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 • C. 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 ingestion 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 Droso phila 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 ingestion, 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; Savoian et al., 1999). Moreover, in cultured cells and in sea urchin embryos, the central spindle is also required for completion of cytokinesis.
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; R aich 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 ingress 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 (A katori 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; M abuchi et al., 1993; M oorman et al., 1996; D rechsel et al., 1997; O ’Connell et al., 1999). Further, a Rho GEF is essential for cytokinesis (Prokopenko et al., 1999; T atsumoto 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 V an A elst and D ’Souza-Schorey, 1997). The requirement for RhoA in cytokinesis may reflect its ability to regulate formin signals since members of the formin gene family are required for cytokinesis in budding yeast (BNI1/BNR1) (I mamura et al., 1997), fission yeast (Cdc12) (Chang et al., 1997), Drosophila (dia) (C astrillon and W asserman, 1994) and C. elegans (cyk-1) (S wan et al., 1998). Several formins also bind to profilin (Chang et al., 1997; E vangelista et al., 1997; I mamura et al., 1997; W atanabe 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 (C arminati and S teamars, 1997; F ujiwara et al., 1999; M iller et al., 1999), spindle orientation in epithelial cells (B usson et al., 1998) and in certain asymmetrically dividing cells, such as the posterior blastomere of the two cell C. elegans embryo (H yman and W hite, 1987; W addle et al., 1994; S kop and W hite, 1998; G önczy et al., 1999a). In these examples, there is evidence that the dynein–dynamin 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 GTPase 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 (G önczy 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. Se verson, D. H amill, and B. B owerman, University of Oregon). The end points of the deficiency TD10 are not molecularly defined, but it uncovers cyk-4, lfl-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 unc-64(e189) glp-1(q339) III; mIs11 IV) to the fully ingressed contractile ring, where it could promote GTP hydrolysis by RhoA, thereby facilitating the completion of cytokinesis.

1A bbreviations used in this paper: G A P, G TPase activating protein; G E F, guanine nucleotide exchange factor; G F P, green fluorescent protein; R N A I, R N A -mediated interference; ts, temperature-sensitive.
Genetic Mapping of CYK-4
The cyk-4 locus maps under the deficiency tD16 which deletes a large fraction of the right arm of LGIII. Embryos derived from mothers with the genotype cyk-4(t1689ts)/tD16 also exhibit extensive furrow ingression. Re-combination mapping using unc-32(e189) cyk-4(t1689ts)/dp yl-2(e364) unc-32(e156) placed cyk-4 distal to (or very close to) unc-25 (23% Dpy non Unc's carried the cyk-4 mutation). Recombination between dpy-18 cyk-4/R7 W7000, which carries several Tc1 elements including one on the cosmid F147, gave rise to 45 Dpy non-Ts animals, three of which lacked the Tc1 insertion on F147, indicating that the cyk-4 gene is distal to this cosmid. Crosses to strains carrying the deficiencies dft03, dft12, and tD10 revealed that cyk-4(t1689ts) is not uncovered by dft12 and is uncovered by both dft12 and tD10. Since tD10 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 A to-ar HBO-103, was reduced to 50% intensity. A 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 s. 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.

RNA Interference
A approximately 500 bp of DNA corresponding to the predicted 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 Ni2+-NTA agarose (Qiagen). Proteins were dialyzed into 50 mM Tris, pH 7.5, 50 mM NaCl, and 5 mM MgCl2 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 MgCl2, and 1 mM DTT and quick frozen. To assess GAP activity, 15 pmol of the GST-GAP 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. A liqouts 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/).

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 BspLUU1 and inserted into the unique NcoI site of MPl7 (see above). This construct was linearized with Xbal, and complex arrays containing linearized genomic DNA and linearized rol-6(su1006) DNA were mixed in a ratio of 1:1000 and injected into unc-32(e1399) cyk-4(t1689ts)/ec1 hermaphrodites. Rolling f1 heterozygotes were singled out at 25°C and rolling unc-2 F2 animals were picked. A line MGl10, cyk-4 xse x1[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 Yuij 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 amnionpropl-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 assayed at 25°C and purified using Ni2+-NTA agarose (Qiagen). Proteins were dialyzed into 50 mM Tris, pH 7.5, 50 mM NaCl, and 5 mM MgCl2 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 MgCl2, and 1 mM DTT and quick frozen. To assess GAP activity, 15 pmol of the GST-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 ingestion 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 ingestion 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-
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.
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 K08E 3.6 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 K08E 3.6 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). A djacent to the COOH-terminal GAP domain is a C1 domain that is predicted to bind to diacylglycerol or phorbol esters. The NH2-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 NH2-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. A005977 (CLOT 94)) that, together with CYK-4 and the previously mentioned human gene, share a common structure consisting of ~650 amino acids, an NH2-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 K08E 3.6 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.
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 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) A n 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 K08E3.3 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.

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.
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. A subset 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 expressing a CYK-4::GFP fusion protein.
pressing a CYK-4:GFP fusion and followed its localization by time-lapse microscopy. The CYK-4:GFP fusion is partially functional since cyk-4xsEx1[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 ingress.

**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-
though staining of some microtubule bundles in the spindle midzone could be detected using an A IR-2 antibody (Fig. 9 G). Thus, recruitment of Z EN-4 to the central spindle is CY K-4-dependent. We next tested whether maintenance of Z EN-4 at division remnants requires functional CY K-4. cyk-4 mutant embryos grown at 16°C were shifted to 25°C for 15 min before fixation and staining with anti-Z EN-4 antibodies. The number of cells and the number of division remnants labeled with the anti-Z EN-4 antibody were counted. embryos maintained at the permissive temperature had a large number of Z EN-4 staining division remnants (Table III), whereas the embryos shifted to the nonpermissive temperature lacked defined staining of division remnants. Thus, both recruitment of Z EN-4 to the central spindle and its maintenance at division remnants is CY K-4-dependent. The reverse experiment was conducted with a temperature-sensitive allele of zen-4(or153ts). In this case, CY K-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 CY K-4 at division remnants is Z EN-4-dependent. We conclude that CY K-4 and Z EN-4/ CeMLP1 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-
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 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/

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**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 |
|-----------------------|-------------|-------------------------------|--------------------|-----------------------------|----------------|
|                       | °C          |                               |                    |                             |                |
| N2 (wild type)        | 25          | 110                           | 137                | 0.80                        | 16             |
| zen-4(or198ts)        | 16          | 61                            | 83                 | 0.73                        | 10             |
| zen-4(or198ts)        | 25          | 11                            | 147                | 0.07                        | 24             |
| (18 min)              |             |                               |                    |                             |                |
| 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.
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 ingestion (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 disassociate. 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-
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 Rho GAPS, 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, ced-10 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 (Dutrarte 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; A dams et al., 1998), and this kinase is essential for cytokinesis. Rho-associated kinase also localizes to this site (Schumacher et al., 1998). This kinase seems to be required primarily for chromosome segregation (Woolard and Hodgkin, 1999), its direct involvement in cytokinesis requires further analysis. INCENP and the contractile ring, in cytokinesis, is an active GTPase activating protein that is required for cell division ring and interacts with profilin. J. Cell Biol. 137:169-182.

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Q uite 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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