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

Yo Tabuse1,*,†, Yasushi Izumi2,*, Fabio Piano3, Kenneth J. Kemphues3, Johji Miwa1 and Shigeo Ohno2

1Fundamental Research Laboratories, NEC Corporation, 34 Miyukigaoka, Tsukuba, Ibaraki 305, Japan
2Department of Molecular Biology, Yokohama City University School of Medicine, 3-9, Fuku-ura, Kanazawa-ku, Yokohama 236, Japan
3Section of Genetics and Development, 435 Biotechnology Building, Cornell University, Ithaca, New York 14853, USA

*These authors contributed equally to this report.
†Author for correspondence (e-mail: ytabuse@frl.cl.nec.co.jp)

Accepted 1 July; published on WWW 25 August 1998

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 distinct.
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 PKCζ (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 mitotic 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 pck-3. They reported that RNA interference of pck-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(bw32)unc-45(sc1, lon-1(e185)par-3(t71)/qcl-1 III, par-5(t55)dpv-20/Dn1, par-6(zu222)unc-101(mf)/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 the T7 gene 10 leader sequence. To express PKC-3, amino acids 66-597 of PKCζ was obtained by 5′¢-rapid amplification of the cDNA end (5′-RACE), using 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).
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 hour 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 CFYVNPLQMSREDSV, 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, pck-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 hour 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 Ca2+-binding domain conserved in the conventional PKC family members. These structural features characterize aPKCs, which are dependent on neither Ca2+ 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 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 pck-3. They found that purified PKC-3 protein requires phosphatidylserine but is independent of Ca2+ and diacylglycerol for its activation, characteristics of aPKCs.

**Antisense pck-3 RNA causes embryonic lethality**

We tested whether pck-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 Kempkes, 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 [pck-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
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 *ykh6* (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 PAR-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 with anti-PKC-3 antibody (Fig. 4B). The immunoprecipitates were co-stained with anti-PAR-3 antibody and revealed that PKC-3 co-precipitated with PAR-3 in a specific complex.

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

<table>
<thead>
<tr>
<th>Template DNA of in vitro transcription</th>
<th>Sense/antisense</th>
<th>No. of animals injected</th>
<th>Total embryos examined</th>
<th>Embryonic lethality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>pkc-3</em></td>
<td>Antisense</td>
<td>8</td>
<td>331</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Sense</td>
<td>7</td>
<td>320</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>8</td>
<td>244</td>
<td>0</td>
</tr>
</tbody>
</table>

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 are mislocalized 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 blastomers. Anterior is to the left; bar in A is approximately 10 μm.
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 pck-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 pck-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
PKC-3 and PAR-3, we investigated whether PKC-3 is required to test further the possible functional interaction between embryo localization to the anterior periphery of the 1-cell associations between the two proteins. 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 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 anti-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 pck-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 pck-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
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 poles of the 1-cell embryo.
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.

We thank Y. Kohara for the cDNA clone yk4h6; T. Miyamoto for her technical assistance with DNA sequencing; K. Nishiwaki and T. Sano for discussions and reading of the manuscript. S. O. was supported by grants from the Ministry of Education, Science, Culture, and Sports of Japan and from the Japanese Society for the Promotion of Science (JSFS). F. P. and K. J. K. were supported by grants from the Cancer Center Research Fund of the Damon Runyon-Walter Winchell Foundation Fellowship, DRC-1404, and NIH grant HD27689.

REFERENCES


