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Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in *Caenorhabditis elegans*

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SUMMARY

Asymmetric cell divisions, critically important to specify cell types in the development of multicellular organisms, require polarized distribution of cytoplasmic components and the proper alignment of the mitotic apparatus. In *Caenorhabditis elegans*, the maternally expressed protein, PAR-3, is localized to one pole of asymmetrically dividing blastomeres and is required for these asymmetric divisions. In this paper, we report that an atypical protein kinase C (PKC-3) is essential for proper asymmetric cell divisions and co-localizes with PAR-3. Embryos depleted of PKC-3 by RNA interference die showing Par-like phenotypes including defects in early asymmetric divisions and mislocalized germline-specific granules (P granules). The defective phenotypes of PKC-3-depleted embryos are similar to those exhibited by mutants for *par-3* and another *par* gene, *par-6*. Direct interaction of PKC-3 with PAR-3 is shown by in vitro binding analysis. This result is reinforced by the observation that PKC-3 and PAR-3 co-localize in vivo. Furthermore, PKC-3 and PAR-3 show mutual dependence on each other and on three of the other *par* genes for their localization. We conclude that PKC-3 plays an indispensable role in establishing embryonic polarity through interaction with PAR-3.

Key word: aPKC, Cell polarity, *par-3*, *Caenorhabditis elegans*, Asymmetry

INTRODUCTION

Asymmetric cell divisions are critically important to generate diverse cell types in the development of multicellular organisms. Polarized distribution of cytoplasmic components and the proper alignment of the mitotic apparatus are prerequisite for asymmetric divisions (Horvitz and Herskowitz, 1992; Strome, 1993; White and Strome, 1996). In *Caenorhabditis elegans*, a series of early asymmetric cleavages produce six founder cells with different cleavage patterns and cell fates. Anterior-posterior (A-P) polarity is established during the first cell cycle and correlates with a dynamic rearrangement of cytoplasm along the A-P axis which is defined by an extrinsic cue provided by sperm (Goldstein and Hird, 1996). Microfilaments accumulate at the anterior periphery (Strome, 1986), central cytoplasm flows toward the posterior pole and cortical cytoplasm flows in the opposite direction (Hird and White, 1993). The first mitotic spindle is placed posteriorly and the first cleavage becomes asymmetric, producing a large cell, AB, in the anterior and a small cell, P1, in the posterior. In concert with the cytoplasmic rearrangement, P granules, germline-specific ribonucleoprotein particles, become localized to the posterior pole (Strome and Wood, 1982; Yamaguchi et al., 1983). P granules are partitioned asymmetrically into the P1 cell and are segregated into blastomeres P2, P3 and P4 through successive divisions. Several other cytoplasmic factors which play roles in cell fate specification are distributed asymmetrically after the first cleavage. For example, SKN-1, which is a putative transcription factor and is required to specify the fate of the EMS blastomere, is present at a higher level in the P1 cell (Bowerman et al., 1992, 1993), and MEX-3, a putative RNA-binding protein, is expressed at a higher level in the AB cell (Draper et al., 1996; Hunter and Kenyon, 1996).

The establishment of the A-P polarity in *C. elegans* embryos is known to require the activities of the maternally expressed *par* genes (Kemphues, 1989; Guo and Kemphues, 1996a; Kemphues and Strome, 1997). Interference of normal functions of these genes causes extensive polarity defects in the 1-cell embryo and results in loss of many early asymmetries (Kemphues et al., 1988; Crittenden et al., 1997; Bowerman et al., 1997). Of the six *par* genes so far identified, *par-1*, *par-2*, *par-3*, *par-5* and *par-6* mutant embryos exhibit the symmetrically placed first cleavage spindle and the equal-sized AB and P1 blastomeres. P granules fail to be distributed exclusively to P blastomeres in *par-1*, *par-3*, *par-4*, *par-5* and *par-6* embryos. Both AB and P1 spindles at the second cleavage of *par-1*, *par-2*, *par-4* and *par-5* embryos are aligned transversely like the wild-type AB spindle, while they are aligned longitudinally in *par-3* and *par-6* embryos. The three *par* genes *par-1*, *par-2* and *par-3* have been molecularly

characterized. PAR-1 contains an amino-terminal serine/threonine kinase domain and a carboxy-terminal domain with binding activity to non-muscle myosin (Guo and Kemphues, 1995, 1996b). PAR-2 is a protein containing a zinc-binding domain of the ring finger class and a myosin-type ATP-binding site (Levitan et al., 1994). PAR-3 is a novel protein (Etemad-Moghadam et al., 1995) with three PDZ domains (Hartmann and Kurzchalia, 1996). Consistent with their role in polarity, the PAR proteins are themselves distributed in a polar fashion in the P lineage blastomeres. PAR-1 and PAR-2 localize to the posterior periphery of the 1-cell and the P1 cell and localize to the ventral periphery of P2 and P3 but are absent from the periphery of all other blastomeres. In contrast, PAR-3 is present in a distribution reciprocal to that of PAR-1 and PAR-2; it is localized to the anterior periphery of 1-cell embryos and P1, localizes to the dorsal side of P2 and P3, and is present uniformly at the periphery of all other blastomeres. (Guo and Kemphues, 1995; Etemad-Moghadam et al., 1995; Boyd et al., 1996).

PAR-3 appears to play a central role in polarity establishment. In *par-3* 1-cell embryos, PAR-1 and PAR-2 are no longer restricted to the posterior pole although they remain peripheral (Etemad-Moghadam et al., 1995), and the *par-3* spindle orientation defect is epistatic to those of the other *pars* (Cheng et al., 1995; K. J. K. unpublished results). Proper distribution of PAR-3 itself, however, is dependent upon the activities of *par-2*, *par-5* and *par-6* (Etemad-Moghadam et al., 1995; Watts et al., 1996; Guo and Kemphues, 1996a).

Insight into how asymmetric cell divisions are controlled can be gained by identifying the proteins with which the PAR proteins interact. Screens for proteins that can bind to the carboxy-terminus of PAR-1 identified a non-muscle myosin which was subsequently shown by RNA-mediated depletion of the protein (RNA interference or RNAi; Rocheleau et al., 1997) to be required for successful asymmetric divisions and for proper distribution of PAR-1, PAR-2 and PAR-3 (Guo and Kemphues, 1996b).

Recently, mammalian atypical protein kinase Cs (aPKCs), PKCζ (Goodnight et al., 1992) and PKCλ (Akimoto et al., 1994), were shown to associate specifically with a mouse protein similar to *C. elegans* PAR-3 (Y. I. et al., unpublished data). Although aPKCs are expressed ubiquitously in animals and have been implicated in mitogenic signal transduction in mammalian cells and have been implicated in mitogenic signal transduction in mammalian cells and the maturation of *Xenopus* oocyte (Berra et al., 1993; Diaz-Meco et al., 1994; Akimoto et al., 1996), their physiological function is totally unknown. The finding of a physical association between aPKCs and a PAR-3 homolog in mammals led us to postulate that aPKC might play a role in establishing embryonic polarity in *C. elegans*.

To test this hypothesis, we obtained a cDNA clone encoding a likely *C. elegans* aPKC from the expressed sequence tag library of Y. Kohara (personal communication) sequenced the cDNA to confirm that it encodes an aPKC molecule and tested its function in early embryos by RNAi. Recently, Wu et al. (1998) have reported the cloning and enzymatic characterization of the same aPKC, which they designated *pkc-3*. They reported that RNA interference of *pkc-3* caused embryonic arrest, but did not report an early phenotype. In this report, we show that embryos depleted of PKC-3 die displaying Par-3-like phenotypes, and that PKC-3 can bind to PAR-3 in vitro and is co-localized with PAR-3 at the anterior cortex of the 1-cell embryo. Furthermore, we found that the two proteins are mutually dependent for their proper localization: PKC-3 localization is disrupted in *par-3* embryos and PAR-3 localization is disrupted in PKC-3-depleted embryos. Finally, other *par* genes that regulate PAR-3 distribution are also required for PKC-3 localization. From these observations, we conclude that *C. elegans* PKC-3 plays an indispensable role in establishing embryonic polarity through interaction with PAR-3.

MATERIALS AND METHODS

Strains and alleles

C. elegans strains were cultured according to standard methods (Brenner, 1974). The *par* mutant strains used in this study are *par-2(lw32)unc-45/sC1*, *lon-1(e185)par-3(it71)/ qC1* III, *par-5(it55)dpy-20/DnT1*, *par-6(zu222)unc-101(m1)/hIn1[unc-54(h1040)]*. The animals showing each marker mutant phenotype were picked up and their embryos were used for immunostaining PKC-3 in these *par* mutants.

Sequence analysis of *pkc-3*

A candidate *C. elegans* aPKC homolog was identified as the cDNA clone yk4h6 (Genbank accession number, D27843 and D27844) in the cDNA sequencing project by Y. Kohara (personal communication). The 5′ portion of the *pkc-3* message, which is not contained in yk4h6, was obtained by 5′-rapid amplification of the cDNA end (5′-RACE), according to the instructions of the manufacturer (Bethesda Research Laboratories), using total RNA prepared from mixed stage wild-type animals as a template. The 5′ portion amplified by PCR was cloned into pBluescript. The entire message corresponding to the cDNA yk4h6 was determined on both strands by the dideoxy termination method. Sequence analysis was done using Genetyx software (Software Development Co., Japan).

RNA interference

In vitro production of sense and antisense RNA and RNA injections were carried out according to the method of Guo and Kemphues (1995) as modified by Rocheleau et al. (1997). The cDNA insert of yk4h6 cloned in pBluescript was PCR-amplified using primers complementary to sequences in the vector. Antisense and sense RNA was produced from the PCR product using Riboprobe or Ribomax in vitro transcription systems (Promega) and the RNA was purified using standard protocols. RNA was microinjected into both distal gonad arms of young adult wild-type (N2) hermaphrodites. 12 hours after injection, the injected worms were transferred to fresh plates and allowed to lay eggs for 10 hours at 20°C. Embryonic lethality of laid eggs was assessed by scoring hatching rate after further incubation for over 24 hours. To examine the early embryonic phenotypes, early embryos were dissected from injected animals a minimum of 12 hours after RNA injection. Embryos were overlaid with an 18×18 mm glass coverslip with Vaseline applied along edges to serve as a spacer and were observed with Nomarski optics.

In vitro binding analysis of PKC-3 and PAR-3

The cDNA expression plasmid, tag-PAR-3 (SRHis-PAR-3), was constructed by fusing amino acids 468-922 of PAR-3 downstream of six histidine residues and a twelve-amino-acid sequence from the T7 gene 10 leader sequence. To express PKC-3, amino acids 66-597 of PKC-3 were fused downstream of the FLAG-tag sequence of an expression vector pME18s-FLAG. COS1 cells were transfected with cDNA expression plasmids by electroporation (BIO-RAD, Gene-Pulser). Transfected cells (10 cm dish) were suspended in 200 μl of a lysis buffer containing 20 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1 mM PMSF, 1.8 μg/ml aprotinin, 1% Triton X-100, 0.1% deoxycholate and

0.1% SDS. After 30 minutes of incubation on ice, the lysates were clarified by centrifugation at 14,000 revs/minute for 30 minutes and immunoprecipitated with anti-T7 tag antibody (Novagen) preabsorbed on Protein G-Sepharose (Pharmacia) for 1 hours at 4°C. The immunocomplexes on Sepharose washed six times with the lysis buffer were separated by SDS-PAGE, transferred to a PVDF membrane, and probed with anti-PKC-3 antibody. In reciprocal experiments, immunocomplexes precipitated with anti-PKC-3 antibody were probed with anti-T7 tag antibody.

Immunostaining of embryos

P granules were visualized using rabbit serum AS798. Although this rabbit was immunized with an unrelated peptide, the serum shows a strong P-granule-specific stain (J. M., unpublished results).

The peptide CFEYVNPLQMSREDSV, which corresponds to a portion of the PKC-3 carboxy-terminus, was synthesized with the addition of an amino-terminal cysteine to facilitate its cross-linking to keyhole limpet hemocyanin (KLH). Rabbit antibodies produced against KLH-coupled antigen were affinity purified.

Affinity-purified rabbit anti-PAR-3 antibodies raised against a GST/PAR-3 fusion protein containing amino acids 660-934 of PAR-3 were described previously (Etemad-Moghadam et al., 1995). Chicken anti-PAR-3 antibodies raised against the same fusion protein and affinity purified were a gift from Diane Morton (Cornell University).

For immunostaining, gravid wild-type worms, *pkc-3(RNAi)* worms, or worms homozygous for various *par* mutations were bisected on polylysine-coated microscope slides to release embryos and were prepared for immunofluorescence microscopy according to Etemad-Moghadam et al. (1995) with slight modifications. The samples were overlaid with coverslips and were placed on dry ice for 15 minutes. After removal of coverslips, the samples were fixed with methanol for 15 minutes and washed with PBS for 5 minutes. The samples were incubated with diluted primary antibodies (rabbit anti-PKC-3 and chicken anti-PAR-3) for 2 hours at room temperature and washed twice for 15 minutes with PBS. The samples were then incubated for 1 hours with secondary antibodies (rhodamine goat anti-rabbit or FITC goat anti-rabbit for PKC-3 and FITC goat anti-chicken for PAR-3). After washing twice with PBS for 15 minutes, samples were overlaid with Vectashield (Vector) and coverslips and viewed using a Zeiss Axioscope. Images were captured with a Princeton Instruments digital camera or photographed using Ektachrome 400 (Kodak).

RESULTS

Identification of *C. elegans* atypical PKC

A candidate *C. elegans* aPKC homolog was identified as the cDNA clone yk4h6 in the cDNA sequencing project by Y. Kohara (personal communication). To confirm whether yk4h6 indeed encodes an aPKC, we determined the sequence of the entire message corresponding to yk4h6. The predicted amino acid sequence showed extensive similarity to mammalian aPKC subfamily members PKCζ (Goodnight et al. 1992) and PKCλ (Akimoto et al. 1994; Fig. 1). The amino terminal half contains one cysteine-zinc finger motif and lacks a potential Ca^{2+}-binding domain conserved in the conventional PKC family members. These structural features characterize aPKCs, which are dependent on neither Ca^{2+} nor diacylglycerol for their activation. The carboxy-terminal half of the predicted yk4h6 protein exhibited about 70% similarity to the kinase domain of aPKCs. The gene corresponding to yk4h6 is located on cosmid clone F09E5 (Genbank accession number U37429), which was sequenced by the *C. elegans* genome sequencing project. Comparison of the genomic and cDNA sequences

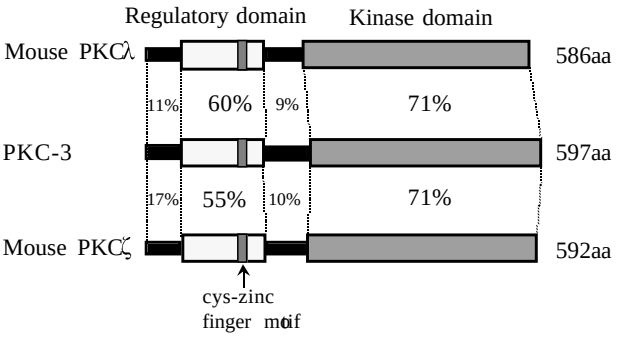

Fig. 1. Comparison of *C. elegans* aPKC, PKC-3, with mouse aPKCs, PKCζ and PKCλ. The stippled box in the predicted regulatory domain at the amino-terminus indicates a region that is highly conserved among these aPKCs and contains a cysteine-rich motif as indicated. The putative kinase domain is shown by the hatched box. The numbers between the drawings represent the percentage of identical amino acids between PKC-3 and each of the mouse aPKCs. The cDNA sequence for *pkc-3* has been deposited in the DDBJ database, accession number AB007320.

revealed that this aPKC gene consists of nine exons as predicted by the genome project. While this paper was in preparation, Wu et al. (1998) reported the cloning and enzymatic characterization of the same PKC, which they have designated *pkc-3*. They found that purified PKC-3 protein requires phosphatidylserine but is independent of Ca^{2+} and diacylglycerol for its activation, characteristics of aPKCs.

Antisense *pkc-3* RNA causes embryonic lethality

We tested whether *pkc-3* might play a role in embryonic polarity by depleting PKC-3 protein from early embryos by RNAi. In RNAi, large amounts of gene-specific in vitro-synthesized antisense or sense RNA is injected into the syncytial gonads of wild-type worms; by an unknown mechanism, the injected RNA leads to specific depletion of the protein encoded by the gene of interest (Guo and Kemphues, 1995, 1996b; Rocheleau et al., 1997; Fire et al., 1998). We microinjected sense or antisense RNA made from yk4h6 into the syncytial region of wild-type hermaphrodite gonads and, after incubation at 20°C for 12 hours, we examined the development of embryos produced from the injected worms [*pkc-3(RNAi)* embryos]. All embryos laid by animals injected with antisense RNA examined failed to hatch (Table 1). As observed previously with other genes, the sense RNA also caused the same embryonic lethal phenotype. Recently, Fire et al. (1998) showed that double-stranded(ds) RNA is more effective than single-stranded(ss) RNA in RNAi. Although the mechanism by which sense and antisense RNA cause the same defects is not fully understood, they suggest that ds RNA present in ss RNA preparations produced by in vitro transcription might play a critical role in RNAi.

Because the cDNA clone yk4h6 contains the entire kinase domain and this domain is 43-50% identical to those of other *C. elegans* PKC such as TPA-1 (Tabuse et al., 1989), PKC-1B (Land et al., 1994) and PKC-2 (Islas-Trejo, A., 1997), we carried out two control experiments to verify the specificity of the effect. First, we injected RNA made from the *tpa-1* gene, a gene with close similarity to PKC-3 over 140 amino acids of the kinase domain, and second, we injected RNA made from

Table 1. Embryonic lethal phenotype caused by antisense and sense RNA of *pkc-3*

Template DNA of in vitro transcription	Sense/antisense	No. of animals injected	Total embryos examined	Embryonic lethality (%)
pkc-3	Antisense	8	331	100
	Sense	7	320	100
None	–	8	244	0

pkc-3 sequences encoding only the first 100 amino acids, a region with no significant similarity to other known *C. elegans* genes. The *tpa-1(RNAi)* worms exhibited no embryonic phenotype, while the worms injected with RNA corresponding to the amino terminus of PKC-3 gave results identical to those using RNA from yk4h6 (data not shown). Although Wu et al. (1998) also have shown that disruption of *pkc-3* function by antisense RNA injection resulted in an embryonic arrest phenotype in *C. elegans*, the early embryogenesis of PKC-3-depleted embryos was not reported.

pkc-3(RNAi) embryos show defects in early embryonic polarity

We next examined the early phenotypes of *pkc-3(RNAi)* embryos to determine whether they show defects in early embryonic polarity. In wild-type embryos, the first division is unequal (Fig. 2A), the second division is asynchronous, and the mitotic spindles in AB and P1 are oriented differently; AB spindle orients perpendicular to the first division axis while P1 orients along the same axis. (Fig. 2D). In *pkc-3(RNAi)* embryos, the first division is equal (*n*=21/23, Fig. 2B), the second division is somewhat synchronous, and both spindles orient along the long axis of the embryo (*n*=23/25, Fig. 2E). The defects of early cleavage patterns caused by *pkc-3(RNAi)* are similar to those described for *par-3* (Kemphues et al., 1988; Cheng et al., 1995, see Fig. 2 C and F) and *par-6* mutations (Watts et al., 1996).

Fig. 3 shows P-granule staining in *pkc-3(RNAi)*and *par-3* embryos at the 4-cell stage. In the wild-type embryo, P granules become localized asymmetrically at the posterior pole prior to the first cleavage and are partitioned into the P1 cell. The granules are partitioned specifically into the P-cell lineage in subsequent unequal cleavages and are present in only P2 at the 4-cell stage (Fig. 3A). P granules, which are mis-partitioned in *par-3* mutants (Kemphues et al., 1988; Cheng et al., 1995, see Fig. 3C), also exhibited defective localization in 14 of the 18 *pkc-3(RNAi)* embryos examined. The staining of P granules was often observed in all four blastomeres of the 4-cell-stage embryo (Fig. 3B).

Although PKC-3-depleted embryos continued to cleave, they failed to execute normal morphogenesis and arrested as amorphous cell masses with a terminal phenotype consistent with that described by Wu et al. (1998). A comparison of *par-3*, *par-6* and *pkc-3(RNAi)* mutant embryos revealed that the *pkc-3(RNAi)* embryos were similar in severity to *par-6* mutant embryos and to weak alleles of *par-3*. To compare the terminal phenotypes of *par-3*, *par-6* and *pkc-3(RNAi)*, we scored gut differentiation, a sensitive indicator of the severity of *par* phenotypes (Cheng et al. 1995). Differentiated gut cells are observed in 30-50% of embryos from strong *par-3* mutant mothers but in 50-70% of embryos from weak *par-3* mutant and *par-6* mutant mothers (Cheng et al., 1995; Watts et al., 1996). Differentiated gut cells were observed in about 90% of arrested *pkc-3(RNAi)* embryos (*n*=64).

PKC-3 physically associates with PAR-3

The observation that PKC-3-depleted embryos displayed Par-3-like phenotypes was consistent with the possible functional relationship between aPKC and PAR-3 that had been proposed based on the binding of the mammalian aPKCs to a mammalian PAR-3-like protein. To determine whether PKC-3 could interact biochemically with PAR-3, a PAR-3/T7 tag fusion protein (aa468-922; tag-PAR-3) containing the expected PKC-3 binding domain was co-expressed with PKC-3 (aa66-597) in COS1 cells. Lysates from transfected cells were immunoprecipitated with anti-T7 tag antibody and probed with anti-PKC-3 antibody raised against a short synthetic peptide corresponding to a portion of the carboxy-terminus of PKC-3. As shown in Fig. 4A, PKC-3 was co-precipitated with tag-PAR-3. A reciprocal experiment showed that tag-PAR-3 was immunoprecipitated

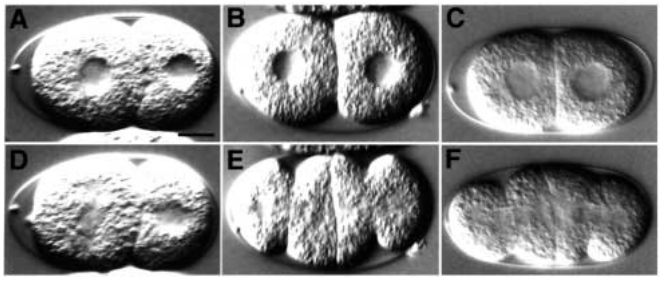

Fig. 2. Defects in early cleavage patterns caused by depletion of PKC-3. Nomarski micrographs of wild-type (A,D), PKC-3-depleted (B,E), and *par-3* mutant embryos (C,F). In wild-type embryos, the first division is unequal giving rise to a large cell, AB, in the anterior and a small cell, P1, in the posterior (A). Anterior is to the left; bar in A is approximately 10 μm.

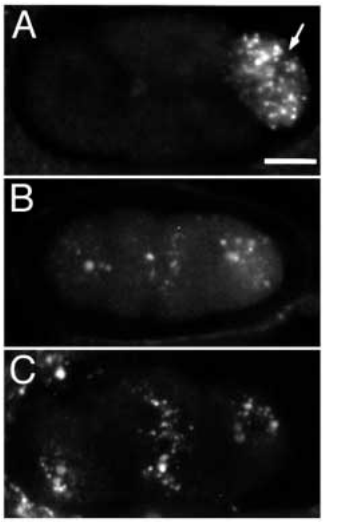

Fig. 3. P-granule distribution in wildtype, *pkc-3(RNAi)* and *par-3* embryos. Wild-type (A), PKC-3-depleted (B), and *par-3 (it71)* mutant embryos (C) at 4-cell stage immunostained with anti-P-granule antibodies are shown. In almost all wild-type 4-cell-stage embryos, P granules become localized exclusively in the P2 cell indicated by an arrow (A). P granules are mislocalized in *pkc-3(RNAi)* and *par-3* embryos and are often detected in all of the blastomeres. Anterior is to the left; bar in A is approximately 10 μm.

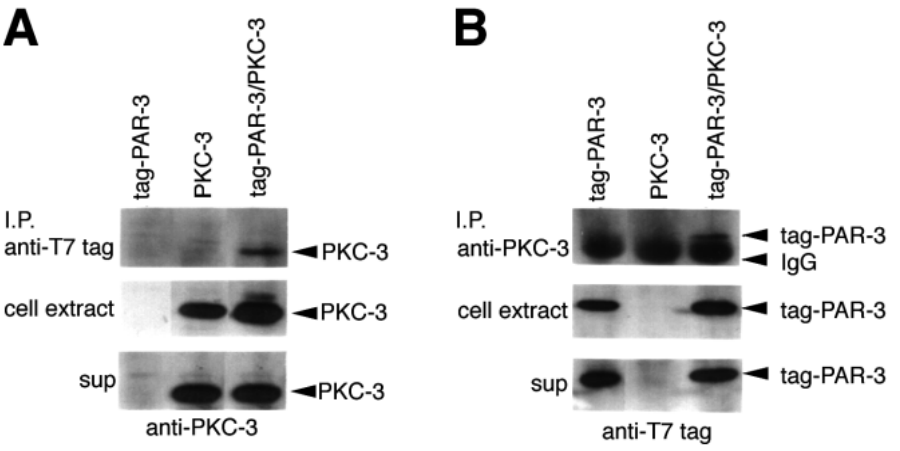

Fig. 4. Association of PKC-3 with PAR-3. COS1 cells were transiently transfected with the expression vectors shown at the top. Cell lysates (cell extract) were clarified by centrifugation (sup) and used as a starting material for immunoprecipitation (I.P.) using the anti-T7-tag (A) and anti-PKC-3 (B) antibodies. The immunoprecipitates were probed with anti-PKC-3 (A) and antiT7-tag (B) antibodies.

with PKC-3 by antibody to PKC-3 (Fig. 4B). These results indicate that PKC-3 can form a complex with PAR-3.

PKC-3 is co-localized with PAR-3 in the 1-cell embryo

The phenotypic similarity between the *pkc-3(RNAi)* and *par-3* embryos, together with the evidence for association of PKC-3 and PAR-3 shown above suggested that the two proteins might co-localize in early embryos. To examine the intracellular distribution of PKC-3, we raised polyclonal antibodies against a short synthetic peptide corresponding to a portion of the carboxy-terminus and affinity-purified them for use in immunofluorescence assays on early wild-type embryos. The antibodies stained the cell periphery of early blastomeres as well as the nuclear envelope. Nuclear envelope staining however, appears to be non-specific. Staining at the periphery was eliminated in *pkc-3(RNAi)* embryos, but the nuclear envelope staining persisted (Fig. 5F). Overall, the peripheral staining pattern in early embryos is similar to that previously reported for PAR-3 (Etemad-Moghadam et al., 1995). The staining in 1-cell embryos is weak and uniform just after the completion of meiosis (Fig. 5A), but increases in intensity and becomes concentrated at the anterior periphery during pronuclear migration. The peripheral PKC-3 staining becomes restricted to about 50% embryo length during the pronuclear meeting (Fig. 5B) and pronuclear fusion stage (n=23). By early anaphase, the staining extends posteriorly beyond the midline of the zygote and covers about 60% of the total length of embryos (n=29, Fig. 5C). In 2- and 4-cell stages, staining is uniform at the periphery of the AB cell, its daughters and the EMS cell, but peripheral staining in P1 and P2 is restricted to the boundaries with other blastomeres (Fig. 5D,E). These results confirm and extend those of Wu et al. (1998) who reported staining at the periphery and the cell boundaries of early blastomeres but did not report asymmetrical staining.

To determine better the extent of overlap between PKC-3 and PAR-3, we examined the intracellular distribution of PKC-3 and PAR-3 in wild-type 1-cell embryos. Fig. 6A-C shows the results of double-label immunofluorescence with anti-PKC-3 (A, red) and anti-PAR-3 (B, green) in a 1-cell embryo at pronuclear meeting. Both PKC-3 staining and PAR-3 staining appear as punctate signals concentrated at the anterior cell periphery. Furthermore, the distribution of PKC-3 overlaps with that of PAR-3 (Fig. 6D-F); overlapping distributions are seen in later stage embryos as well (data not shown). Fig. 6F shows an enlargement of a tangential optical section of the anterior periphery of an embryo doubly-stained for PKC-3 and PAR-3. The punctate staining for each of the proteins resolves as a

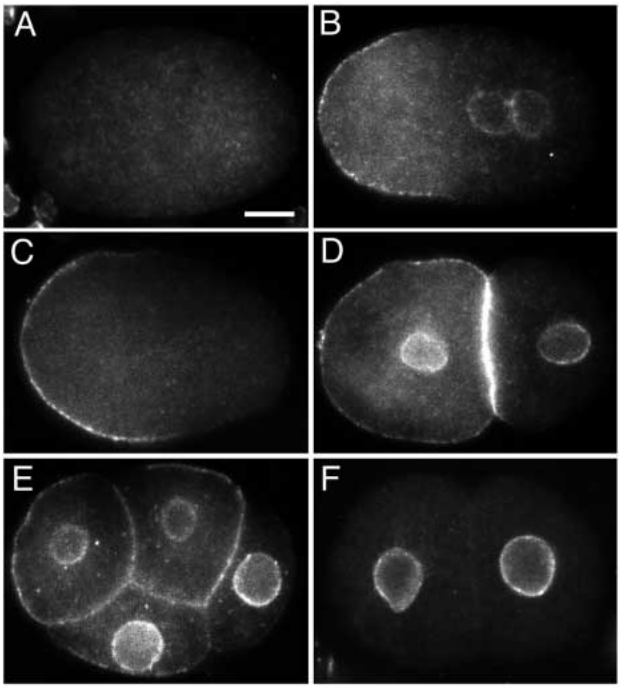

Fig. 5. Asymmetric distribution of PKC-3 in early embryos. Wild-type embryos at various embryonic stages were stained for PKC-3. (A) 1-cell embryo prior to pronuclear decondensation. PKC-3 staining is weak and uniform throughout the cytoplasm. (B) 1-cell embryo at pronuclear meeting. PKC-3 becomes restricted to the anterior periphery. (C) Anaphase-stage embryo. PKC-3 can be seen at the anterior periphery. (D) 2-cell-stage embryo. PKC-3 is present uniformly at the periphery of the AB cell and at the boundary of AB and P1 blastomeres. (E) 4-cell-stage embryo. PKC-3 can be seen at the periphery of somatic cells ABa, ABp and EMS but peripheral staining of the germ-line cell P2 is restricted to the boundaries with ABp and EMS. (F) 2-cell stage *pkc-3(RNAi)* embryo. The staining at the cell periphery is eliminated but the nuclear envelope staining is apparent (see text). Anterior is to the left; bar in A is approximately 10 μm.

pattern of somewhat irregular patches of varying size. Some patches are positive for both proteins (yellow/orange; arrows in Fig. 6F) consistent with the hypothesis that the proteins form molecular complexes in vivo. Most patches, however, are detectable by only one of the antibodies. In the optical section shown, there are approximately 120 patches positive for both, 360 positive for PAR-3 only and 350 positive for only PKC-3. The doubly labeled patches do not appear to be due to chance overlap of two completely independent protein distributions; reversing the orientation of the PKC-3 image relative to the PAR-3 image resulted in only 12 apparently overlapping patches. In fact, significant overlap is only seen when the two images are in their original register. To determine to what extent the non-overlapping staining was due to artifacts of the staining or detection procedures, we compared these results to those from embryos doubly labeled with chicken anti-PAR-3 and rabbit anti-PAR-3 antibodies. In this experiment, almost all patches showed overlap (not shown). Thus, the partial overlap of PKC-3 and PAR-3 distributions may reflect dynamic associations between the two proteins.

Mutual dependence of PKC-3 and PAR-3 for their localization to the anterior periphery of the 1-cell embryo

To test further the possible functional interaction between PKC-3 and PAR-3, we investigated whether *par-3* is required for the proper distribution of PKC-3. We stained PKC-3 in *par-3(it71)* embryos, which lack detectable PAR-3 (Etemad-Moghadam et al., 1995). In all embryos examined (n=80; 37 1-cell, 24 2-cell and 19 4-cell-stage embryos), no localized PKC-3 peripheral staining was detected, although faint cytoplasmic staining remained (Fig. 7A; compare to Fig. 6A). This result indicates that wild-type *par-3* is required for proper localization of PKC-3. Next, we examined the PAR-3 distribution in the PKC-3-depleted embryos. In the absence of PKC-3, PAR-3 showed a cell-cycle-dependent mislocalization (Fig. 7B,C). For example, in 18/24 *pkc-3(RNAi)* embryos scored between meiosis and pronuclear meeting, the PAR-3 protein appeared asymmetric and peripheral. In all cases, however, the signal was weaker and not as tightly restricted as in wild-type embryos (compare Fig. 7B to Fig. 6B). This staining is lost as the cell cycle progresses; none of the 22 *pkc-3(RNAi)* 1-cell embryos scored at metaphase and anaphase had a detectable signal (Fig. 7C). In subsequent stages of early development, weak peripheral staining, especially at cell boundaries, is seen in all blastomeres in early stages of the cell cycle, but staining becomes undetectable at late cell-cycle stages (data not shown). Thus, PKC-3 has a role in establishing or maintaining the asymmetric distribution of PAR-3. Overall, these observations lead us to propose that PKC-3 and PAR-3 cooperate to achieve a mutually dependent anterior peripheral localization in 1-cell embryos.

par-2, *par-5* and *par-6*, which regulate PAR-3 distribution, are also required for proper localization of PKC-3

Several other *par* genes, *par-2*, *par-5* and *par-6*, were previously shown to be required for proper localization of PAR-3 (Etemad-Moghadam et al., 1995; Watts et al., 1996). In *par-6* 1-cell embryos, PAR-3 distribution is cytoplasmic and uniform rather than peripheral and asymmetric. In *par-2* and *par-5* 1-cell embryos, although PAR-3 is detected peripherally, its localization is not restricted to the anterior half and extends to the posterior part of the embryo. At the 2-cell stage, PAR-3 is distributed all around the cortex of both blastomeres of *par-2* and *par-5* embryos. If PKC-3 localization is dependent upon PAR-3, then PKC-3 should be mislocalized in mutant backgrounds in which PAR-3 is mislocalized.

Indeed, the distribution of PKC-3 parallels the distribution of PAR-3 in these mutant backgrounds. In *par-6* embryos, no peripheral staining of PKC-3 is detected although faint and uniform staining persists in the cytoplasm (Fig. 8A). In *par-2* embryos, PKC-3 is present at the cortex in a gradient along the A-P axis, but its distribution extends to about 70% of the egg length during pronuclear migration and pronuclear fusion (n=24). As the cell cycle proceeds, the distribution of PKC-3 extends over 90% of the egg length by late anaphase (n=8, Fig. 8B). As a result of the uniform distribution of PKC-3 in the *par-2* 1-cell embryo, all blastomeres exhibited peripheral PKC-3 staining in 80% of 2-cell embryos (n=25, Fig. 8C) and 92% of 4-cell embryos (n=24). The PKC-3 distribution patterns of *par-5* embryos are very similar to those exhibited by *par-2* embryos, though the extent of the localization defect is

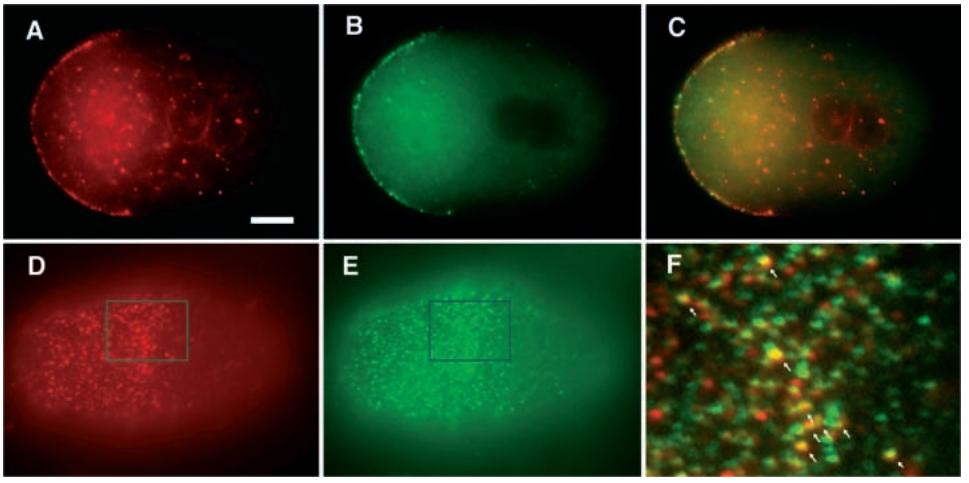

Fig. 6. Co-localization of PKC-3 and PAR-3 in *C. elegans* 1-cell embryos. (A-C)Medial optical section of a late pronuclear stage 1-cell embryo doubly stained with anti-PKC-3 antibody (A, red) and anti-PAR-3 antibody (B, green) are shown. (C) The merged view of the same doubly stained embryo is shown. (D,E) Tangential optical section of an early pronuclear stage embryo doubly stained with anti-PKC-3 (D, red) and anti-PAR-3 (E, green). The embryo is slightly flattened, making a large area of the cell periphery parfocal. (F) Merged and enlarged view of the boxed area in D and E. Arrows indicate patches positive for both PKC-3 and PAR-3 (yellow/ orange). Anterior is to the left; bar in A represents approximately 10 μm.

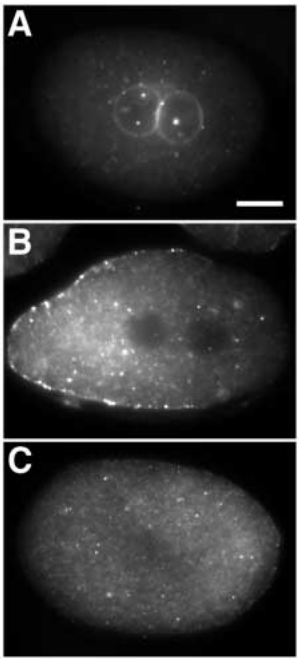

Fig. 7. Mutual dependence of PKC-3 and PAR-3 for their asymmetric localization. (A) Pronuclear fusion stage *par-3* (*it71*) embryo immunostained for PKC-3; compare to the staining of the wild-type embryo (Fig. 6A). Pronuclear migration stage (B) and early anaphase 1-cell stage (C) *pkc-3(RNAi)* embryo immunostained for PAR-3. Anterior is to the left; bar in panel (A) is approximately 10 μm.

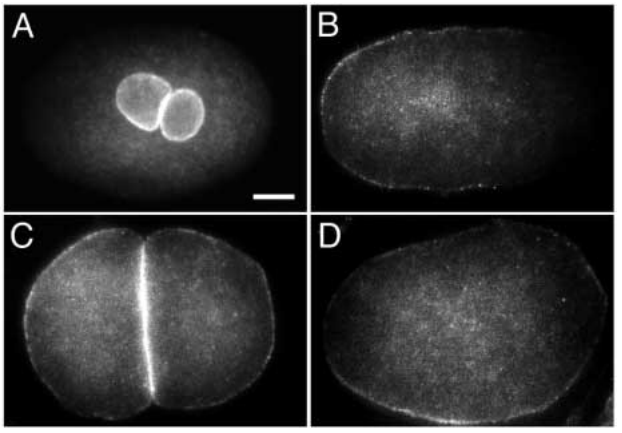

Fig. 8. Mis-localization of PKC-3 in *par-2*, *par-5* and *par-6* embryos. (A) Immunostaining of pronuclear fusion stage *par-6*(*zu222*) embryo. (B) *par-2* (*lw32*) embryo at the anaphase of the first cell cycle. (C) 2-cell stage *par-2* (*lw32*) embryo at the early anaphase of the second cell cycle. (D) *par-5* (*it55*) embryo at the anaphase of the first cell cycle. Anterior is to the left; bar in A is approximately 10 μm.

somewhat more severe in *par-5* than *par-2* background. In *par-5* embryos, PKC-3 is distributed to about 80% of the egg length during pronuclear migration and fusion (n=12), and covers almost the entire cortex of the 1-cell embryo by late anaphase (n=9). All blastomeres showed peripheral PKC-3 staining in 90% of 2-cell embryos (n=30, Fig. 8D) and 80% of 4-cell embryos (n=25). These results are consistent with the notion that PKC-3, cooperating with PAR-3, plays a role in *par* gene pathway to establish early embryonic polarity.

DISCUSSION

We have demonstrated here that an atypical protein kinase C, PKC-3, is required for establishing asymmetry in early *C. elegans* embryos. We found that embryos depleted of PKC-3 by RNA interference died, displaying early polarity defects, that PAR-3 and PKC-3 are co-localized in vivo and can associate in vitro, that the two proteins are mutually dependent for their asymmetric peripheral localization and that the two proteins are both dependent upon *par-2*, *par-5* and *par-6* for proper distribution. These results provide strong evidence that PKC-3 cooperates with PAR-3 to establish embryonic polarity in *C. elegans*.

In *C. elegans* embryos, the establishment of anterior-posterior polarity occurs during the first cell cycle and depends on the functions of *par* genes and non-muscle myosin (Kemphues et al., 1988; Guo and Kemphues, 1996a,b; Kemphues and Strome, 1997). Without the normal functions of these genes, the first cleavage is often equal, cytoplasmic components such as P granules, SKN-1 and MEX-3 are mislocalized, spindles misalign at the second cleavage and cell fates are drastically altered. The asymmetric distribution of the *par* proteins at the cell periphery leads to the hypothesis that these genes are acting as components of a signaling system that creates cytoplasmic differences between anterior and posterior poles of the 1-cell embryo, perhaps by locally modifying the cortical cytoskeleton.

The discovery that PKC-3 acts with PAR-3 to establish polarity is consistent with this hypothesis and provides the first example of a specific in vivo role for an atypical protein kinase. Vertebrate atypical PKCs have been shown to be involved in cell proliferation and in insulin-induced oocyte maturation in culture (Berra et al., 1993; Diaz-Meco et al., 1994; Akimoto et al., 1996) but their role in intact animals is not known. Wu et al. (1998) showed that PKC-3 is required for embryonic viability in *C. elegans* and here we show that it plays an important role in asymmetric cell division. Because PKC-3 is expressed at later stages as well (Wu et al., 1998), other roles for the protein are likely.

The exact functional relationship between PKC-3 and PAR-3 is not clear. Three possibilities are suggested by our data. First, PAR-3 could be acting to recruit PKC-3 to the cell periphery where it acts as a signaling molecule. This idea is supported by the observation that PKC-3 does not become peripheral in the absence of PAR-3. It seems unlikely, however, that PAR-3 is functioning exclusively through PKC-3 because the gut differentiation phenotype of *pkc-3(RNAi)* is not as severe as that of *par-3* mutants. Although this difference in severity could be due to incomplete depletion of PKC-3, peripheral PKC-3 is undetectable in *pkc-3(RNAi)* embryos. Thus, it appears that there is residual PAR-3 activity in the absence of detectable PKC-3. Furthermore, an exclusively 'downstream' role for PKC-3 is not consistent with the cell-cycle-dependent mislocalization of PAR-3 in *pkc-3(RNAi)* embryos. This phenotype suggests a second possibility: that PKC-3, like the *par-6* product, acts to recruit PAR-3 to the cell periphery or maintain it there throughout the cell cycle or both. It could do this by phosphorylating PAR-3 directly or by modifying the cortical cytoskeleton. The two possibilities discussed above are not mutually exclusive; PAR-3 could recruit PKC-3 to the cell periphery where its kinase activity has the dual effect of providing a signal leading to anterior/posterior differences as well as maintaining PAR-3 at

the periphery. A third possibility is that PKC-3 and PAR-3, perhaps along with the product of the *par-6* gene, act together to form a functional complex whose stability or localization requires the presence of both.

In conclusion, starting with the discovery of an interaction between mammalian aPKCs and a mammalian protein similar to PAR-3 (Y. I. et al., unpublished data), we have shown that the *C. elegans* atypical protein kinase C, PKC-3 is required to establish polarity in the *C. elegans* embryo and it does this via its interaction with PAR-3. This leads us to suggest that aPKC/PAR-3-mediated signaling cascades may also function in cellular polarity in other species.

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REFERENCES

Akimoto, K., Mizuno, K., Osada, S., Hirai, S., Tanuma, S., Suzuki, K. and Ohno, S. (1994). A new member of the third class in the protein kinase C family, PKC λ, expressed dominantly in an undifferentiated mouse embryonal carcinoma cell line and also in many tissues and cells. *J. Biol. Chem*. **269**, 12677-12683.

Akimoto, K., Takahashi, R., Moriya, S., Nishioka, N., Takayanagi, J., Kimura, K., Fukui, Y., Osada, S., Mizuno, K., Hirai, S., Kazlauskas, A. and Ohno, S. (1996). EGF or PDGF receptors activate atypical PKCλ through phosphatidylinositol 3-kinase. *EMBO J.* **15**, 788-798.

Berra, E., Diaz-Meco, M. T., Dominguez, I., Municio, M. M., Sanz, L., Lozano, J, Chapkin, R. S. and Moscat, J. (1993). Protein kinase C ζ isoform is critical for mitogenic signal transduction. *Cell* **74**, 555-563.

Bowerman, B., Eaton B. A. and Priess, J. R. (1992). *skn-1*, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. *Cell* **68**, 1061-1075.

Bowerman, B., Draper, B. W., Mello, C. C. and Priess, J. R. (1993). The maternal gene *skn-1* encodes a protein that is distributed unequally in early *C. elegans* embryo. *Cell* **74**, 443-452.

Bowerman, B., Ingram, M. K., and Hunter, C. P. (1997). The maternal *par* genes and the segregation of cell fate specification activities in early *Caenorhabditis elegans* embryos. *Development* **124**, 3815-3826.

Boyd, L., Guo, S., Levitan, D., Stinchcomb, D. T. and Kemphues, K. J. (1996). PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in *C. elegans* embryos. *Development* **122**, 3075-3084.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Cheng, N. N., Kirby, C. M. and Kemphues, K. J. (1995). Control of cleavage spindle orientation in *Caenorhabditis elegans*: the role of the genes *par-2* and *par-3*. *Genetics* **139**, 549-559.

Crittenden, S. L., Rudel, D., Binder, J., Evans, T. C. and Kimble, J. (1997) Genes required for GLP-1 asymmetry in the early *Caenorhabditis elegans* embryo. *Dev Biol*. **181**, 36-46.

Diaz-Meco, M. T., Lozano, J., Municio, M. M., Berra, E., Frutos, S., Sanz, L. and Moscat, J. (1994). Evidence for the in vitro and in vivo interaction of Ras with protein kinase C ζ. *J. Biol. Chem*. **269**, 31706-31710.

Draper, B. W., Mello, C. C., Bowerman, B., Hardin, J. and Priess, J. R. (1996). The maternal gene *mex-3* encodes a KH domain protein and regulates blastomere identity in early *C. elegans* embryo. *Cell* **87**, 205-216.

Etemad-Moghadam, B., Guo, S. and Kemphues, K. J. (1995). Asymmetrically distributed PAR-3 protein contributes to cell polarity and spindle alignment in early *C. elegans* embryos. *Cell* **83**, 743-752.

Fire, A., Xu, S., Montogomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Goldstein, B. and Hird, S. N. (1996). Specification of the anteroposterior axis in *Caenorhabditis elegans*. *Development* **122**, 1467-1474.

Goodnight, J., Kazanietz, M. G., Blumberg, P. M., Mushinski, J. F. and Mischak, H. (1992). The cDNA sequence, expression pattern and protein characteristics of mouse protein kinase C ζ. *Gene* **122,** 305-311.

Guo, S. and Kemphues, K. J. (1995). *par-1*, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell* **81**, 611-620.

Guo, S. and Kemphues, K. J. (1996a). Molecular genetics of asymmetric cleavage in the early *Caenorhabditis elegans* embryo. *Cur. Opin. Genet. Dev.* **6**, 408-415.

Guo, S. and Kemphues, K. J. (1996b). A non-muscle myosin required for embryonic polarity in *Caenorhabditis elegans*. *Nature* **382**, 455-458.

Hartmann, E. and Kurzchalia, T. (1996). Are there similarities between the polarization of the *C. elegans* embryo and of an epithelial cell? *Trends in Cell Biology* **6**, 131-132.

Hird, S. N. and White, J. G. (1993). Cortical and cytoplasmic flow polarity in early embryonic cells of *Caenorhabditis elegans. J. Cell Biol.* **121**, 1343-1355.

Horvitz, H. R. and Herskowitz, I. (1992). Mechanisms of asymmetric cell division: two Bs or not two Bs, that is the question. *Cell* **68**, 237-255.

Hunter, C. P. and Kenyon, C. (1996). Spatial and temporal controls target *pal-1* blastomere-specification activity to a single blastomere lineage in *C. elegans* embryo. *Cell* **87**, 217-226.

Islas-Trejo, A., Land, M., Tcherepanova, I., Freedman, J. H. and Rubin, C. S. (1997). Structure and expression of the *Caenorhabditis elegans* protein kinase C2 gene. *J. Biol. Chem.* **272**, 6629-6640.

Kemphues, K. J., Priess, J. R., Morton, D. G. and Cheng, N. (1988). Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. *Cell* **52**, 311-320.

Kemphues, K. J. (1989). *Caenorhabditis*. In *Frontiers in Molecular Biology: Genes and embryos* (ed D. M. Glover and B. D. Hames) pp 95-126. London: IRL Press.

Kemphues, K. J. and Strome, S. (1997). Fertilization and establishment of polarity in the embryo. In *C. elegans II* (ed. Riddle, D. L., Blumenthal, T, Meyer, B. J. and Priess, J. R.) pp 335-359. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Land, M., Islas-Trejo, A., Freedman, J. H. and Rubin, C. S. (1994). Structure and expression of a novel, neuronal protein kinase C (PKC1B) from *Caenorhabditis elegans*. *J. Biol. Chem.* **269,** 9234-9244.

Levitan, D. J., Boyd, L., Mello, C. C., Kemphues, K. J. and Stinchcomb, D. T. (1994). *par-2*, a gene required for blastomere asymmetry in *Caenorhabditis elegans*, encodes zinc-finger and ATP-binding motifs. *Proc. Natl. Acad. Sci. USA* **91**, 6108-6112.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.-H., Ali, M., Priess, J. R. and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* **90**, 707-716.

Strome, S. and Wood, W. B. (1982). Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **79**, 1558-1562.

Strome, S. (1986). Fluorescence visualization of the distribution of microfilaments in gonads and early embryos of the nematode *Caenorhabditis elegans. J. Cell Biol.* **103**, 2241-2252.

Strome, S. (1993). Determination of cleavage planes. *Cell* **72**, 3-6.

Tabuse, Y., Nishiwaki, K. and Miwa, J. (1989). Mutations in a protein kinase C homolog confer phorbol ester resistance on *Caenorhabditis elegans. Science* **243**, 1713-1716.

Watts, J. L., Etemad-Moghadam, B., Guo, S., Boyd, L., Draper, B. W., Mello, C. C., Priess, J. R. and Kemphues, K. J. (1996). *par-6*, a gene involved in the establishment of asymmetry in early *C. elegans* embryos, mediates the asymmetric localization of PAR-3. *Development* **122**, 3133-3140.

White, J. and Strome, S. (1996). Cleavage plane specification in *C. elegans*: how to divide the spoils. *Cell* **84**, 195-198.

Wu, S.-L., Staudinger, J., Olson, E. N. and Rubin, C. S. (1998). Structure, expression, and properties of an atypical protein kinase C (PKC3) from *Caenorhabditis elegans*. *J. Biol. Chem*. **273**, 1130-1143.

Yamaguchi, Y., Murakami, K., Furusawa, M. and Miwa, J. (1983). Germline-specific antigens identified by monoclonal antibodies in the nematode *Caenorhabditis elegans*. *Dev. Growth Differ.* **25**, 121-131.