (amino acids 407–669) was cloned into pGEX4T-1. Proteins were expressed at 25°C and purified using GSH-agarose (Sigma Chemical Co.). Proteins were dialyzed into 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, and 1 mM DTT and quick frozen. To assess GAP activity, 15 pmol of the GTPases were loaded with 1 pmol α-[^{32}P]GTP in 20 mM Tris, pH 7.6, 4 mM EDTA, 25 mM NaCl, 1 mM DTT, 1 mM ATP, and 0.1 mg/ml BSA at room temperature. The sample was placed on ice and $MgCl_2$ was added to 17 mM. GST-CYK-4-GAP was added at the indicated concentrations and, at intervals, samples were taken by dilution into 2% SDS, 20 mM EDTA. Aliquots were spotted onto TLC plates (PEI-cellulose; Machery-Nagel) and developed in 1 M LiCl. The plates were dried and exposed using a Storm PhosphorImager (Molecular Dynamics) and the data analysis was performed using the public domain NIH Image program (developed at the US National Institutes of Health; http://rsb.info.nih.gov/nih-image/).

Results

cyk-4 Mutants Initiate, but Do Not Complete, Cytokinesis

The *cyk-4(t1689ts)* allele was isolated in a screen for maternal effect embryonic lethal mutations on chromosome III (Gönczy et al., 1999b). The cyk-4 locus is defined by a single, temperature-sensitive (ts), allele. The phenotype of embryos derived from homozygous *cyk-4* hermaphrodites at the restrictive temperature (hereafter referred to as *cyk-4* mutant embryos) during the first division is shown in Fig. 1 B. *cyk-4* mutant embryos appear normal until cytokinesis, except that defects in polar body extrusion are frequently observed (data not shown). The first cleavage furrow forms at the correct time and place, it ingresses extensively, but, invariably, it regresses. Quantitation of the extent of furrow ingression in *cyk-4* mutant embryos reveals that, on average, furrows ingress to 73 ± 13% (n = 14) of the egg diameter. A multipolar spindle develops in the second cell cycle and the process of furrow ingression and regression occurs again. This pattern is repeated until the embryos become grossly disorganized.

Although the majority of cell divisions in worm development occur early in embryogenesis, germline cells and many cells in the nervous system are produced during postembryonic development (Sulston and Horvitz, 1977). A number of mutations in genes required for cell division cause worms to become sterile and uncoordinated (O'Connell et al., 1998; Woollard and Hodgkin, 1999). To determine if *cyk-4* is required postembryonically, we per-

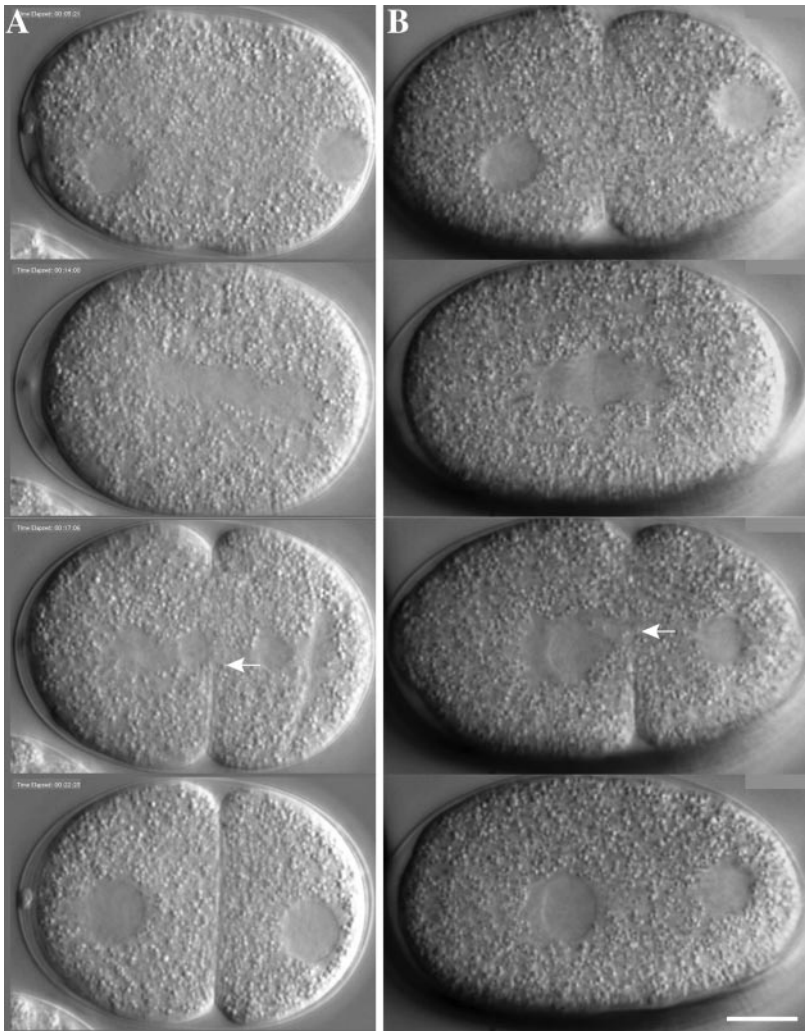

Figure 1. cyk-4(t1689ts) mutant embryos fail to complete cytokinesis. Wild-type embryos (A) and embryos from *cyk-4* mutant hermaphrodites (B) were dissected from young adults, mounted on agarose pads, and observed by time-lapse Nomarski microscopy. The ingressing cleavage furrow is indicated (arrows). Bar, 10 μm.

formed temperature shift experiments with worms homozygous for the *cyk-4(t1689ts)* allele (Table I). Homozygous animals grown at 16°C are viable and fertile. Homozygous *cyk-4* animals that were shifted to 25°C at the L4 stage produce embryos with the cytokinesis defects described above. Homozygous animals shifted to 25°C at earlier stages, either fail to hatch or become sterile and uncoordinated, depending on the time of the temperature shift. Thus, cyk-4 is required postembryonically, perhaps because of its role in cell division. Unexpectedly, animals shifted at the L2/L3 stages become highly uncoordinated adults, even though most motor neurons would be expected to have completed all their divisions at the time of the shift. This may suggest that CYK-4 has additional roles besides its role in cell division. However, we cannot rule out the possibility that some of the cells in the ventral nerve cord divide later in *cyk-4* mutants.

To investigate why *cyk-4* mutant embryos fail to complete cytokinesis, we localized actin and tubulin in wild-type and *cyk-4* mutant embryos. Both wild-type and mu-

Table I. Phenotype of cyk-4 Mutant Animals at the Restrictive Temperature

Genotype	Stage at time of shift to 25°C	Percent viable	Phenotype of surviving progeny	N
N2 (wild type)	embryos	98.8	wild-type	485
cyk-4	(not shifted)	70.2	fertile	198
cyk-4	embryos	1.5	sterile, highly Unc	324
cyk-4	L1 larvae	100.0	sterile, highly Unc	89
cyk-4	L2/L3	100.0	sterile, highly Unc	55
cyk-4	L4	100.0	fertile, lay dead embryos	52
cyk-4, xsEx1[cyk-4:GFP]	embryos	59.4	fertile	350

Except for the N2 control, the complete genotype of the strain was *unc-32(e189) cyk-4(t1689ts)*. Gravid hermaphrodites were allowed to lay embryos for 2 h at the permissive temperature. The adult was removed and the number of embryos was counted. After 24 h at the indicated temperature, the number of unhatched embryos was counted.

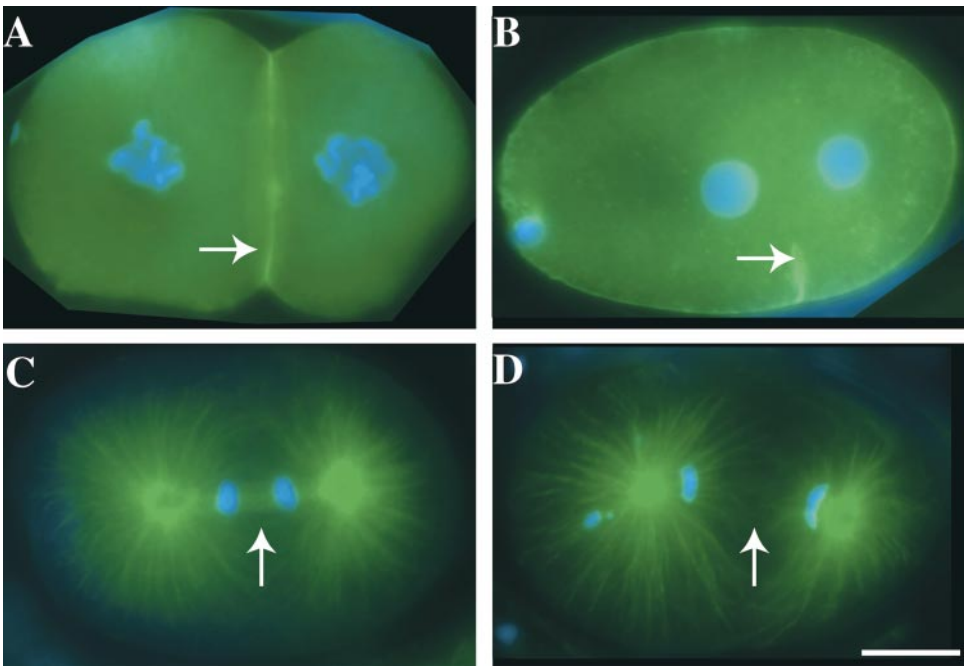

Figure 2. *cyk-4* mutant embryos produce ingressing cleavage furrows, but do not form a prominent central spindle. Wild-type (A and C) and *cyk-4* mutant embryos (B and D) were fixed and stained for actin (green) and DNA (blue) (A and B), and tubulin (green) and DNA (blue) (C and D). Bar, 10 μm.

tant embryos contained ingressing cleavage furrows that stain with an antiactin antibody (Fig. 2, A and B). The metaphase spindles of *cyk-4* mutant embryos appeared normal (data not shown). However, spindle morphology during early anaphase was significantly different in mutant embryos as compared with the wild type. In wild-type embryos (20/20), prominent microtubule bundles form between the separating masses of chromatin, forming the central spindle (Fig. 2 C). In cyk-4 mutant embryos (9/10), such bundles were largely reduced and disorganized (Fig. 2 D). We conclude that CYK-4 is required for the organization of the central spindle during anaphase. Since the central spindle is required for cytokinesis, it is possible that the *cyk-4* mutant embryos fail to complete cytokinesis because they fail to assemble the central spindle.

Cloning of the cyk-4 Gene

To investigate the molecular basis for the phenotypes described above, we first sought to map the *cyk-4* locus and clone the affected gene (for details see Materials and Methods). The *cyk-4* gene maps distal to *unc-64* on the extreme right arm of LG III (Fig. 3). The *cyk-4* gene was identified by functional rescue of the zygotic requirement for *cyk-4* using pools of cosmids. A pool of three cosmids (ZK520, W06F12, and K08E3) allowed *cyk-4* homozygotes to hatch and develop to adulthood at 25°C. These cosmids were injected individually and cosmid K08E3 contained rescuing activity. Further subcloning revealed that a 4.9-kb genomic fragment, predicted to contain the complete K08E3.6 gene and no other intact gene, could rescue the *cyk-4* zygotic and germline phenotypes. Finally, the coding region was amplified from DNA derived from *cyk-4(t1689)* homozygotes and sequenced and a single point mutation was identified that differed from the sequence provided by the genome project; this mutation was not observed in another line derived from the same parental strain. These data show that the defect in *cyk-4(t1689ts)* embryos is due to a point mutation in the K08E3.6 gene.

The predicted protein product of the *cyk-4* gene has a COOH-terminal domain that contains the consensus motifs of GTPase activating proteins for Rho family GTPases (Fig. 3). Adjacent to the COOH-terminal GAP domain is a C1 domain that is predicted to bind to diacylglycerol or phorbol esters. At the NH_2 terminus of the protein is a 90–amino acid region predicted to form a coiled-coil domain. The S15L point mutation found in *cyk-4(t1689ts)*, is located NH_2-terminal to the coiled-coil domain. Human and mouse proteins with structural similarities to *cyk-4* have been described (Toure et al., 1998; Wooltorton et al., 1999). These genes are expressed in a variety of proliferating tissues. In addition, the *Drosophila* sequence database contains an entry (accession no. AC005977 (CLOT 94)) that, together with CYK-4 and the previously mentioned human gene, share a common structure consisting of ~650 amino acids, an NH_2-terminal coiled-coil domain and a conserved COOH terminus containing C1 and GAP domains. The structural conservation of CYK-4 suggests that its function is conserved among metazoans.

Since the *cyk-4(t1689ts)* mutation may contain residual activity, we used RNA-mediated interference (RNAi) to deplete embryos of CYK-4 protein. RNAi of the predicted open reading frame K08E3.6 generated multinucleate embryos, which exhibited a similar phenotype to that of the *cyk-4* mutant including loss of the central spindle (Fig. 4 D) and incomplete cytokinesis (not shown). Interestingly, the gonads of *cyk-4(RNAi)* animals become disorganized 30 h postinjection (Fig. 4 B) and irregularly sized embryos are produced, suggesting that CYK-4 acts not only during embryonic and zygotic development, but also in the female germline.

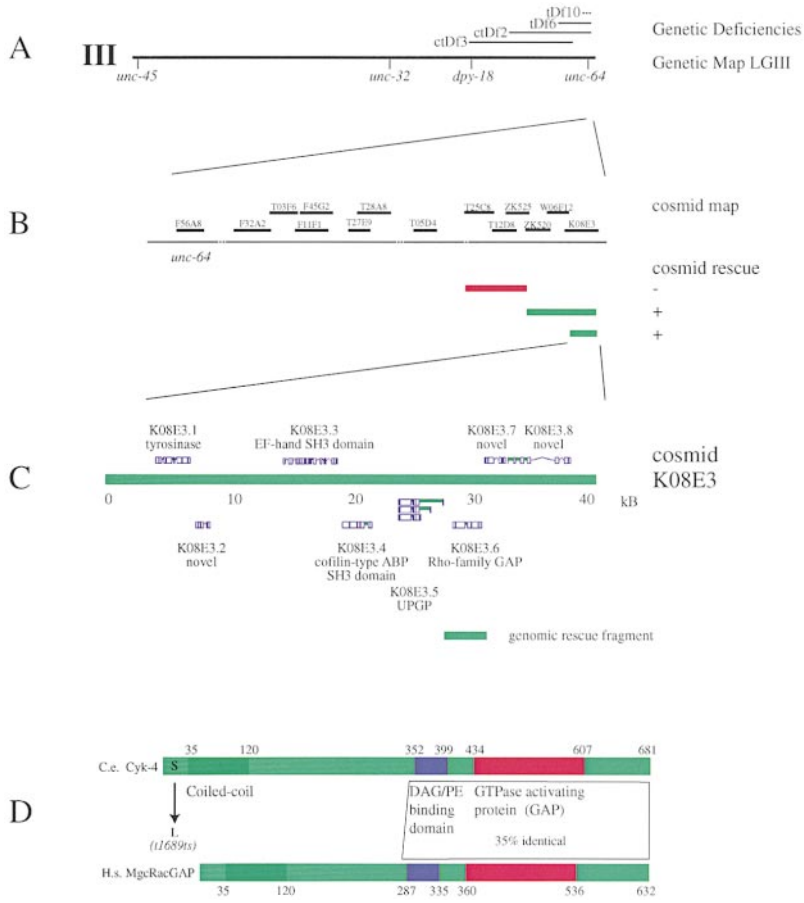

Figure 3. Positional cloning of the *cyk-4* locus. (A) A schematic of LGIII showing the positions of various loci and the extent of the deficiencies. *cyk-4* is uncovered by tDf10, tDf6, and ctDf2, but not ctDf3. (B) An enlargement of the physical map from the *unc-64* locus until the end of LGIII. The ability of various cosmid pools to rescue *cyk-4* is indicated. (C) A map of the predicted genes on K08E3. All the predicted genes except for K08E3.1 and K08E3.5 were inactivated by RNAi and only RNAi of K08E3.6 produced multinucleate embryos. (D) A schematic representation of the domain structure of CYK-4 and its human orthologue. The position of the point mutation identified in *cyk-4(t1689ts)* is indicated.

Biochemical Activity of the CYK-4 GAP Domain

The presence of a Rho family GAP domain suggests that CYK-4 may regulate one or more GTPases of the Rho branch of the GTPase superfamily. To determine whether CYK-4 is active as a GAP and whether its GAP activity is restricted to particular members of the Rho subfamily, a recombinant fusion protein containing GST and the CYK-4

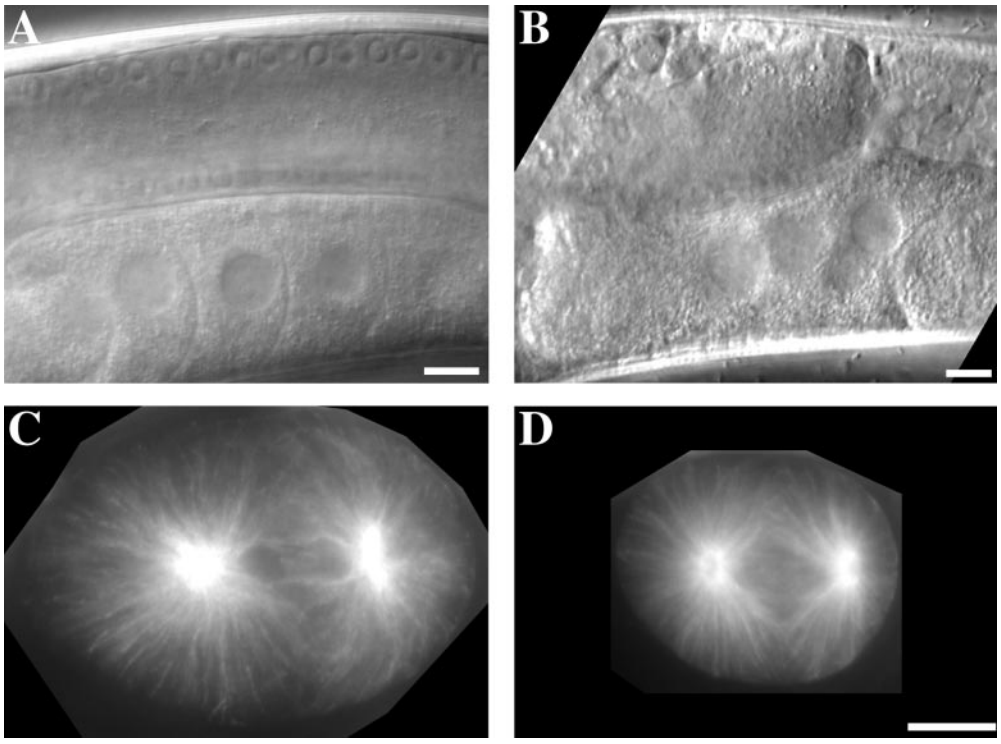

Figure 4. cyk-4(RNAi) causes disorganization of the proximal gonad and affects formation of the central spindle. Young adults were injected with *cyk-4* dsRNA, and the injected animals were analyzed 30 h after injection (B). The gonad of an uninjected worm is shown for comparison (A). Wild-type embryos (C) and embryos from *cyk-4(RNAi)*–injected animals (D) were fixed and stained for tubulin. Bars, 10 μm.

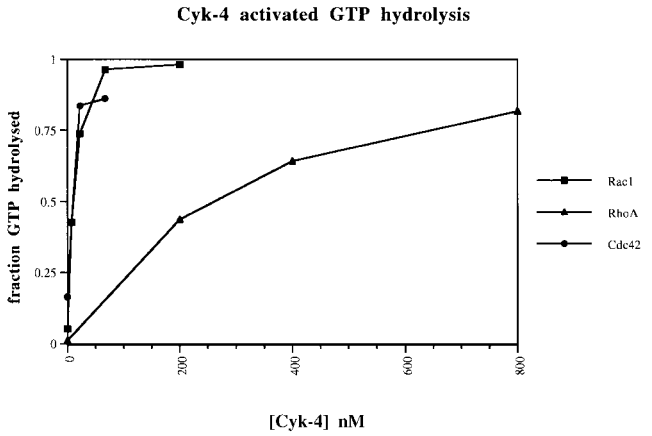

Figure 5. CYK-4 enhances GTP hydrolysis by Rho, Rac, and Cdc42. The GTPases were preloaded with α-[^{32}P]GTP, and then GST-CYK-4-GAP or GST was added at the indicated concentration. Samples were taken at 2-min intervals, the labeled nucleotide was resolved by thin layer chromatography, and the fraction of GTP and GDP was quantitated. This graph shows the fraction of GTP hydrolyzed at the 2-min time point as a function of CYK-4 concentration. These data are representative of at least three independent experiments.

GAP domain was prepared and GTP hydrolysis assays were performed with recombinant *C. elegans* Rho, Rac, and Cdc42. The GAP domain of CYK-4 promotes GTP hydrolysis by all three tested GTPases (Fig. 5). However kinetic differences were observed. At the conditions used in the assays, we found that the CYK-4 GAP domain is more active towards Rac and Cdc42 than towards Rho. The human orthologue has a similar activity profile in vitro (Toure et al., 1998). However, since CYK-4 has activity towards all three GTPases, these data are not sufficient to determine the in vivo targets of the CYK-4 GAP domain.

We next used RNAi to determine which, if any, of the Rho family GTPases are required for cytokinesis in the *C. elegans* embryo. We performed RNAi experiments with RhoA, Rac1, Cdc42, and three additional GTPases found in the genome that fall into the Rho subfamily. Approximately 90% of *Rho(RNAi)* embryos exhibit cytokinesis defects in the first and/or second cell cycle (Table II and Fig. 6). In most embryos, furrow ingression was inhibited. Interestingly, central spindles assemble in *RhoA(RNAi)* embryos (data not shown). In contrast, 88% of *Cdc42 (RNAi)* embryos complete cytokinesis normally. However, a distinct defect in the early embryo is observed in 54% of *Cdc42(RNAi)* embryos; defects in spindle positioning are observed in P0 and/or P1. A minority (12%) of *Cdc42(RNAi)* embryos fail to initiate cytokinesis; in most cases, these embryos appear osmotically swollen even when provided with osmotic support. *Rac(RNAi)* embryos hatch with high efficiency and did not exhibit a detectable phenotype in the early embryo. RNA interference experiments with the additional GTPases either alone or in combinations did not reveal any additional defects in the early embryo. Thus, RhoA is the only member of the Rho family that is clearly required for cytokinesis and is, therefore, likely to be the critical target for the CYK-4 GAP domain.

The Subcellular Localization of CYK-4

We next determined the subcellular localization of the CYK-4 protein. CYK-4 localization is cell cycle–dependent (Fig. 7). In interphase cells, CYK-4 is present in the cytoplasm and slightly concentrated in the nucleus. CYK-4 is also highly concentrated in a spot at the anterior of the embryo, DNA labeling reveals that this localization corresponds to the site of polar body extrusion. As embryos enter mitosis, CYK-4 protein concentrates around the mitotic spindle. In early anaphase, CYK-4 concentrates to the central spindle. As the cleavage furrow ingresses, CYK-4 becomes highly concentrated on the central spindle into a structure that often appears ring shaped (not shown). Upon completion of cytokinesis, CYK-4 staining persists at division remnants. CYK-4 is occasionally observed in cytoplasmic, ringlike structures averaging 1.2 μm in diameter (not shown).

To determine if CYK-4 localizes to the central spindle before the onset of cleavage furrow ingression, we investigated the dynamics of CYK-4 localization in live embryos. To accomplish this goal, we generated a transgenic line ex-

Table II. RNAi Phenotype of Rho Family Members

Injected dsRNA	Locus	Percent cytokinesis defective (N)	No. of time-lapse recordings	No. of cytokinesis-defective	Additional phenotypes
RhoA	Y51H4A.B	95 (175)	15	15 (100%)	
Rac-1	C09G12.8B	Not emb. lethal	5	0	
Cdc42	R07G3.1	12 (74)	22	3 (14%)	symmetric 1st division (5/22); no rotation in P1(12/22); rotation of AB (2/22)
Others	F22E12.2	Not emb. lethal			
	Y32F6B.3	Not emb. lethal			
	K03D3.9	Not emb. lethal			

Young adult hermaphrodites were injected with the indicated dsRNAs and broods of laid embryos were scored for embryonic lethality. dsRNAs that induced embryonic lethality were further characterized by dissecting embryos and scoring for multinucleate embryos and by performing time lapse recordings and evaluating cytokinesis, spindle orientation in the single cell embryo (symmetric or asymmetric first cleavage), and spindle positioning in the P1 and AB blastomeres.

Figure 6. rhoA(RNAi) causes cytokinesis defects and *cdc42 (RNAi)* causes defects in spindle positioning. Young adults were injected with the indicated dsRNAs (Table II), and the embryos produced by the injected worms were analyzed by time-lapse Nomarski microscopy. Sequential images from a (A) wild-type, (B) *rhoA(RNAi)*, and (C) a *cdc42(RNAi)* embryo are shown. Bar, 10 μm.

pressing a CYK-4:GFP fusion and followed its localization by time-lapse microscopy. The CYK-4:GFP fusion is partially functional since *cyk-4 xsEx1[cyk-4:GFP]* animals are viable and fertile at 25°C, whereas the parental *cyk-4* strain is inviable at 25°C. However, the fusion construct does not fully rescue the mutation, since ~40% of embryos produced by this line fail to hatch (Table I).

We used low light level fluorescence microscopy to visualize CYK-4:GFP in living embryos. Embryos were imaged using a multimode imaging system whereby a series of z-sections and a Nomarski image were recorded every 10 s. The fluorescent images from each time point were projected to form a single image. These recordings reveal that CYK-4 accumulates on the central spindle before the initiation of furrowing (Fig. 8; 3:40). CYK-4 localized to the central spindle becomes compressed into a bright spot that persists at the division remnant. The remnant persists for several cell cycles, although instances were observed where the remnant (sometimes from the polar body) detaches from the cortex and is observed as a discrete spot in the cytoplasm. This detachment of CYK-4 from division remnants likely accounts for the CYK-4 rings seen in fixed specimens. We conclude that CYK-4 localization on the central spindle precedes furrow ingression.

CYK-4 and ZEN-4/CeMKLP1 Are Functionally Interdependent

There are remarkable similarities between CYK-4 and the kinesin-like protein ZEN-4/CeMKLP1 (Powers et al., 1998; Raich et al., 1998). *zen-4* mutant embryos also initiate, but fail to complete, cytokinesis. They also fail to assemble a robust central spindle in early anaphase. Furthermore, ZEN-4 localizes to the central spindle and persists at division remnants after completion of cytokinesis. To test whether these proteins functionally interact, we first assessed whether CYK-4 and ZEN-4/CeMKLP1 colocalize. Embryos expressing CYK-4:GFP were fixed and CYK-4 and ZEN-4 were localized simultaneously (using an anti-GFP antibody to detect CYK-4:GFP). The two proteins colocalize both on central spindle structures and on division remnants (Fig. 9, A–C). We next investigated if ZEN-4 localization requires functional CYK-4 protein. ZEN-4 staining of *cyk-4* mutant embryos reveals that ZEN-4 localization to the central spindle is absent (Fig. 9 E), al-

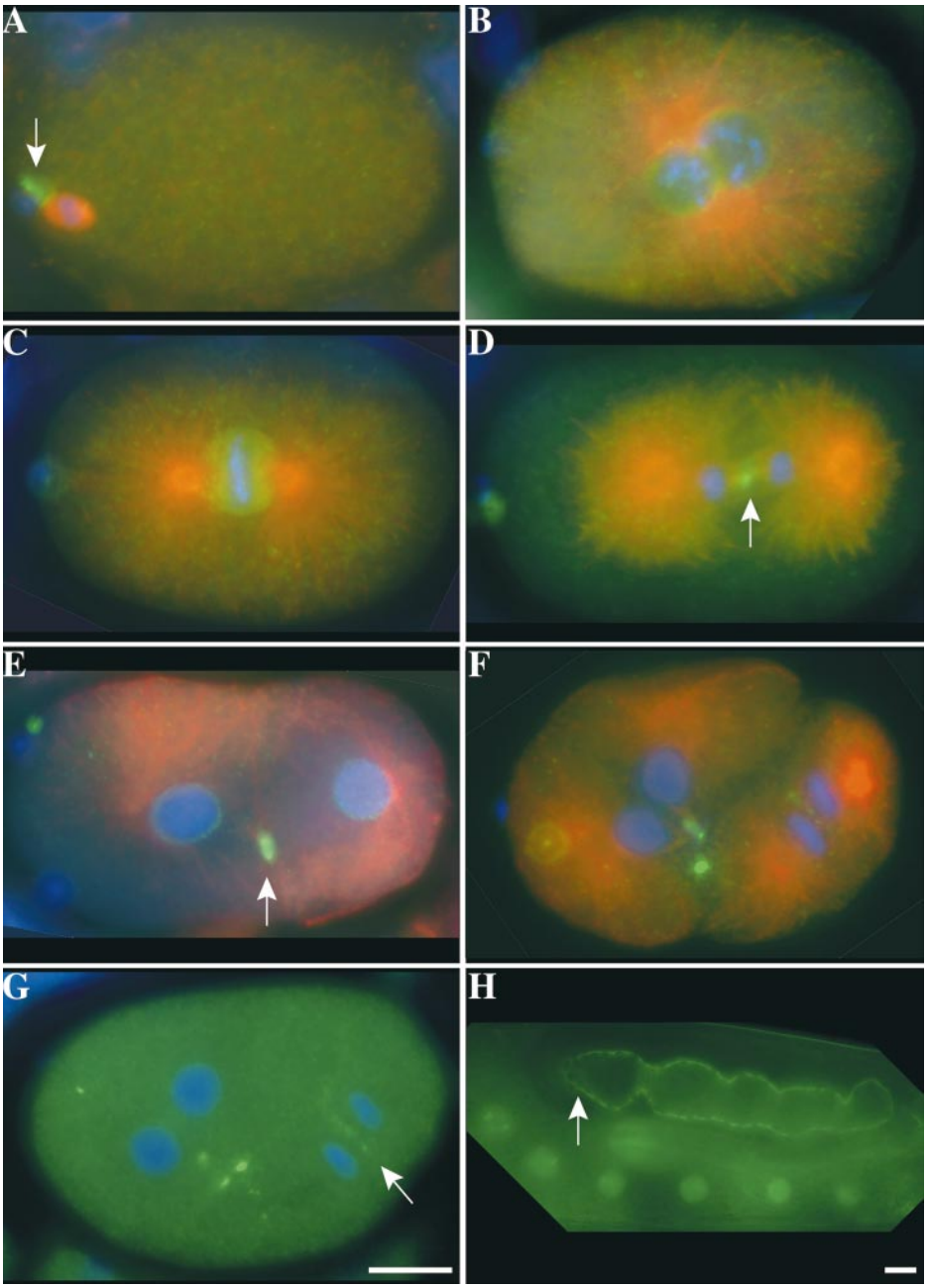

Figure 7. CYK-4 localizes to the central spindle and division remnants. (A–F) Wild-type embryos were fixed and stained for CYK-4 (green), tubulin (red), and DNA (blue). The localization of CYK-4 to the central spindle (arrow) in a single cell (D) and a two cell embryo (F) is shown. The localization of CYK-4 to the division remnant from the polar body (A), and between the AB and P1 blastomeres is indicated with arrows (E). (G) An embryo from a line expressing CYK-4:GFP is stained with anti-GFP antibodies. The same structures are seen as with CYK-4 antibodies (arrow). (H) The intrinsic fluorescence of the gonad of a worm expressing CYK-4:GFP. CYK-4 is seen at the incomplete membranes of the syncytial gonad (arrow) and in oocyte nuclei. Bars, 10 μm.

though staining of some microtubule bundles in the spindle midzone could be detected using an AIR-2 antibody (Fig. 9 G). Thus, recruitment of ZEN-4 to the central spindle is CYK-4–dependent. We next tested whether maintenance of ZEN-4 at division remnants requires functional CYK-4. *cyk-4* mutant embryos grown at 16°C were shifted to 25°C for 15 min before fixation and staining with anti–ZEN-4 antibodies. The number of cells and the number of division remnants labeled with the anti–ZEN-4 antibody were counted. Embryos maintained at the permissive temperature had a large number of ZEN-4 staining division remnants (Table III), whereas the embryos shifted to the nonpermissive temperature lacked defined staining of division remnants. Thus, both recruitment of ZEN-4 to the central spindle and its maintenance at division remnants is CYK-4–dependent. The reverse experiment was conducted with a temperature-sensitive allele of *zen-4(or153ts)*. In this case, CYK-4 staining at division remnants was observed in *zen-4* mutant embryos at the permissive temperature, but this staining disappeared upon a brief shift to the nonpermissive temperature (Fig. 9, H and I and Table III). Thus, maintenance of CYK-4 at division remnants is ZEN-4–dependent. We conclude that CYK-4 and ZEN-4/CeMKLP1 colocalize, and that the two proteins are interdependent for their localization.

We were interested in determining if embryos carrying mutations in both *cyk-4* and *zen-4* are distinguishable from the single mutants. Two strains were built: one strain was homozygous for *zen-4(or153ts)* and heterozygous for *cyk-4(t1689ts)*, and the second strain was homozygous for *cyk-*

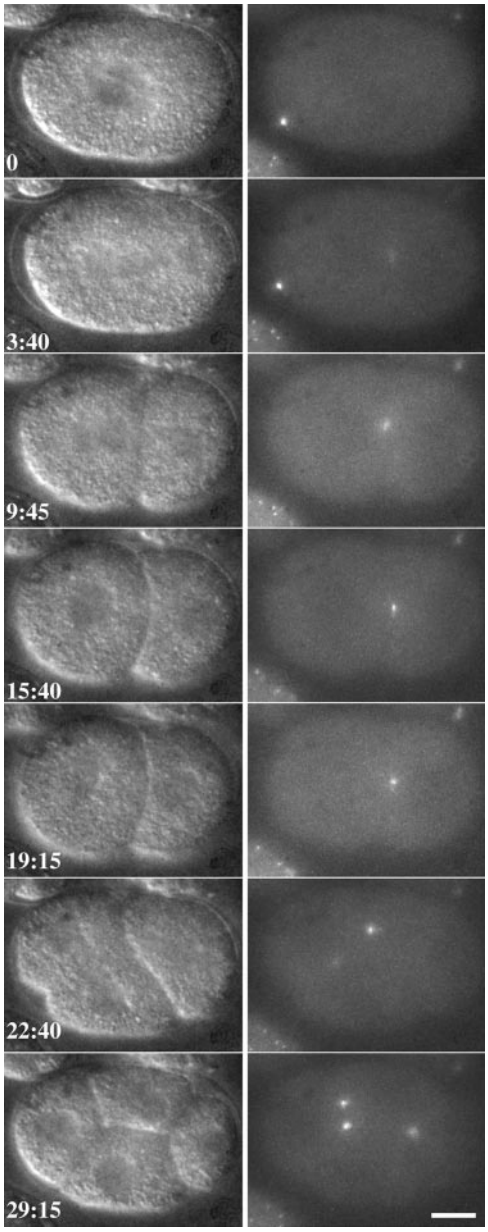

Figure 8. Time-lapse analysis of CYK-4:GFP. An embryo from a line expressing CYK-4:GFP was imaged using low light level microscopy. The central spindle localization of CYK-4 is observed before furrow ingression. Bar, 10 μm.

4(t1689ts) and heterozygous for *zen-4(or153ts)*. Both strains were viable at 16°C, but they failed to produce doubly homozygous larvae. We found that worms of genotype *unc-32(e189) cyk-4(t1689ts) /qC1* III, *zen-4(or153ts)* laid a fraction of embryos that arrested during embryonic development, typically before the comma stage. Thus, *cyk-4(t1689ts)* and *zen-4(or153ts)* are synthetically lethal.

Discussion

The division of a cell into two daughters requires dynamic interactions between the microtubule-based mitotic spindle and the actin-based contractile ring. In animal cells, the position of the cleavage furrow, an actomyosin-based structure, is determined by the mitotic spindle in a manner that is poorly understood. In recent years, it is has become clear that the central spindle also plays an important role in cytokinesis. To gain insight into this fundamental cellular process, we have analyzed the cytokinesis-defective mutant, *cyk-4*. Cytological analysis reveals that *cyk-4* mutant embryos fail to assemble the central spindle. Though lacking a central spindle, *cyk-4* mutant embryos furrow extensively, but they fail to complete cytokinesis. The mutation responsible for the *cyk-4* phenotype was found to be a missense mutation in a gene encoding a Rho family GAP that, in vitro, stimulates GTP hydrolysis by Rho, Rac, and Cdc42. CYK-4 localizes to the central spindle and to cell division remnants. CYK-4 colocalizes with the ZEN-4/CeMKLP1 kinesin-like protein. Moreover, CYK-4 and ZEN-4/CeMKLP1 are interdependent for their localization. We conclude that the CYK-4 GAP and the ZEN-4/CeMKLP1 kinesin-like protein cooperate to assemble the central spindle. Furthermore, we propose that a concentrated source of CYK-4 GAP on the central spindle could downregulate the RhoA GTPase and thereby promote the late stages of cytokinesis.

CYK-4–dependent Assembly of the Central Spindle: A Model

In *cyk-4* mutant embryos the robust microtubule bundles that constitute the central spindle do not form. Instead, the spindle develops into two mitotic asters separated by a few overlapping, disorganized, microtubules. A similar phenotype is observed in *zen-4* mutant embryos (Powers et al., 1998; Raich et al., 1998). Thus, both the ZEN-4/

Table III. Cyk-4 and Zen-4 Are Interdependent for Their Localization to Division Remnants

Genotype	Temperature	Total no. of stained remnants	Total no. of cells	Average no. of remnants/cell	No. of embryos
	℃				
N2 (wild type)	25	110	137	0.80	16
zen-4(or198ts)	16	61	83	0.73	10
zen-4(or198ts)	25 (18 min)	11	147	0.07	24
N2 (wild type)	25	150	205	0.73	22
cyk-4(t1689ts)	16	61	98	0.62	10
cyk-4(t1689ts)	25° (15 min)	10	176	0.06	19

Wild-type and *zen-4(or153ts)* embryos grown at the indicated temperatures were fixed and stained for Cyk-4. Similarly, wild-type and *cyk-4(t1689ts)* embryos, grown at the indicated temperatures, were fixed and stained for Zen-4/CeMKlp1. The no. of cells in each embryo and the no. of remnants staining with Cyk-4 or Zen-4 antibodies was counted.

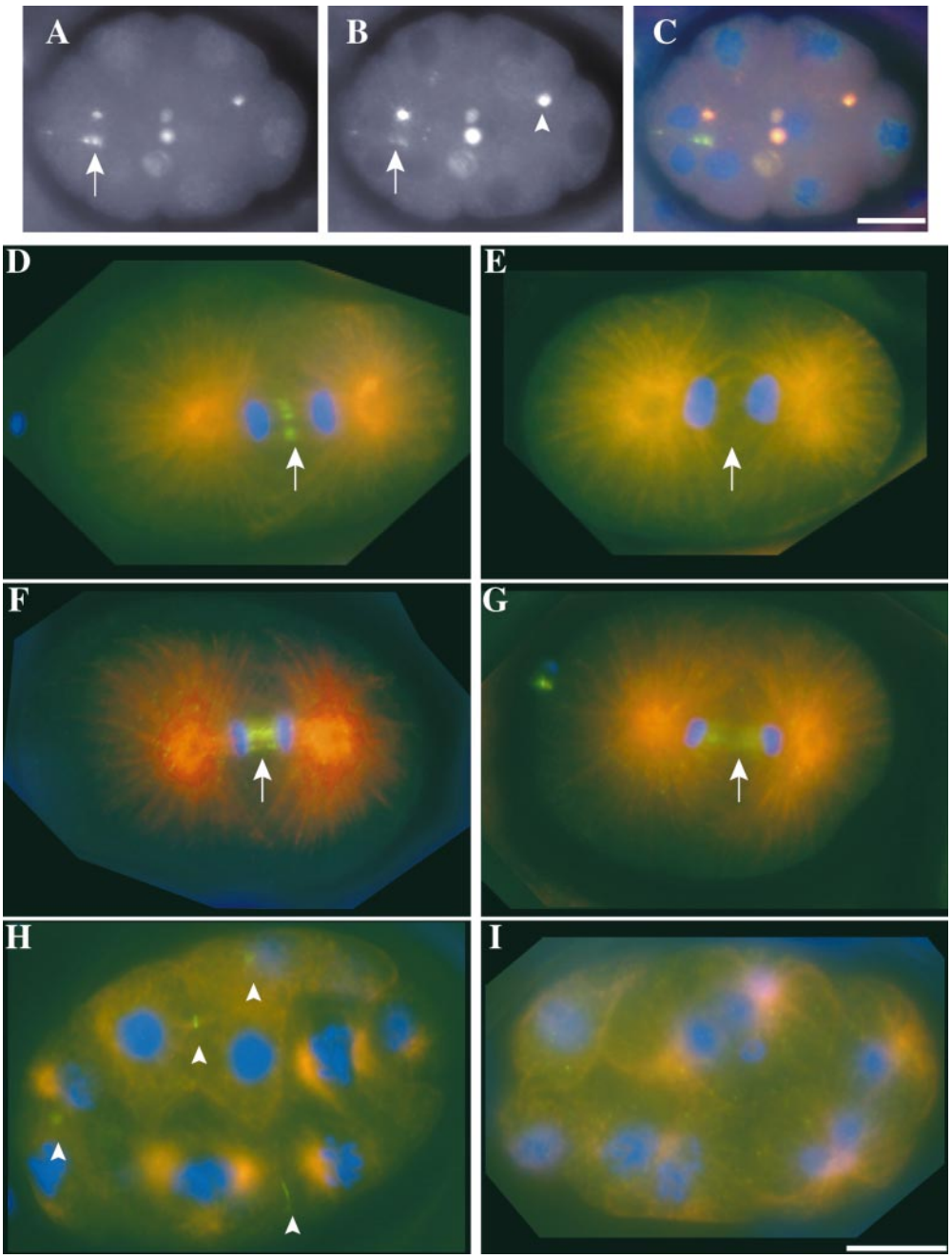

Figure 9. CYK-4 and ZEN-4/CeMKLP1 colocalize and are interdependent for their localization. An embryo expressing CYK-4:GFP stained for GFP (A), ZEN-4/CeMKLP1 (B), and the merged image (C). CYK-4 and ZEN-4/CeMKLP1 colocalize at division remnants (arrow) and central spindle (arrowheads) structures. ZEN-4 localization to the central spindle is CYK-4–dependent (arrow). Wild-type (D) and *cyk-4(t1689ts)* embryos (E) were fixed and stained for ZEN-4/CeMKLP1 (green), tubulin (red), and DNA (blue). AIR-2 localization to the central spindle is CYK-4–independent (arrow). Wild-type (F) and *cyk-4(t1689ts)* embryos (G) were fixed and stained for AIR-2 (green), tubulin (red), and DNA (blue). CYK-4 maintenance to division remnants (arrowheads) is ZEN-4/CeMKLP1–dependent. *zen-4(or153ts)* worms were maintained at 16°C and either fixed immediately (H) or shifted to 25°C for 18 min (I). Embryos were fixed and stained for CYK-4 (green), tubulin (red), and DNA (blue). Bars, 10 μm.

CeMKLP1 kinesin-like protein and the CYK-4 GAP are essential for this microtubule bundling. The *Drosophila* orthologue of ZEN-4/CeMKLP1 is also required for cytokinesis, though it seems to be required for all aspects of furrow ingression (Adams et al., 1998). Members of the MKLP1 subfamily of kinesin-like proteins have microtubule bundling activity in vitro (Nislow et al., 1992; Kuriyama et al., 1994). However, in vivo, ZEN-4–mediated microtubule bundling requires CYK-4.

How could CYK-4 and ZEN-4 cooperate to assemble the central spindle? We propose that a complex containing multiple motor proteins could specifically localize to overlapping, antiparallel microtubules (Fig. 10). If such a motor complex transits along a microtubule, it might continue to an end and dissociate. However, if such a motor complex transited along a microtubule in the vicinity of an antiparallel microtubule, the complex might bind simultaneously to both microtubules and attempt to move alternately in opposite directions, the net result being that the complex would concentrate in the region of microtubule overlap. Since CYK-4 does not have a microtubule motor domain, yet it is essential for the formation of the central spindle, we propose that CYK-4 forms a complex with multiple ZEN-4 homodimers that localizes to and stabilizes overlapping antiparallel microtubules (Fig. 10).

Is the RhoGAP activity of CYK-4 necessary to promote microtubule bundling by ZEN-4? We propose that central spindle assembly is unlikely to require CYK-4 GAP activ-

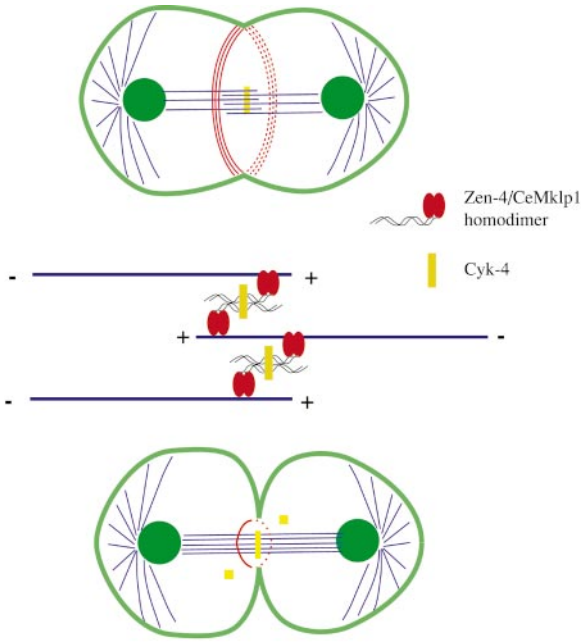

Figure 10. Model for the function of CYK-4 in central spindle formation and cytokinesis. See text for details.

ity. This is suggested by two lines of evidence. First, central spindle assembly is defective in the *cyk-4(t1689ts)* allele that carries a missense mutation at amino acid 15. This substitution is distant from the COOH-terminal GAP domain, and in vitro, the NH_2 terminus of CYK-4 is dispensable for GAP activity. Thus, this allele would be predicted to retain catalytic activity in vivo and, therefore, GAP activity is not sufficient for central spindle assembly. Moreover, Rho RNAi experiments reveal that central spindle assembly is Rho-independent, suggesting that Rho GAP activity is not required for this process. Thus, CYK-4 may act to promote central spindle assembly, independent of its GAP activity.

The Function of the CYK-4 Gap Domain

If CYK-4 function in central spindle assembly is independent of the Rho GTPase, what is the function of the CYK-4 GAP domain? CYK-4 is likely bifunctional, one function being to promote the assembly of the central spindle, the second function being to promote GTP hydrolysis by Rho family members. These two functions might be related in that the first function would serve to concentrate CYK-4 at a site where GAP activity is required. We speculate that CYK-4 GAP activity is required late in cytokinesis, to promote GTP hydrolysis by a Rho family GTPase whose downregulation causes disassembly of the contractile ring and cell separation (Fig. 10).

Which GTPase might CYK-4 act on to promote cytokinesis? The CYK-4 GAP domain has all the hallmarks of a Rho family GAP and we, therefore, expect that it will act on this subfamily of the GTPase superfamily. Like many other RhoGAPs, the GAP domain of CYK-4 is promiscuous in its ability to promote GTP hydrolysis on Rho, Rac, and Cdc42 (Lamarche and Hall, 1994). The strongest piece of evidence that CYK-4 acts on Rho is based on the observation that, of the GTPases tested, RhoA is the only one that is clearly essential for cytokinesis. The requirement for Rho in cell division is well documented in a variety of experimental systems. To date, there is no evidence that Rac is required for cytokinesis and our data, using RNAi to deplete Rac, also failed to detect a role for this GTPase in this process. Moreover, it has been recently reported that *ced-10* mutants, which are defective in corpse engulfment subsequent to apoptosis and distal tip cell migration, contain mutations in the *rac* gene (Reddien and Horvitz, 2000). *ced-10* mutants do not have any gross phenotypes indicative of a role in cytokinesis. Moreover, Rac1-deficient mice are gastrulation-defective, but the embryos do not contain multinucleate cells, which would indicate a cell division defect (Sugihara et al., 1998). The sum of these data argues that Rac is not an essential target of CYK-4 during cytokinesis. With regard to Cdc42, previous studies have implicated this GTPase in cytokinesis (Dutartre et al., 1996; Drechsel et al., 1997). Superficially, the weakly penetrant cytokinesis phenotype observed in *Cdc42(RNAi)* embryos is consistent with these earlier data. However, the *Cdc42(RNAi)* embryos that are cytokinesis-defective are also osmotically swollen and, therefore, the cytokinesis defect may be indirect. Thus, at this juncture, it appears most likely that RhoA is the key substrate for the CYK-4 GAP activity.

Further support for the hypothesis that completion of cytokinesis requires downregulation of RhoA by CYK-4 would be supported by experiments in which the *cyk-4* phenotype is phenocopied by RhoA mutants that are hydrolysis-defective. However, the genetic tools necessary to express such dominant mutants in the early *C. elegans* embryo are currently unavailable. It is surprising that the CYK-4 GAP domain is less active towards RhoA as compared with Rac or Cdc42, if indeed RhoA is the relevant target of its GAP activity. One possible explanation is that full-length CYK-4 has a different activity profile as compared with the isolated GAP domain. A more interesting possibility is that CYK-4 localization is important for CYK-4 GAP activity. The phenotype of *cyk-4* mutant embryos suggests that CYK-4 needs to act when the contractile ring is in close proximity to the central spindle. Since CYK-4 is concentrated on the central spindle at this time, its high local concentration might overcome its lower activity towards RhoA.

The Central Spindle: At the Center of Cytokinesis

There appear to be at least two microtubule-dependent steps in cytokinesis: (1) contractile ring positioning and (2) completion of cytokinesis. In some cells, both processes are dependent on the central spindle. An important open question is whether these two reactions are mechanistically similar. While assembly of the contractile ring requires activation of RhoA, we have shown previously that the position of the contractile ring is specified in a RhoA-independent manner in *Xenopus* embryos (Drechsel et al., 1997). We have shown here that a Rho GAP is required for the late stages of cytokinesis, suggesting that the second process, completion of cytokinesis, does involve RhoA. Therefore, we believe that the two microtubule-dependent steps in cytokinesis are distinct.

CYK-4 and ZEN-4 are not the only components of the central spindle, a number of other components, some of which are required for cytokinesis, are also present at this site. Polo kinase is known to associate with MKLP1 and to concentrate in the central spindle (Lee et al., 1995; Adams et al., 1998), and this kinase is essential for cytokinesis. Rho-associated kinase also localizes to this site (Kosako et al., 1999). The AIR-2 aurora-like kinase localizes to the central spindle (Schumacher et al., 1998). This kinase seems to be required primarily for chromosome segregation (Woollard and Hodgkin, 1999), its direct involvement in cytokinesis requires further analysis. INCENP and the TD-60 antigen also localize to the central spindle, and there is evidence that they may play a role in cytokinesis (Eckley et al., 1997; Mackay et al., 1998; Martineau-Thuillier et al., 1998; Savoian et al., 1999). Interestingly, a Rho GEF that is required for cytokinesis, ECT2, also accumulates on the central spindle (Tatsumoto et al., 1999), however the *Drosophila* orthologue, Pebble, does not localize in this manner (Prokopenko et al., 1999). Further studies are necessary to understand the specific functions of all of these cytokinesis regulators and to determine which of these proteins are functionally interdependent as we have shown is the case for CYK-4 and ZEN-4/CeMKLP1.

Concluding Remarks

In this manuscript, we have described an initial phenotypic, molecular, biochemical, and cell biological analysis of the *cyk-4* gene. These studies indicate that this protein is an active GTPase activating protein that is required for cytokinesis, likely by its ability to regulate the RhoA GTPase. Quite surprisingly, one additional function of this protein is to promote assembly of the central spindle. Thus, CYK-4 is a key molecule required for cytokinesis that regulates both the structure of the late mitotic spindle and the function of the contractile ring.

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