Development 126, 127-135 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV5238

PAR-6 is a conserved PDZ domain-containing protein that colocalizes with PAR-3 in *Caenorhabditis elegans* embryos

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Accepted 12 October; published on WWW 3 December 1998

SUMMARY

The *par* genes are required to establish polarity in the *Caenorhabditis elegans* embryo. Mutations in two of these genes, *par-3* and *par-6*, exhibit similar phenotypes. A third gene, *pkc-3*, gives a similar phenotype when the protein is depleted by RNA interference. PAR-3 and PKC-3 protein are colocalized to the anterior periphery of asymmetrically dividing cells of the germline lineage and the peripheral localizations of both proteins depends upon the activity of *par-6*. Here we report the molecular cloning of *par-6* and the immunolocalization of PAR-6 protein. We found that *par-6* encodes a PDZ-domain-containing protein and has homologues in mammals and flies. Moreover, we discovered that PAR-6 colocalizes with PAR-3 and that *par-3* and *pkc-3* activity are required for the peripheral localization of PAR-6. The localization of both PAR-3 and PAR-6 proteins is affected identically by mutations in the *par-2*, *par-4* and *par-5* genes. The co-dependence of PAR-3, PAR-6 and PKC-3 for peripheral localization and the overlap in their distributions lead us to propose that they act in a protein complex.

Key words: *Caenorhabditis elegans*, PDZ domain, Asymmetry, PKC-3, *par*, PAR-6

INTRODUCTION

The first asymmetric cell division of the *C. elegans* zygote provides an opportunity to study mechanisms for generating cellular asymmetries (reviewed by Guo and Kemphues, 1996). The first cleavage bisects the asymmetrically placed spindle and produces daughter cells, AB and P_1, with differences in behavior and in fate (Sulston et al., 1983).

Consistent with these differences, AB and P_1 have different cytoplasmic compositions with respect to molecules with potential or demonstrated roles in regulating cell fates. Some molecules are restricted to or enriched in AB (GLP-1; Evans et al., 1994; MEX-3; Draper et al., 1996); other molecules are restricted to or enriched in P_1 (PIE-1; Mello et al., 1996; MEX-1; Guedes and Priess, 1997; SKN-1; Bowerman et al., 1993; P granules; Strome and Wood, 1982).

Asymmetric distribution of most of these molecules depends upon the activities of the *par* genes (partitioning defective). Mutations in these genes lead to somewhat gene-specific alterations in the behavior and fates of AB and P_1 and frequently cause mislocalization of P granules, GLP-1, SKN-1, PAL-1 and PIE-1 (Bowerman et al., 1993, 1997; Crittenden et al., 1994; Kemphues et al., 1988; Tenenhaus et al., 1998; Watts et al., 1996). Analysis of *par* gene phenotypes has led to the proposal that the *par* genes are required to establish anteroposterior (A/P) polarity in the zygote (for review, see Guo and Kemphues, 1996).

Three of the PAR proteins themselves exhibit asymmetric distributions. PAR-1, a putative serine/threonine kinase (Guo and Kemphues, 1995), and PAR-2, a protein containing a putative ATP-binding site and a zinc-binding motif of the 'RING finger' class (Levitan et al., 1994), are localized to the posterior periphery (Boyd et al., 1996) whereas PAR-3, a novel protein (Etemad-Moghadam et al., 1995) containing three PDZ domains (Kurzchalia and Hartmann, 1996), is localized to the anterior periphery of a 1-cell wild-type embryo. It appears that the asymmetry of PAR-3 and PAR-2 are interdependent and PAR-1 is restricted to the posterior periphery by PAR-3 (Boyd et al., 1996; Etemad-Moghadam et al., 1995).

Mutations in the *par-3* and *par-6* genes disrupt many embryonic asymmetries in similar ways (Etemad-Moghadam et al., 1995; Kemphues et al., 1988; Watts et al., 1996). For example, PAR-1 and PAR-2 become evenly distributed around the periphery of *par-3* and *par-6* embryos and P granules are localized incompletely or not at all. The first mitotic spindle stays in the center of the embryos, resulting in AB and P_1 blastomeres of equal size. These two cells lack many of the asymmetries typical of wild type; both blastomeres divide longitudinally and they often express equivalent levels of SKN-1(Bowerman et al., 1997; Watts et al., 1996).

Mutations in *par-6* affect the distribution of PAR-3. Although steady-state levels of PAR-3 are not affected in *par-6* mutants, PAR-3 is not stably localized to the periphery of *par-6* embryos. Most interphase and prophase blastomeres have faint and usually symmetric PAR-3 staining while most metaphase, anaphase and telophase blastomeres have no

detectable peripheral staining. This result indicates that PAR-6 is required for the stable peripheral localization of PAR-3. Taken together with the similarity of the mutant phenotypes, it suggests that *par-3* and *par-6* may function closely together to establish and maintain embryonic asymmetries (Watts et al., 1996).

Recently, Tabuse et al. (1998) discovered that another protein, an atypical protein kinase C (PKC-3), is also required for the stable peripheral localization of PAR-3. Embryos depleted of PKC-3 by RNA interference (RNAi) show unstable peripheral localization of PAR-3. Moreover, PKC-3 is essential for proper asymmetric cell division; *pkc-3 (RNAi)* embryos show defects similar to those in *par-3* and *par-6* mutants. In addition, PKC-3 colocalizes with PAR-3 and its peripheral localization requires *par-3* and *par-6* (Tabuse et al., 1998). It appears, therefore, that PKC-3 plays a similar role to PAR-3 and PAR-6 in establishing embryonic polarities.

As a step to understand better the relationship between PAR-3, PKC-3 and PAR-6, we initiated molecular analysis of the *par-6* gene. In this paper, we report that the *par-6* gene encodes a conserved 309 amino-acid protein, which contains one PDZ domain. We also discovered that PAR-6 co-localizes with PAR-3 and is dependent upon PAR-3 and PKC-3 for its peripheral localization. Combined with previous results, these findings indicate that the peripheral localizations of PAR-3, PKC-3 and PAR-6 are interdependent and suggest that the three proteins might act in a complex.

MATERIALS AND METHODS

Strains and alleles

C. elegans strains were cultured according to standard methods (Brenner, 1974). The N2 Bristol strain was used as the wild-type strain. The mutant strains used are listed as follows: KK788 *par-6(zu170) unc-101(m1) / hInI [unc-54(h1040)]* I, KK818 *par-6(zu222) unc-101(m1) / hIn1 [unc-54(h1040)]* I, KK830 *unc-101(m1) par-6(zu174)/ hIn1 [unc-54(h1040)]* I, PS968 *sy216/ hCI I,* KR2839 *hDf15 (h1486) unc-75 (e950)/hInI [unc-54(h1040)]* I*;* KK747 *par-2(lw32) unc-45(e0286ts) / sC1 dpy-01(e0001)* III, KK639 *lon-1(e185) par-3(it 71)/qC1* III; KK254 *dpy-20(e1282) par-5(it55) / DnT1* IV; KK288 *rol-4(sc8) par-1(b274) / nT1* V, KK575 *dpy-21(e428) par-4(it120) /* + + V; KK423 *dpy-21*(*e428*) *par-4*(*it57*ts). The term '*par* embryos' refers to embryos laid by the homozygous *par* mothers. The deficiency *hDf15* was a gift from Anne Rose, University of British Columbia, and the deficiency *sy216* (Lee et al., 1994) was a gift from Paul Sternberg, California Institute of Technology.

PCR-based deletion mapping

Using a protocol described by Williams (1995), single homozygous deletion embryos (*sy216* or *hDf15*) were picked from among the unhatched embryos of deletion-heterozygous mothers and tested by the polymerase chain reaction (PCR) for the presence of nucleotide sequences marking the ends of cosmids spanning the genomic region containing *par-6*. The following primers were used (listed by cosmids): W02D9-L: GGTTATCCGTTTACGTGCAC. W02D9-R: TTCATGACTTCCTCGTGCG. F07H6-L: GTTCCGGTTGCTAC-TATACG. F07H6-R: GGAATAGCCATGAAAGAAGC. R02E6-L: GTGCACAAATGGCTCAGAGG. R02E6-R: CCAGAGTATGCTC-TACGTTC. W01F5-L: CAGAACTGTGGACTTGCAG. W01F5-R: GCGTCAAATCAATGGCTTG. W02A11-L: CAATGGGACAACC-TAGAACC. W02A11-R: CTCCGAGGTTACAGTAATCC.

For each set of primers, at least six single embryo PCR assays were done for both *sy216* and *hDf15* deletions. PCR amplification using primers from WO2D9, F07H6, R02E6 and W01F5 cosmids gave negative results with *sy216* and *hDf15* embryos while the primers of W02A11 gave positive results. We could not distinguish the difference in the right end breakpoints of *sy216* and *hDf15* deletions, so we show only deletion *sy216* in Fig. 1.

Germline transformation rescue of *par-6* and RNA interference assay

The YAC Y4C12 DNA was recovered according to the protocol descibed previously (Mello et al., 1996) except that the YAC band cut from the gel was purified using a Geneclean® spin filter kit (Bio 101) and concentrated by Speedvac to a final concentration of 100 ng/μl. The cosmid and YAC strains were kindly provided by Alan Coulson at the Sanger Centre in Cambridge, England.

Cosmid or YAC DNA (100 ng/μl) was mixed with plasmid DNA pRF4 carrying the dominant marker, *rol-6*(*su1006*) (200-300 ng/μl), and injected into the distal arms of the gonad using the protocol of Mello et al. (1991). Rol non-Unc progeny were picked to separate plates to identify germline transformants. To assay for rescue of *par-6*, Unc Rol and Unc non-Rol segregants from germline-transformed lines were tested for the production of viable progeny. At least 20 Unc Rol worms were tested from each line.

Sense or antisense RNA from yk74e7 and yk10f4 was made and injected according to the method of Guo and Kemphues (1995) except that we used a Ribomax kit (Promega) to produce sense or antisense RNA. Embryonic lethality (failure to hatch) of the eggs laid by the injected worms was scored after 24 hours. Early embryos were cut from injected animals, mounted on 5% agar pads for microscopy and videotaped. The cDNA clones were kindly provided by Yuji Kohara, National Institute of Genetics, Mishima, Japan.

Molecular analyses

Southern blots were performed according to standard protocols (Sambrook et al., 1989). Allele-specific DNA polymorphisms were detected using yk74e7 cDNA as probes. To amplify the fragments containing the Tc1 transposon insertion site from *par-6* alleles, we used a *par-6*-specific primer, *par-6*-2, (GGATCCGTAAAACCTGC-CAACCAACG) and a Tc1-specific primer (TGGGTATTCCTTG-TTCGAAGC) (Rosenzweig et al., 1983). DNA from single worms was extracted and subjected to the PCR reaction as described above and cloned and sequenced.

Total RNA was extracted from adult N2 and *glp-4* (*bn2*) worms grown at 25°C using a published protocol (Krause, 1995). Northern blots containing total RNA isolated from gravid wild-type worms were prepared according to standard protocols (Sambrook et al., 1989) and probed with the labeled cDNA yk74e7.

Reverse transcription PCR (RT-PCR) was conducted according the published method (Krause, 1995). RNA was made from about 100 wild-type worms and homozygous worms of each *par-6* allele. To detect *par-6* transcript, the primers *par-6*-5′: GGGATCCGT-ATGTCCTACAACGGCTCC and *par-6*-3′: TCGACTCAGTCC-TCTCCACTGTC were used for PCR amplification. The *par-3*-specific primers, *par-3*-1: GCTCTACAACAGGCGAATAC and *par-3*-2: CTGCTAATCGTCAGTCTCAC (Etemad-Moghadam et al., 1995), were used as controls. RT-PCR analyses were performed on three independently extracted RNA samples from each of wild type and the three *par-6* alleles.

Mouse and fly EST cDNA clones were identified using the standard BLASTP program and ordered from Genome Systems Inc. Their sequences were obtained and confirmed on both strands.

Antibodies and immunostaining

Full-length PAR-6 was expressed from pQEPAR6, a plasmid derived from the pQE32 vector (Qiagen) with *par-6* cDNA inserted in the *Bam*HI-*Sal*I site. The fusion protein was purified using the Qiagen denaturing purification protocol. Polyclonal antibodies recognizing PAR-6 fusion proteins were produced at Cornell Univeristy Research

Animal Resources Facility. Two rabbits, #7053 and #7115, were injected with three 500 μg doses of purified PAR-6 fusion proteins (1 mg/ml) at 2-week intervals, serum collected after 2 months and anti-PAR-6 antibodies were affinity purified as previously described (Guo and Kemphues, 1995).

To assay the specificity of the antibodies, embryos from approximately 100 adult wild-type, homozygous *unc-101 par-6 (zu222)* and 200 adult *lon-1 par-3 (it71)* hermaphrodites analyzed by western blot following the method of Etemad-Moghadam et al. (1995). Blots were probed with purified anti-PAR-6 antibodies or monoclonal anti-α-tubulin.

Immunostaining of embryos was done according to the published protocol (Guo and Kemphues, 1995). Slides were mounted and viewed by fluorescence or confocal microscopy. For each stage of N2 embryos, we examined at least 15 embryos from 10 slides. *pkc-3 (RNAi)* embryos were prepared according to the method of Tabuse et al. (1998). The fluorescence images were obtained with a CCD camera (Princeton Instruments).

RESULTS

Molecular cloning of *par-6*

We identified the *par-6* gene via a map-based cloning approach summarized in Fig. 1. Previously, *par-6* was mapped on LGI, 0.7 map unit to the right of *unc-101* (Watts et al., 1996) and we determined that *par-6* was deleted by *sy216* and *hDf15*. Using PCR-based deletion mapping, we were able to locate *par-6* between cosmids W05A3 and W02A11. We confirmed this mapping result by obtaining rescue of the *par-6* maternal effect lethal phenotype with YAC Y4C12 via germline transformation. The cosmids in this region were then tested in the transformation rescue assay; the results are summarized in Fig. 1. Two overlapping cosmids, T26E3 and R09B9, rescued *par-6* while the flanking cosmids T06G6 and R02E6 did not. All tested *par-6* homozygous worms carrying the rescuing cosmid (Rol Unc phenotype) produced some viable progeny (*n*=18 from each of two RO9B9 lines and one T26E3 line); sibling *par-6* worms lacking the transgene (Unc nonRol) produced dead embryos (*n*=20 tested from each line). Over 80% of the progeny (*n*=420) from Rol Unc worms hatched and 50% of the hatched embryos grew up to adults, of which 75% were fertile. Larval arrest and sterility among the progeny of rescued animals was expected since rare escapers from *par-6* mothers exhibit these phenotypes (Watts et al., 1996).

T26E3, which rescued *par-6*, and T06G6, which overlapped T26E3 but did not rescue *par-6*, have been sequenced by the *C. elegans* genome sequencing project (Wilson et al., 1994). Two putative genes predicted by genefinder are present on the region of T26E3 that does not overlap T06G6 (T26E3.1 and T26E3.2). These genes were represented in Dr Yuji Kohara's EST library by cDNAs yk10f4 and yk74e7, respectively. To test whether one of these might be *par-6*, we used a reverse genetic assay – RNA interference (Guo and Kemphues, 1995; Rocheleau et al., 1997; Fire et al., 1998). The embryos laid by worms injected with the RNA made from yk74e7 mimicked the *par-6* phenotype whereas embryos from worms injected with yk10f4 did not. This result suggested that yk74e7 cDNA represents the *par-6* gene.

All three alleles of *par-6* were generated in mutator lines where the Tc1 transposon was actively transposing (Watts et al., 1996). To determine whether Tc1 insertions were the cause

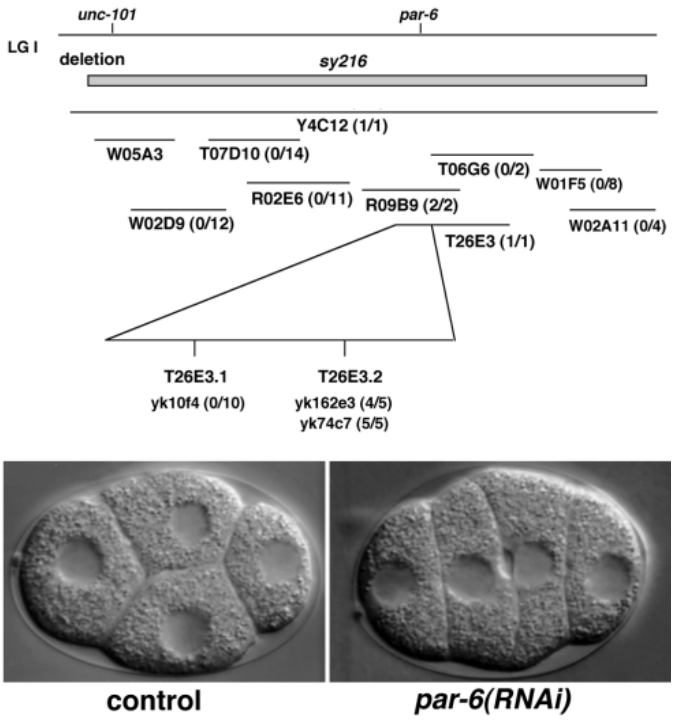

Fig. 1. Identification of the *par-6* gene. The top line is the genetic map; shaded bar below shows the extent of the *sy216* deletion with respect to the cosmids and YAC shown below it. Parentheses adjacent to cosmid and YAC names indicate the results of germline transformation experiments (number of rescuing lines/total number of lines tested). cDNA clones representing two candidate genes on cosmid T26E3 are listed, with results of RNA interference experiments in parentheses beside them (number of Par-6-like embryos/total embryos examined). The photographs at the bottom show a control 4-cell embryo (left) and a 4-cell embryo from a mother injected with RNA from yk74c7 (right).

of the *par-6* mutations, we used yk74e7 as a probe on Southern blots to search for polymorphisms associated with the *par-6* alleles. All three alleles of *par-6* exhibited new restriction fragments about 1.8 kb larger than the original band, consistent with Tc1 insertion (Rosenzweig et al., 1983). To determine the precise location of the insert, we used a Tc1-specific primer and a *par-6*-specific primer to amplify DNA fragments by PCR from single worms homozygous for each of the three *par-6* alleles. Products were obtained from all mutant alleles but not from wild type. The sequences of these products revealed that the Tc1 insertion site in *zu174* and *zu222* is 60 bp downstream of the stop codon and the *zu170* insertion is 72 bp downstream of the stop codon (Fig. 2). The result confirms the identity of T26E3.2 as *par-6*.

Northern blots probed with yk74E7 showed a single abundant germline-enriched 1.5 kb transcript (data not shown). yk74E7 carries a 1.47 kb insert containing a 930 bp open reading frame, which encodes a protein of 309 amino acids with an estimated molecular mass of 34 kDa (Fig. 2). The putative start codon is preceded by an in-frame stop codon 12 nucleotides upstream, and a *C. elegans* translation initiation consensus sequence is found just upstream of this ATG (Krause, 1995, Fig. 2). Together, these data support the view that *par-6* cDNA clone yk74e7 is full length or nearly full length.

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GAAAAAATCG ATATTTCCCA CGAAAATTGT GCATTATTTC CGGCTCAACG CGGAGCTGTC

ACCTGTAACT TGTAATTATG TCCTACAACG GCTCCTACCA TCAAAATCAT CATTCAACAC
    ***          M   S Y N   G S Y H   Q N H   H S T

TTCAAGTCAA ATCGAAATTT GATTCTGAAT GGCGTCGTTT CTCAATACCA ATGCATTCTG
L Q V K   S K F   D S E   W R R F   S I P   M H S

CGTCTGGTGT CTCTTACGAT GGGTTCCGGA GCCTTGTCGA GAAGCTTCAT CACCTGGAAA
A S G V   S Y D   G F R   S L V E   K L H   H L E

GTGTCCAATT CACACTGTGC TACAACTCGA CTGGCGGTGA TCTTCTACCA ATCACAAATG
S V Q F   T L C   Y N S   T G G D   L L P   I T N

ACGATAACCT CCGAAAATCG TTCGAATCGG CTCGCCCACT GCTCCGTTTG CTCATTCAAC
D D N L   R K S   F E S   A R P L   L R L   L I Q

GCCGCGGTGA ATCATGGGAA GAGAAATATG GTTATGGTAC AGACTCTGAT AAACGATGGA
R R G E   S W E   E K Y   G Y G T   D S D   K R W

AGGGAATTTC ATCACTTATG GCACAAAAAC CACCGAAACG CAGTTATTCA ATCTCGAATC
K G I S   S L M   A Q K   P P K R   S Y S   I S N

CCGAAGATTT TCGACAGGTC TCTGCAATCA TCGACGTGGA CATTGTGCCA GAAGCTCATC
P E D F   R Q V   S A I   I D V D   I V P   E A H

GCCGTGTCCG TCTCTGCAAG CACGGACAAG AACGACCACT TGGATTCTAT ATTCGTGACG
R R V R   L C K   H G Q   E R P L   G F Y   I R D

GAACATCGGT TCGAGTGACA GAACGAGGCG TCGTCAAAGT TTCAGGAATT TTCATTTCCC
G T S V   R V T   E R G   V V K V   S G I   F I S

GGCTTGTCGA TGGTGGGCTC GCCGAGTCCA CAGGCCTTCT TGGTGTTAAT GACGAGGTGC
R L V D   G G L   A E S   T G L L   G V N   D E V

TCGAGGTGAA TGGAATTGAG GTTCTCGGAA AGACGCTGGA TCAGGTCACT GATATGATGG
L E V N   G I E   V L G   K T L D   Q V T   D M M

TCGCCAATGC TCATAACTTG ATTATTACTG TAAAACCTGC CAACCAACGA AACACACTGT
V A N A   H N L   I I T   V K P A   N Q R   N T L

CACGAGGACC GTCACAACAA GGAACACCGA ACGCCAGTGA GATGTCGGCC GCCACCGCAG
S R G P   S Q Q   G T P   N A S E   M S A   A T A

CCGCTACAGG TGGAATTCAG CGCCCGATGA AAATGAACGG AAGCTCCGAC GGCAGTTATC
A A T G   G I Q   R P M   K M N G   S S D   G S Y

ATCCGAAACA GCACGACGCA AATGATTCGG ACAGTGGAGA GGACTGAAAA AACTCTTTTC
H P K Q   H D A   N D S   D S G E   D    (zu174 & zu222)

AGCCATTTTT CCTCGATTTT TTCAGAAAAA AAAACCACAA AATGTGACCA TTTTTATACA
          (zu170)

TAATTATATT ATTTATATTC TCGCTAAAAC CACACACACA CACAACCAAA ACCGCTTAAT
TTATTTATTT TCCCCTTTTT CTCCTAATTT TTAATCAAAT TTGTCGGCTT TCTCTTCACT
TTTTTGCGCA CAATTTTCAC TTTTATTTCC CCCGTTTCTC GCCCCAAAAT GTGCCTTCCT
TTCGCCCTTT TTATGTGATT TTTCCCCTTC AGAGCTCTCT GAGCCCATAC TTTTTTTTTC
GAACTTTGGC TCAAATTTTT TGACAACAGA CACGACATAC AATTTTTCCC CTGAAATTCA
CATTATTAGT GAGAATAAAT TATTTATTTC AA
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Fig. 2. *par-6* cDNA sequence. The TAA marked by asterisks in the sequence is the in-frame stop codon preceding the putative ATG. A possible translation initiation consensus sequence is shown in bold. The PDZ domain of PAR-6 is underlined. The bases preceding the Tc1 transposon insertion sites in *par-6* mutations are indicated in large font. GenBank Accession number: AF070968.

The Tc1 transposons appear to prevent accumulation of *par-6* transcripts. Using RT-PCR, we detected *par-6* transcripts in N2 worms but not in *par-6* worms of all three alleles. In control experiments, RT-PCR using *par-3*-specific primers detected *par-3* messages in the same RNA samples (data not shown).

par-6 encodes a PDZ-containing protein that is conserved in worm, fly and mouse

Protein database searches reveal that PAR-6 contains one PDZ domain. PDZ domains are protein motifs of approximately 100 amino acids that are found in a growing number of proteins and mediate protein-protein interactions (Ponting et al., 1997). The PDZ domain of PAR-6 shares most similarity to the PDZ domain of Tax clone 40, a human protein that interacts with Tax protein of Human T-CELL Leukemia virus (HTLV) (Rousset et al., 1998).

The alignment of PAR-6 PDZ with PSD-95 PDZ3 shows that the amino acids forming the β-sheets and α-helix structures in PSD-95 PDZ3 (Doyle et al., 1996; Fig. 3) are well conserved in PAR-6 PDZ, suggesting the overall secondary structure of the PDZ domain of PAR-6 would be similar to PSD-95 PDZ3.

In our database searches, we identified *Drosophila melanogaster* and *Mus musculus* EST cDNA clones. We obtained these cDNA clones, determined their complete sequence and found that the fly and mouse cDNAs show 47% and 45% overall similiarity with *C. elegans* PAR-6, respectively. The conservation is greatest among these homologues over a 115 amino acid region containing the PDZ domain; worm PAR-6 is 88% and 80% identical to the fly and mouse proteins, respectively (Fig. 3B). In addition to the PDZ domain, the N-terminal portions are also quite similar among the three proteins. When compared with the worm PAR-6 sequence over amino acids 14-96, the fly and mouse protein are 52% and 43% identical (Fig. 3B).

PAR-6 is localized asymmetrically in the zygote

We obtained polyclonal antibodies from two rabbits using bacterially expressed 6His-PAR-6 fusion proteins as antigen. The antiserum from rabbit 7053 gave the best specificity and was used for all analysis unless indicated otherwise. As shown in Fig. 4A, a major band of 34 kDa, the predicted size for the *par-6* gene product, was recognized by the affinity-purified antibodies in wild-type embryo extracts but was absent from embryos of *par-6* (*zu222*) homozygous hermaphrodites.

We stained early embryos in gravid worms and examined the pattern of expression of the *par-6* gene product. We found that the distribution of the PAR-6 signal was identical when we used purified antiserum from either of the two immunized rabbits. The staining pattern that we describe is not observed with the preimmune serum and is absent from *par-6* (*zu222*) embryos (*n*=42) (Fig. 5G), *par-6 (zu170)* (*n*=23) and *par-6 (zu174)* (*n*=26) embryos (not shown). Therefore, we are confident that the staining pattern that we describe here represents the distribution of the PAR-6 protein.

Antibody staining is detectable throughout the cytoplasm and at the periphery of blastomeres up to about the 50-cell stage. The peripheral staining is asymmetric in the cells of the germline lineage P_0, P_1, P_2 and P_3.

Asymmetric peripheral localization of PAR-6 develops as the newly fertilized egg, P_0, progresses through the cell cycle. Localized antibody staining is not detectable in unfertilized mature oocytes, nor in newly fertilized eggs undergoing the first meiotic division. We first detect patchy PAR-6 staining at the periphery of the embryo after completion of meiosis I, as determined by the presence of a single polar body. 70% of embryos in meiosis II exhibit patchy PAR-6 staining all around the periphery with no obvious asymmetry (*n*=20) (Fig. 5A). When the female pronucleus completes metaphase II as indicated by the presence of a second polar body, peripheral PAR-6 is restricted to the anterior 85% of embryo length (EL;

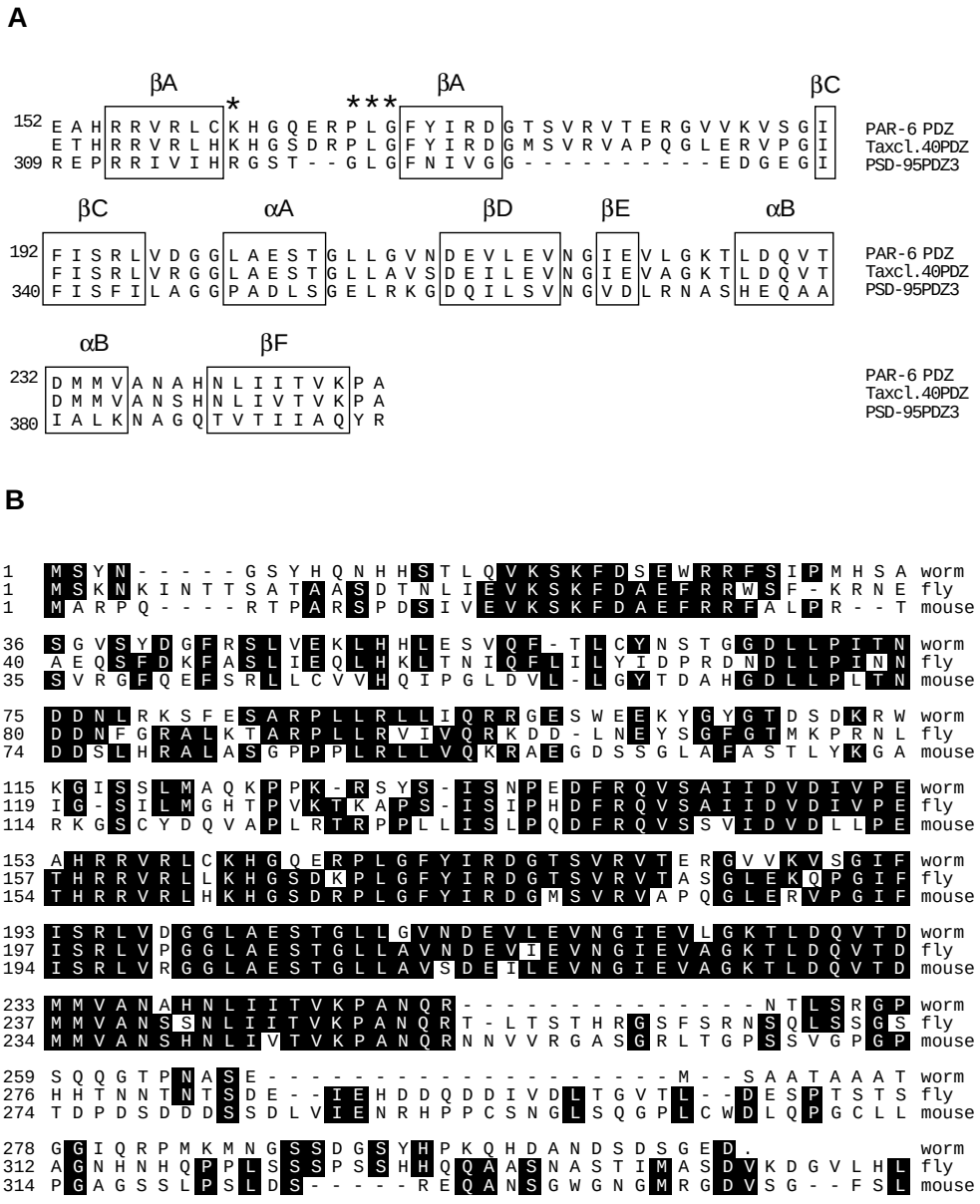

Fig. 3. Comparisons of PAR-6 with other proteins. (A) Sequence alignment of PAR-6 PDZ with other PDZs. The predicted secondary structure elements in the PAR-6 PDZ were derived from its alignment with PSD-95 PDZ3 (Doyle et al., 1996) and are shown as boxes. Tax clone 40 is not a complete mRNA sequence (Rousset et al., 1998); thus, the protein sequence is not numbered. The predicted hydrophobic pocket of PSD-95 PDZ3 which can bind to the C-terminal peptide is indicated with asterisks. (B) Amino acid sequence comparison of worm PAR-6 and its putative homologues identified in fly and mouse: fly EST clone #LD08317 (GenBank Accession numbers: AA264648 and AF070969) and mouse EST clone #440139 (GenBank Accession numbers: AA016558 and AF070970). The predicted mouse and fly proteins contain 347 and 352 amino acids, respectively. The alignment was generated using the CLUSTAL method (Higgins and Sharp, 1989).

0% is anterior) (*n*=8) (not shown). In embryos between prophase and telophase of the first mitosis, the peripheral PAR-6 is restricted to the anterior 55% EL of the embryos (*n*=24) (Fig. 5B). During the first cleavage PAR-6 staining is detectable in the advancing furrow.

Asymmetry of PAR-6 is also observed in P_1 and P_2, the germline cells in 2-cell and 4-cell embryos, respectively. Just after the first cleavage, strong peripheral staining is present where P_1 apposes AB. The strength of this signal suggests that protein is present along this boundary in both cells. As the P_1 cell progressed into prophase, PAR-6 distribution extends posteriorly, covering the anterior 30% of 13 of 16 P_1 cells scored (Fig. 5C, arrows). Starting in late metaphase and through anaphase, the distribution of PAR-6 in P_1 becomes skewed to the ventrolateral periphery (Fig. 5D), the part of the cell fated to give rise to EMS. PAR-6 is distributed uniformly at the AB periphery throughout the 2-cell stage. In the 4-cell stage, PAR-6 peripheral staining is strong in ABa, ABp and EMS, but PAR-6 can be detected only faintly around the periphery of P_2 (Fig. 5E) until metaphase of P_2 at the 7-cell stage. At this time PAR-6 is asymmetrically localized to the dorsal side of P2, the part of the cell fated to give rise to C (Fig. 5F). The protein is distributed asymmetrically in P_3 as well (data not shown). Peripheral PAR-6 could be detected till about the 50-cell stage. Overall, the localization pattern of PAR-6 is very similar to that of PAR-3 (Etemad-Moghadam et al., 1995).

PAR-3 and PAR-6 co-localize at the cell periphery

To determine if the localization of PAR-3 and PAR-6 are coincident, we stained wild-type embryos simultaneously with rabbit anti-PAR-6 and chicken anti-PAR-3 antibodies. PAR-3 and PAR-6 distributions overlapped at all stages in early embryos. Fig. 6A-C shows confocal images of a wild-type 1-cell embryo stained for both proteins. Tangential optical

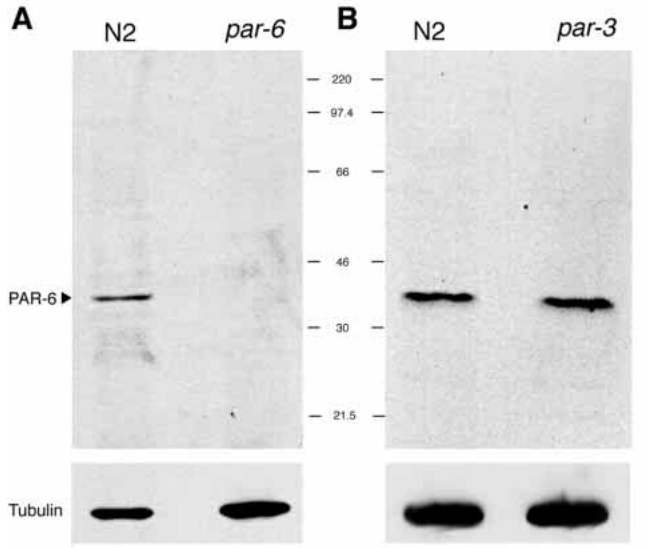

Fig. 4. Western Blot analysis of PAR-6. (A) Western blot of total proteins from extracts of embryos of wild-type (N2) and *par-6 (zu222)* homozygotes probed with anti-PAR-6 antibodies. Monoclonal anti-α-tubulin antibodies were used as a loading control. The position of molecular markers in kDa is shown on the right. (B) Western blot of total proteins from extracts of embryos of wild-type (N2) and *par-3 (it71)* homozygotes probed with anti-PAR-6 antibodies.

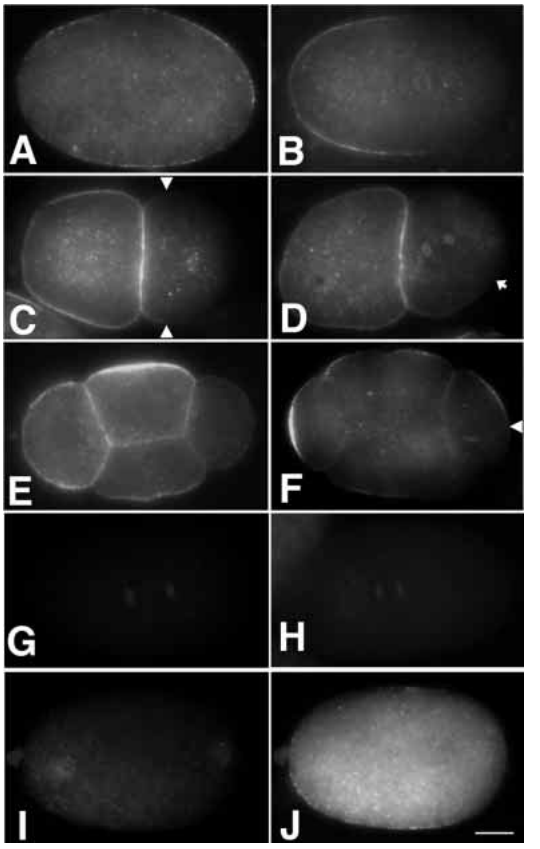

Fig. 5. PAR-6 distribution in early embryos. Micrographs of embryos, wild type (A-F), *par-6 (zu222)* (G), *par-3 (it71)* (H), co-stained with anti-PAR-6 and DAPI. The *pkc-3 (RNAi)* embryos were co-stained with anti-PAR-6 (I), anti-PAR-3 (J), and DAPI. (A) 1-cell embryo in meiosis II. (B) 1-cell embryo at anaphase of the first mitosis. (C) 2-cell embryo, when P_1 is in prophase. Arrows indicate the posterior extent of PAR-6 signal in P_1. (D) Late 2-cell embryo, when P_1 is in anaphase. Arrowhead indicates posterior extent of PAR-6 signal on the ventral side. (E) 4-cell embryo. Note the weak signal at the periphery of prophase stage P_2 cell. (F) Seven cell embryo, when P_2 is in metaphase. Arrowhead indicates the ventral boundary of PAR-6 staining. (G) Late 1-cell stage *par-6*(*zu222*) embryo. (H) Late 1-cell stage *par-3 (it71)* embryo. (I) Anti-PAR-6 staining in an early 1-cell *pkc-3(RNAi)* embryo. (J) Anti-PAR-3 staining in the same *pkc-3(RNAi)* embryo shown in I. Note the weak and patchy signal of PAR-3 on the periphery. The high levels of cytoplasmic staining in this embryo are due to a higher background staining with this antibody. Scale bar, 9 μm.

sections viewed at high magification revealed that the staining patterns of both PAR-3 and PAR-6 resolve as irregular dots ranging from 100 nm to about 1.5 μm across what we will refer to as protein clusters (Fig. 6D-F). The PAR-6 protein clusters are consistently more diffuse than PAR-3 clusters (compare Fig. 6D to 6G). Furthermore, there appear to be more PAR-6 protein clusters on the periphery than PAR-3 clusters; of five embryos scored, there were an average of 320 PAR-6 clusters and 180 PAR-3 clusters. Approximately 45% of the peripheral clusters detected with the anti-PAR-6 antibody also showed signal with the anti-PAR-3 antibody and about 80% of the clusters detected with anti-PAR-3 antibody also showed signal with anti-PAR-6. Thus PAR-6 is colocalized with PAR-3 in about 40% of the peripheral protein clusters detected by the two antibodies.

We noticed differences of intensity between PAR-6 and PAR-3 in clusters that stained with both. To test to what extent the variablility in the signals were due to the difference in secondary antibodies or the variability in staining or detection, we stained embryos with affinity-purified rabbit and chicken anti-PAR-3 antibodies simutaneously and did a similar analysis as described above for PAR-3 and PAR-6. Although the intensities of signals revealed by the two different antibodies were not always equal for any individual cluster, we found that over 90% of the protein clusters gave signals from both antibodies (n=4 1-cell embryos; mean of 190 clusters/embryo; Fig. 6G-I). This result indicates that the differences in the pattern of anti-PAR-6 and anti-PAR-3 staining may reflect true differences in the distributions of the proteins in vivo.

PAR-3 and PKC-3 are required for the peripheral localization of PAR-6

Previous work had shown that *par-6* is required for the peripheral localization of PAR-3 and PKC-3 (Watts et al., 1996; Tabuse et al., 1998). To determine if PAR-3 and PKC-3 are required for the proper localization of PAR-6, we examined PAR-6 distribution in *par-3* (*it71*) and *pkc-3 (RNAi)* mutants.

Peripheral PAR-6 is not detectable at any point in the cell cycle in either *par-3* (n=110) or *pkc-3(RNAi)* (n=90) embryos at any stage (Fig. 5H,I). PAR-6 protein, however, was present in normal amounts in *par-3* mutants as determined by western blot (Fig. 4B). We could not determine whether PAR-6 protein is present in normal amounts in *pkc-3 (RNAi)* because RNAi does not produce sufficient numbers of embryos for western blot analysis. These results suggest that *par-3* and *pkc-3* are

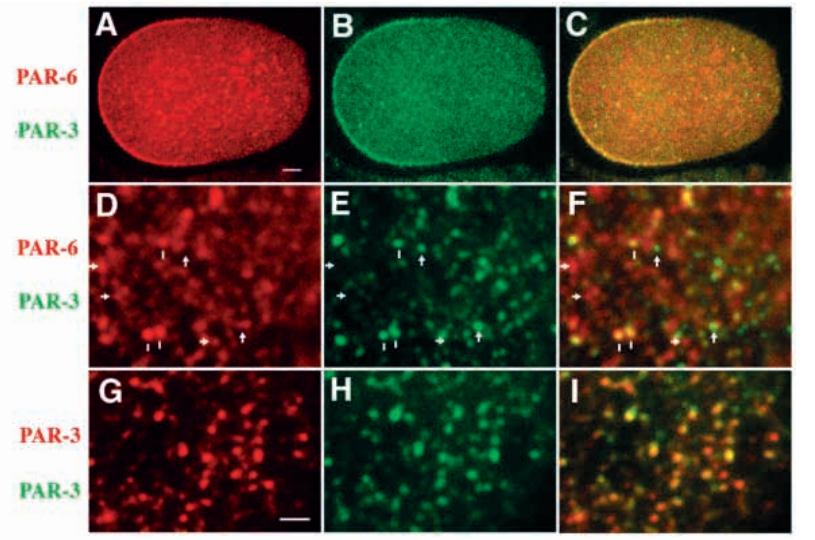

Fig. 6. Co-localization of PAR-6 and PAR-3 in wild-type 1-cell embryos. (A-C) Medial confocal optical cross section of an embryo stained with rabbit anti-PAR-6 (A; red) and chicken anti-PAR-3 (B; green). (C) Merged image of A and B. Regions of overlap are yellow or orange. (D-F) High magnification images of a tangential optical section of a 1-cell embryo taken using a conventional optical microscope. PAR-6 staining is shown in red (D); PAR-3 is shown in green (E) and the merged image in F. Peripheral protein clusters containing both PAR-3 and PAR-6 signals are indicated by vertical lines. Clusters giving strong signal with one antibody and no or weak signal with the other are indicated by arrows: high PAR-3, vertical arrows; high PAR-6, horizontal arrows. The merged image of D and E is shown in F. (G-I) Same as D-F except that staining patterns in G and H are from rabbit anti-PAR-3 (red) and chicken anti-PAR-3 (green). Scale bars, 5 μm.

required for recruiting PAR-6 to the periphery or maintaining it there. Consistent with previous results (Tabuse et al., 1998), in *pkc-3(RNAi)* embryos early in the cell cycle, PAR-3 can still be detected as a weak and patchy peripheral signal (Fig. 5J).

par-2, *par-4* and *par-5* are required for the proper asymmetry of PAR-6

If *par-3* is required for the peripheral localization of PAR-6, we reasoned that mutations in *par-2* and *par-5*, which affect the localization of PAR-3 (Etemad-Moghadam et al., 1995), might affect that of PAR-6 as well. Therefore, we stained *par-2* and *par-5* embryos with antibodies against PAR-6 and PAR-3. In *par-2* embryos, the distributions of both PAR-6 and PAR-3 extended to 85%-95% EL of the 1-cell embryos (n=16) even in metaphase and later stages of mitosis (Fig. 7A,B). In twelve of eighteen *par-5 (it55)* 1-cell embryos, PAR-6 and PAR-3 were detected in a somewhat patchy distribution all around the periphery without obvious A/P asymmetry (Fig. 7C,D). In the remaining six embryos, the distributions of the two proteins extended more into the posterior but some A/P asymmetry was retained. In both *par-2* and *par-5* mutants, regardless of the extent of mislocalization, the colocalization of PAR-3 and PAR-6 is not distinguishably different from wild type.

For completeness, we also examined the localization of PAR-6 in *par-1* and *par-4* mutants. While PAR-6 is localized like wild-type in *par-1* mutants, we found that PAR-6 is mislocalized in *par-4* (Fig. 7E). During prophase, metaphase and early anaphase of the first mitosis, PAR-6 signal extends to about 70-80% EL (n=14). This was unexpected since a previous analysis had reported normal distribution of PAR-3 in *par-4* mutants (Etemad-Moghadam et al., 1995). When we checked the staining of PAR-3 in *par-4*, we found that PAR-3 shows the same pattern of mislocalization as PAR-6 (Fig. 7E,F), indicating that *par-4* plays a role in restricting these two proteins to the anterior of the zygote (see Discussion).

DISCUSSION

The *par-6*, *par-3* and *pkc-3* genes play an important role in the establishment of polarity in *C. elegans* embryos (Etemad-Moghadam et al., 1995; Kemphues et al., 1988; Tabuse et al., 1998; Watts et al., 1996). The *par-3* gene encodes a PDZ domain-containing protein that is localized to the cell periphery at one pole of asymmetrically dividing cells of the germline lineage (Etemad-Moghadam et al., 1995) where it is proposed to interact with the atypical protein kinase, PKC-3 (Tabuse et al., 1998). The strong similarity of the *par-3*, *par-6* mutant and *pkc-3(RNAi)* phenotypes, together with the observation that *par-6* is required for the localization of PAR-3 and PKC-3 proteins argues that *par-6* is functionally related to the other two proteins (Tabuse et al., 1998; Watts et al., 1996). We have described the cloning of the *par-6* gene and the discovery that it encodes a conserved PDZ-containing protein that has

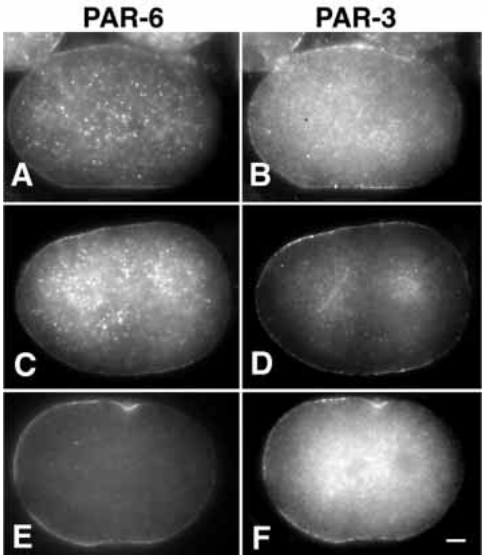

Fig. 7. Distribution of PAR-6 and PAR-3 in *par-2*, *par-4* and *par-5*. (A,B) 1-cell stage *par-2* embryo showing extension of peripheral PAR-3 and PAR-6 into the posterior (compare to Fig. 5B). (C,D) 1-cell stage *par-5* embryo showing lack of asymmetry of cortical staining. (E,F) Late 1-cell stage *par-4* embryo showing extension of peripheral PAR-3 and PAR-6 into the posterior. The cleavage furrow has initiated. Scale bar, 5 μm.

homologues in *Drosophila* and mouse. Furthermore, we have shown that PAR-6 co-localizes with PAR-3 and that *par-3* and *pkc-3* are required for the peripheral localization of PAR-6. Based on these results, we propose that PAR-6, PAR-3 and PKC-3 are components of an anteriorly localized peripheral protein complex.

PAR-6, PAR-3 and PKC-3 may form a protein complex

The colocalization of PAR-3 and PAR-6 in peripheral protein clusters is consistent with their similar phenotypes and raises the possibility that PAR-3 and PAR-6 form protein complexes in vivo. Complex formation may be mediated by PDZ domains, protein motifs that promote protein-protein interaction at specialized sites in the submembranous regions of a variety of cell types (Ponting et al., 1997). Because PDZ domains promote formation of clusters via PDZ/PDZ interactions and interactions of PDZ domains with other protein motifs, such a complex might also include other proteins. Indeed, as described above, PKC-3 could also be a component of the complex.

The functional relationships among PAR-6, PAR-3 and PKC-3 are unclear. Each is required for the stable peripheral localization of the others (Watts et al., 1996; Tabuse et al., 1998; this report). A working model can be proposed based on the observations that PAR-3 is transiently present at the cell periphery in the absence of either PAR-6 or PKC-3, but both PAR-6 and PKC-3 absolutely require PAR-3 (Watts et al., 1996; Tabuse et al., 1998; this report). We propose that PAR-3 can localize to the periphery early in the cell cycle independently of PAR-6 and PKC-3. PAR-3 then recruits PAR-6 and PKC-3, which can act together to stabilize the complex at the periphery.

The function of this hypothetical protein complex remains to be determined. One possibility consistent with the presence of multiple PDZ domains and the involvement of a protein kinase C is that this complex may function in intracellular signaling to establish embryonic polarity. Ina^{D}, a protein containing five PDZ domains, has been shown to act as a scaffold for the formation of a protein complex that functions in phototransduction in the *Drosophila* eye and is localized to the rhabdomeres of photoreceptors (Tsunoda et al., 1997). By analogy, the PDZ domains of PAR-6 and PAR-3 might play an important role in assembling a signaling complex at the anterior periphery of *C. elegans* embryos.

PAR-6 appears to be highly conserved across species. The conservation is not restricted to the PDZ domain, but extends across the entire protein, implying that the binding partners and biochemical activity of the worm, fly and mouse proteins are conserved as well. Because PKC-3 has all the features of mammalian atypical protein kinases (Tabuse et al., 1998; Wu et al., 1998) and a potential mammalian homologue of PAR-3 has been identified through interactions with an atypical protein kinase C (Izumi et al., 1998), we speculate that the function of the PAR-6/PAR-3/PKC-3 interactions may also be conserved.

The roles of PAR-2, PAR-4 and PAR-5 in establishing PAR-6/PAR-3 asymmetry

PAR-2, PAR-4 and PAR-5 are essential for establishing or maintaining proper asymmetry of PAR-6 and PAR-3. As expected from the co-dependence of PAR-3 and PAR-6 for their localization, the PAR-6 distribution is altered in *par-2* and *par-5* embryos in the same way that PAR-3 distribution is altered. In addition, we found that both PAR-3 and PAR-6 distributions are altered in *par-4* 1-cell embryos. A previous report (Etemad-Moghadam et al., 1995) that *par-4* did not affect PAR-3 distribution was based on experiments with the temperature-sensitive *par-4* allele *it57*, while our analysis used *it120*, a stronger *par-4* mutation. The allele differences may explain the differences in our findings. Because PAR-3 and PAR-6 are still co-localized in *par-2*, *par-4* and *par-5* embryos, these genes appear not to be required for PAR-3/PAR-6 complex formation.

Although *par-2*, *par-4* and *par-5* all affect localization of both PAR-3 and PAR-6, these genes affect polarity in different ways. In *par-2* and *par-4* mutants, PAR-6 and PAR-3 extend beyond the normal A/P boundary yet still retain some A/P asymmetry. Therefore, PAR-2 and PAR-4 may play a role in reinforcing and sharpening the A/P boundary but not in generating the A/P asymmetry of PAR-3 and PAR-6. Although their effects on PAR-3 and PAR-6 are similar, mutations in *par-4* and *par-2* have distinguishable phentoypes (Kemphues et al., 1988; Morton et al., 1992) and, although *par-2* mutants can be suppressed by lowering the levels of *par-6* (Watts et al., 1996), *par-4* mutants cannot (T. H. unpublished results). The *par-5* gene appears to function differently from either *par-2* or *par-4* in localizing PAR-3 and PAR-6. Because *par-5* mutations lead to wide distribution of PAR-3 and PAR-6 with no obvious polarity, PAR-5 may have a more primary role in establishing A/P polarity of PAR-3 and PAR-6 in 1-cell embryos.

In summary, PAR-6 is a PDZ domain-containing protein that co-localizes with PAR-3. The three proteins, PAR-6, PAR-3 and PKC-3, are co-dependent for their stable peripheral localization and may be part of a protein complex that functions to establish polarity in early *C. elegans* embryos. The conservation of PAR-6 across species leads us to speculate that such a complex may play roles in establishing cellular asymmetries in other animals.

The authors thank Alan Coulson for cosmid strains and Yuji Kohara for providing the cDNA clones. Some genetic strains were provided by the Caenorhabditis Genetics Center, which is supported by the National Institutes of Health National Center for Research Resources. We are grateful to Diane Morton and Kathy Baker for providing us with chicken anti-PAR-3 antibodies. We would like to thank Aaron Schetter for helping to sequence the *Drosophila* EST cDNA clone. We also want to thank Daryl Hurd and Fabio Piano for critically reading the manuscript, Fang Yu, Michael Goldberg and Dan Starr for useful discussions, and Carol Bayles for assisting with microscopy and confocal imaging. T. H. is a recipient of Wu Memorial Award. This work was supported by NIH grant HD27689 to K. J. K.

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