

## The *C. elegans par-4* gene encodes a putative serine-threonine kinase required for establishing embryonic asymmetry

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### SUMMARY

During the first cell cycle of *Caenorhabditis elegans* embryogenesis, asymmetries are established that are essential for determining the subsequent developmental fates of the daughter cells. The maternally expressed *par* genes are required for establishing this polarity. The products of several of the *par* genes have been found to be themselves asymmetrically distributed in the first cell cycle. We have identified the *par-4* gene of *C. elegans*, and find that it encodes a putative serine-threonine kinase with similarity to a human kinase associated with Peutz-Jeghers Syndrome, LKB1 (STK11), and a *Xenopus* egg and embryo kinase, XEEK1. Several strong *par-4* mutant alleles are missense mutations that alter conserved residues within the kinase domain, suggesting that kinase activity is essential

for PAR-4 function. We find that the PAR-4 protein is present in the gonads, oocytes and early embryos of *C. elegans*, and is both cytoplasmically and cortically distributed. The cortical distribution begins at the late 1-cell stage, is more pronounced at the 2- and 4-cell stages and is reduced at late stages of embryonic development. We find no asymmetry in the distribution of PAR-4 protein in *C. elegans* embryos. The distribution of PAR-4 protein in early embryos is unaffected by mutations in the other *par* genes.

Key words: *Caenorhabditis elegans*, *par-4*, Asymmetry, Polarity, Maternal effect, Serine-threonine kinase

### INTRODUCTION

Asymmetries established during the first cell cycle of the *C. elegans* embryo are necessary for proper patterning and establishment of differential cell fates in the early blastomeres (see Bowerman, 1998; Rose and Kemphues, 1998 for recent reviews). The posterior pole of the zygote is determined by the position of the sperm at fertilization (Goldstein and Hird, 1996). In response to this polarity cue, a dramatic reorganization of cytoplasm occurs that correlates with the emergence of a number of visible asymmetries, including a transient accumulation of foci of filamentous actin at the anterior pole (Strome, 1986) and localization of P granules to the posterior pole (Hird et al., 1996; Strome and Wood, 1982, 1983). The first mitotic spindle forms along the anteroposterior axis and migrates to the posterior leading to asymmetric placement of the cleavage furrow. This results in production of a larger blastomere, AB, in the anterior and a smaller blastomere, P1, in the posterior. In addition to their size difference, the AB and P1 blastomeres exhibit different cell cycle rates, different spindle orientations and different cell fate potentials (Sulston et al., 1983). Differences in cell fate potentials are reflected in differences in the distribution of the known cell fate regulators SKN-1, GLP-1, MEX-3, PIE-1 and

PAL-1 in AB and P1 (Bowerman et al., 1993; Draper et al., 1996; Evans et al., 1994; Hunter and Kenyon, 1996; Mello et al., 1996).

Maternal effect lethal mutations in the *par* (partitioning defective) genes disrupt many of the asymmetries established during the first cell cycle of embryogenesis (Cheng et al., 1995; Kemphues et al., 1988; Kirby et al., 1990; Morton et al., 1992; Watts et al., 1996), including P granule distribution, cleavage spindle placement and cell cycle asynchrony. The products of several of the *par* genes have been identified and have been shown to be asymmetrically distributed in the 1-cell embryo. PAR-1, a putative serine-threonine kinase, and PAR-2, a protein containing a zinc-binding motif of the ring finger class, are distributed to the posterior periphery of the 1-cell embryo (Boyd et al., 1996; Guo and Kemphues, 1995). PAR-3 and PAR-6 proteins, which contain PDZ domains, are distributed at the anterior cell cortex (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999). An atypical protein kinase C, PKC-3, has recently been identified, that co-localizes with PAR-3 and PAR-6, and gives a phenotype similar to *par-3* and *par-6* mutants upon RNA interference (Tabuse et al., 1998).

Mutations in the *par-4* gene, like mutations in the other *par* genes, affect several aspects of polarity, but with some unique features (Morton et al., 1992). Unlike other *par* mutants,

placement of the first cleavage spindle is normal in *par-4* mutant embryos, resulting in daughter cells with wild-type size asymmetry. However, these two cells divide synchronously and produce 4-cell embryos with P granules and GLP-1 protein in all cells (Crittenden et al., 1997; Morton et al., 1992). *par-4* mutations prevent differentiation of intestinal markers in the embryo and affect differentiation of both body wall and pharyngeal muscle cells (Kemphues et al., 1988; Morton et al., 1992). Mutations in the *par-4* gene also result in reduced asymmetry of PAR-3 and PAR-6 proteins at the cortex of the 1-cell embryo (Hung and Kemphues, 1999). Temperature shifts with a temperature-sensitive allele of *par-4* provide evidence that the requirement for PAR-4 expression for embryo viability and for intestinal development begins during oogenesis and ends before the 4-cell stage of embryogenesis (Morton et al., 1992).

To gain further understanding of the role of the *par-4* gene in *C. elegans* development, we have begun a molecular analysis of the gene and its product. We report here the identification of the gene, its predicted protein product and the distribution of the protein in early *C. elegans* embryos.

## MATERIALS AND METHODS

### Strains and maintenance

*C. elegans* strains were cultured according to standard methods (Brenner, 1974). The N2 Bristol strain was used as the standard wild-type strain. All *par-4* alleles with the designation *zu* were isolated in the laboratory of Jim Priess (Fred Hutchinson Cancer Research Center) during a screen for recessive, nonconditional, maternal-effect, embryonic lethal mutations derived from a transposon-mobilized strain (Mello et al., 1994). The alleles *zu160*, *zu187*, *zu198*, *zu178*, and *zu182* failed to complement *par-4(it57)*. These mutations were backcrossed 6-10 times to homozygous *egl-23(n601); him-3(e1147); par-4(it138)* was identified in a screen for UV/psoralen-induced maternal effect lethal mutations (Lesilee Rose and K. J. K., unpublished). *par-4(lw38)* was generated using gamma irradiation (Jocelyn Shaw, University of Minnesota). The *par-4* alleles, *it33*, *it47ts*, *it57ts*, *it72*, *it74*, *it75*, *it83*, *it84* and *it88*, have been described previously (Morton et al., 1992). The temperature-sensitive (*ts*) mutants were maintained as homozygous stocks at 16°C and shifted up to 25°C for 24 hours prior to examination for mutant phenotypes. Other *par-4* mutations were marked with *dpy-21(e428)* and maintained in *trans* to a wild-type chromosome. Other strains used in this study include: *rol-4(sc8) par-1(b274)/DnT1 V*, *par-2(lw32) unc-45(e2867ts)/qC1III*, *lon-1(e185)par-3(it71)/qC1III*, *dpy-20(e1282) par-5(it55)/DnT1(IV)*, and *par-6(zu222) unc-101(ml)/hIn1 I*.

For tests of *it57ts* male fertility, *it57 L1* larvae produced at 16°C were raised at 25°C and mated to *unc-51(e369) dpy-21(e428) V* hermaphrodites at 25°C. Six *it57ts* males were mated with 3-6 hermaphrodites for each cross. Outcross progeny were identified as non-Unc non-Dpy worms.

### Cloning of the 3.2 kb *Bgl*II polymorphic fragment from *par-4(zu187)*

DNA from *dpy-21(e428) par-4(zu187)/++ V* was isolated, restricted to completion with *Bgl*II and separated on a 0.8% low-melting temperature agarose gel. DNA of approximately 2.8-3.5 kb was isolated from the gel and the overhanging ends were partially filled in with G and A. These fragments were ligated with  $\lambda$ -ZAP II vector (Stratagene), cut with *Xho*I and partially filled in with C and T. After packaging (Gigapack, Stratagene), the phage library was screened

using TcI sequences as a probe. Several positive phage were subjected to *in vivo* excision, producing a Bluescript plasmid containing the cloned insert. These plasmids were used to probe Southern blots to confirm that they contained the novel TcI insertion.

### Southern analysis and northern analysis

Genomic DNA from mixed stage worms was prepared for Southern blots by the proteinase K method (Sulston and Hodgkin, 1988) or with a PureGene DNA isolation kit (Gentra Systems). After restriction digestion with the appropriate enzymes, the DNA was separated on a 0.8% agarose gel and transferred to Hybond N+ membranes (Amersham) using standard techniques (Ausubel et al., 1987).

Total RNA from young adult worms was prepared using the glass bead method (Lee et al., 1993). mRNA was isolated using a PolyAtract mRNA Isolation kit (Promega). The mRNA was separated on a formaldehyde gel and transferred to Hybond N+ membranes (Amersham) using standard techniques (Ausubel et al., 1987). Probes for Southern and northern analysis, as well as for genomic and cDNA library screens were prepared using a random primer labeling kit (Boehringer-Mannheim).

### Sequence and 5' end analysis

The 5' end of the *par-4* gene was isolated by RACE-PCR (5' Amplifinder, Clontech) using primer 102C (GGGTTTAATGTCCTTATGCG) to initiate cDNA synthesis and ligating the cDNA to an anchor sequence. Primer 102B (GCCGGTTCATGTGCGAC) and a primer corresponding to the anchor sequence were used to amplify the cDNA by the polymerase chain reaction (PCR). To obtain the rest of the *par-4* coding sequence, we used the primers 102DMA-R (ATGTAGACTTTT-CCCTTAGCCGG) and 7238-R (AGGTGGC-CAGACTAC-AAACTACA) to initiate reverse transcription (RT) of *C. elegans* RNA and amplified the cDNA with these primers and forward primers 102J (GGAGCACAAGCAAACCTCTG) or 102DMA (CCGG-CTAAGGGAAAAGTCTACAT) by PCR. These 102DMA-R-102J and 7238R-102DMA products were cloned into the pCRII vector (Invitrogen) and designated pDMAR-J and pDM1.2, respectively. To obtain additional sequence within the 3'UTR, reverse transcription was initiated with primer 7764R (CGTTTTTAA-GCCAATTTTCGTAT) and amplified with this primer and primer 7287 (TCTCGTGATCTTTCGGTCTCGTA) by PCR. DNA sequencing of plasmid clones was carried out by the Cornell University Biotechnology Program Automatic Sequencing Facility or manually using a Sequenase kit (United States Biochemicals) following the manufacturer's instructions. Sequencing of PCR products followed the procedure described by Levitan et al., (1994). Primers were synthesized by the Cornell University Biotechnology Program DNA synthesis facility. For sequence analysis of each of the *par-4* mutant alleles, *it33*, *it47ts*, *it57ts*, *it75*, *it83*, *it88* and *it138*, RNA from approximately 50-100 homozygous worms was used for RT-PCR with the primer pairs 7238R-102DMA and 102DMAR-102J. The RT-PCR products were cloned into pCR2.1 using a TA cloning kit (Invitrogen) and sequenced from primers within the vector as well as *par-4*-specific primers, including: 7013R (TTGAGCCGGG-AAATGAGC), DM5135 (CGGATATGTGGAGTGCAGGTGTCA), 102M (CTCGACATGGAACCGGCTCGACGTC), and 102G (GTAGAATCTGAAGCAATGCGTC). Because 102DMA covers the same part of the gene as 102DMA-R, we would not identify any nucleotide changes that fell in this 24 nucleotide region. We also would not detect sequence changes beyond the primers used for amplification; before nucleotide 25 of the coding region or beyond nucleotide 1956, in the 3' untranslated region. Sequence analyses were performed from the RT-PCR products of at least two RNA preparations for each allele. Because the observed nucleotide change in *dpy-21 par-4(it33)* worms was the same as that in *dpy-21 par-4(it83)* worms, we also sequenced the original isolates of each strain and verified that the identity did not arise from a stock-keeping

error; we found the same nucleotide change in each of these strains as well.

We used genome sequence information provided by the *C. elegans* DNA Sequencing Consortium to design some of our primers and to determine splice sites for the *par-4* gene (Consortium, 1998). Sequence comparisons were performed using BLAST (Altschul et al., 1990). Alignment of protein sequences was performed using the program MegAlign (CLUSTAL method; Higgins et al., 1992) in the DNASTar package with sequences from GenBank or EMBL databases. The *par-4* sequence reported in this paper has been deposited in the GenBank database (accession no. AF160189).

### RNA interference

The RT-PCR product of primers 7238R and 102DMA was subcloned from the PCRII vector into pBluescript (Stratagene) at the *EcoRI* site. This sequence begins at nucleotide 760 of the coding sequence and continues into the 3'UTR. The DNA was linearized with *XbaI* for transcription with T7 polymerase or with *HindIII* for transcription by T3 polymerase. Transcription was performed with a Ribomax kit (Promega). Sense and antisense RNA were combined at 37°C for 5-10 minutes prior to injection. Injections were performed as described previously (Guo and Kemphues, 1996). Injected worms were allowed to lay eggs for at least 48 hours before scoring embryo viability. Photography, video analysis and gut granule assays were performed as previously described (Morton et al., 1992).

### Antibody production and immunostaining

Primers GCGGCCCGGGGCTCGAATTCATGTGC (reverse) and GCGGCCCGGGGACATCCTCGGGAGCACA (forward) were used to synthesize a PCR product corresponding to *par-4* sequence encoding amino acids 6-169 and containing synthetic *SmaI* restriction sites at the ends. The product was linked to the pGex 2T vector (Pharmacia) to produce a glutathione-S-transferase (GST) fusion protein (Smith and Johnson, 1988). Polyclonal antibodies recognizing the PAR-4-GST fusion protein were produced in two rabbits, 5-307 and 5-308, by the Cornell University Research Animal Resources Facility. Antisera were affinity purified as described (Guo and Kemphues, 1995) or by blot affinity purification (Olmsted, 1986).

Protein extracts were prepared from *C. elegans* animals as described previously (Etemad-Moghadam et al., 1995). Worms of a given genotype were individually picked and pooled to prepare extracts. Homozygous *par-4* progeny of *dpy-21(e428) par-4/++* worms were identified based upon their Dpy phenotype. Extracts of approximately 100 worms were run on 8% polyacrylamide gels and transferred to nitrocellulose membranes (Schleicher and Schuell) by standard methods. Embryo extracts were made following hypochlorite treatment of approximately 350 adult hermaphrodites. Blots were incubated with affinity-purified antibody from either rabbit 5-307 or 5-308 or monoclonal anti-tubulin (gift from M. Fuller) and horseradish peroxidase-linked anti-rabbit IgG (anti-mouse for tubulin) and detected using the ECL (Amersham) or Super Signal (Pierce) systems. We find that the 5-308 antibody gives a stronger signal, but also cross-reacts with a bacterial band that appears at about 79 kDa. 5-307 antibody gives a weaker signal, but no cross-reacting bands are seen on western blots.

For immunofluorescence, worms were fixed on slides as described (Guo and Kemphues, 1995). Antiserum was diluted in PBS-T and incubated with the sample at 37°C for 1 hour. After washing, slides were incubated with secondary antibody (fluorescein conjugated goat anti-rabbit) or Texas Red-conjugated goat anti-rabbit, (Jackson ImmunoResearch) at 37°C for 1 hour. P-granule staining was done with either K76 or OIC1D4 antisera (kindly provided by Susan Strome), using fluorescein-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). Samples were viewed with a Zeiss photomicroscope II equipped with epifluorescence or with a BioRad confocal microscope.

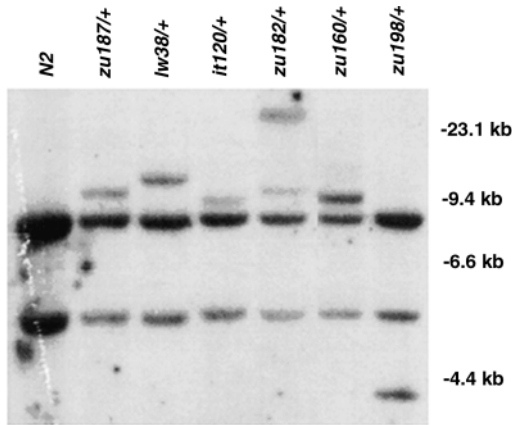
## RESULTS

### Identification of the *par-4* gene

We used a transposon-tagging approach to identify DNA encoding *par-4*. Mutations isolated in *C. elegans* mutator strains are often Tc1 insertions into the gene (Collins et al., 1987). DNA from a mutator-induced *par-4* mutant, *zu187*, was found to carry a novel 3.2 kb *BglII* restriction fragment that hybridized with Tc1 transposon sequences. This restriction fragment was present in three independently outcrossed lines, but not in N2 DNA. The DNA containing the novel Tc1 was cloned as described in Materials and Methods. The resulting plasmid was partially sequenced and an open reading frame was identified in a fragment flanking the Tc1 insertion. The fragment flanking the Tc1 insertion was used as a probe in extensive screens of available genomic and cDNA libraries. No positive clones were identified, so we used RACE-PCR to verify that RNA corresponding to this fragment is present in *C. elegans*. A 0.8 kb 5' PCR product was obtained ending with a 22-nucleotide variant of the *C. elegans* SL-2 spliced leader, designated SLb (Ross et al., 1995). SL2-type leader sequences have been shown to be *trans*-spliced to downstream genes of operons in *C. elegans* (Spieth et al., 1993). Shortly following this sequence are two ATGs in frame with an open reading frame extending the length of the PCR product. The sequence contained 90 nucleotides identical to the genomic sequence flanking the novel Tc1 insertion from *zu187*. The RACE-PCR clone hybridizes to a transcript of about 3 kb on a northern blot as do DNA fragments on either side of the *zu187* Tc1. The 0.8 kb 5' PCR sequence also hybridizes to the yeast artificial chromosome (YAC) clone Y59A8 (not shown), consistent with the position of *par-4* obtained from physical mapping data.

The 0.8 kb 5' sequence was used as a probe to examine other *par-4* alleles by Southern analysis. An *XbaI* digest reveals polymorphisms in five independent *par-4* alleles (Fig. 1), providing additional evidence that this sequence of DNA encodes a portion of the *par-4* gene.

Although no existing *C. elegans* cDNAs were found containing *par-4* sequences, BLAST searches revealed a very high similarity to cDNA pk03b02 from *C. briggsae*. This clone was isolated from a mixed stage cDNA library made by P. Kuwabara (Kuwabara and Shah, 1994) and was partially sequenced as part of the *C. elegans* sequencing project (M. Marra, personal communication). The 1.7 kb PCR product was obtained from Dr Marra and sequenced. This sequence was found to be approximately 75% identical to the putative *par-4* sequence over 642 nucleotides and extended another 850 nucleotides. We compared this *C. briggsae* sequence with *C. elegans* sequences on Y59A8 and Y37H2 YACs determined by the Genome Sequencing Consortium and identified additional candidate exons of the *C. elegans par-4* gene. We designed primers corresponding to the matching *C. elegans* sequences and were able to produce RT-PCR products that linked our *par-4* 5' coding sequences to additional downstream coding sequences (Materials and Methods). By comparing the sequences of these RT-PCR products with genomic sequences we were able to determine the structure of the *C. elegans par-4* gene. The *par-4* gene contains eleven exons in a region of more than 8 kb on chromosome V. The *par-4* RT-PCR products



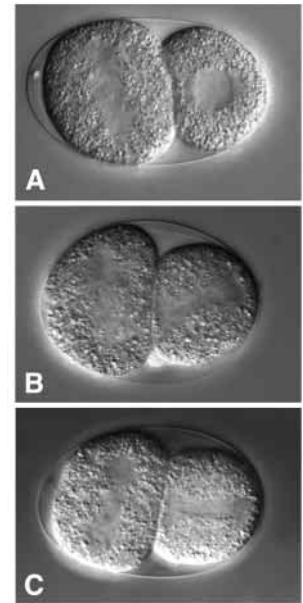
**Fig. 1.** Southern blot of genomic DNA from wild-type and heterozygous *par-4* worms. DNA was digested with *Xba*I and the filter probed with the *par-4* 5' RACE-PCR sequence. DNA from the heterozygous mutant strains contains both wild-type and unique fragments that hybridize to the probe.

show a single open reading frame encoding a 617 amino acid protein, followed by a 3'UTR of at least 450 nucleotides.

### RNA-mediated interference produces *Par-4* phenotypes

For verification that the sequence that we isolated corresponds to the *par-4* gene, we used RNA interference (RNAi; Fire et al., 1998) to test whether RNA transcribed from a 1.2 kb RT-PCR product corresponding to the 3' portion of the coding region (pDM1.2, see Materials and Methods) could cause *par-4*-like embryo defects. RNAi-induced phenocopies have previously been used to demonstrate gene identity (Guo and Kemphues, 1996). *par-4* mutations are strict maternal effect lethal mutations, resulting in 100% lethality of embryos produced by homozygous mutant mothers. We observed 80% lethality in embryos produced by nine worms injected with pDM1.2 transcripts versus 1% embryo lethality from uninjected worms ( $n=6$ ) carried through the same procedure. Early embryos injected with pDM1.2 transcripts exhibited characteristic Par-4 phenotypes, being smaller and rounder than normal, showing extensive cytoplasmic mixing and blebbing, excessive swing of the cleavage spindle at first division and asymmetric first division but synchronous subsequent cell divisions (Fig. 2). Spindle orientation at the second division was the same as that seen in wild-type and most *par-4* mutant embryos, with the anterior cell spindle elongating transverse to the long axis of the embryo and the posterior cell spindle rotating and elongating parallel to the long axis of the embryo ( $n=8$ ). Arrested embryos of injected animals developed into a mass of many differentiated cells, but showed no morphogenesis, typical of those produced by *par-4* mutant mothers. We observed intestinal differentiation in approximately 10% of arrested embryos ( $n=89$ ) of injected worms. Because no embryos produced by worms carrying strong alleles of *par-4* develop intestinal granules, we conclude that RNAi with pDM1.2 did not completely deplete PAR-4 in the gonads and embryos of injected worms.

We previously reported that approximately 20% of *par-4(it33)* embryos show an abnormal spindle orientation in the posterior cell at the second division, such that both anterior and



**Fig. 2.** Effect on asynchronous cleavage by RNA interference with pDM1.2 transcripts. Wild-type (A), *par-4(it75)* (B), and PAR-4-depleted (C) embryos at second cleavage. The anterior cell, AB (left), divides before the posterior cell, P1, in wild-type embryos. Both cells divide synchronously in *par-4(it75)* mutant and PAR-4 depleted embryos.

posterior cells divide transverse to the long axis of the embryo (Kemphues et al., 1988). Because this phenotype did not appear in embryos from RNAi-treated worms, we re-examined the spindle orientation in 2-cell embryos of *it33* and additional *par-4* mutants. Consistent with our earlier results, we found that the posterior cell spindle failed to rotate in 6 of 35 *par-4(it33)* embryos (21%), resulting in an abnormal spindle orientation. However, this phenotype was seen in only 1 of 31 *par-4(it75)* embryos and was not seen in 24 *par-4(it83)* embryos. Because *par-4(it75)* worms produce no detectable PAR-4 protein, and because the mutated nucleotide in *par-4(it83)* is identical to that mutated in *it33* (see below), we conclude that the P1 spindle rotation defect sometimes seen in the *par-4(it33)* mutant strain is due to genetic background effects.

### The *par-4* gene encodes a serine-threonine kinase

The RT-PCR sequences corresponding to the *par-4* gene reveal a single open reading frame encoding a protein of 617 amino acids. Comparison of this sequence with known sequences of other proteins reveals that PAR-4 is likely to be a serine-threonine protein kinase. The predicted PAR-4 protein contains the signature substrate specificity sequence of serine-threonine kinases as well as all of the invariant amino acids associated with protein kinases (Hanks and Hunter, 1995). The PAR-4 protein also contains an acidic domain preceding the kinase domain and a unique C-terminal sequence following the kinase domain. BLAST searches (Altschul et al., 1990) reveal that PAR-4 is similar to three vertebrate serine-threonine kinases, human serine-threonine kinase 11 [STK11, also named LKB1 (Hemminki et al., 1998; Jenne et al., 1998)], mouse LKB1, which is 90% identical to human LKB1/STK11 (Smith et al., 1999), and *Xenopus* Egg and Embryo Kinase 1 (XEEK1, Su et al., 1996). The similarity between PAR-4 and these proteins is restricted to the kinase domain, and is especially high in the putative catalytic loop region of the kinase. We have aligned the sequence of PAR-4 with human STK11/LKB1 and XEEK1, and find that within the kinase domain, there is 42% amino acid

1	M D A P S T S S G A Q S K L L M P G D D E A D E D H Q N R G D P N L Q Q K Q K I	PAR-4
1	M -	STK11
1	M L C P S S -	XEEK1
41	Q L N V D P D Y D D D E D D D C F I D G C E A S A P I T R E L V D G A I E R R S	PAR-4
8	Q L G M -	STK11
14	E I G F -	XEEK1
81	K D R N V K M S I G V Y D E Y D D D D D E E E T E E D Q R R R F V E G I R N I	PAR-4
18	- -	STK11
21	- -	XEEK1
121	R H K Q Q E S F D L E E H P I P V E S E A M R Q F I N I Q V N N A M M F N Q D N	PAR-4
22	- -	STK11
25	- -	XEEK1
161	S E F Q H I E F E P I V K Q K G P K I I E G Y M W G G Q I G T G S Y G K V K E C	PAR-4
39	- -	STK11
42	- -	XEEK1
201	I D M Y T L T R R A V K I M K Y D K L R K I T N G W E N I R S E M S I L R R M N	PAR-4
67	L D S E T L C R R A V K I L K K K K L R R I P N G E A N V K K E I Q L L R R L R	STK11
70	L D S D T L C R R A V K I L K K K K L R R I P N G E A N V K K E I Q L L R R L R	XEEK1
241	H R N V I K L I E I F N I P A K G K V Y M V M E Y C I G S V Q Q L L D M E P A R	PAR-4
107	H K N V I Q L V D V L Y N E E K Q K M V M V M E Y C V C G M Q Q E M L D S V P E K	STK11
110	H R N V I Q L V D V L Y N E E K Q K M Y M V M E Y C V C G M Q Q E M L D S V Q D K	XEEK1
281	R L T I G E S H A I F I E L I D G L N Y L H S K R V S H K D I K P G N L L V S I	PAR-4
147	R F P V C Q A H G Y F C Q L I D G L E Y L H S Q G I V H K D I K P G N L L T T	STK11
150	H F P V F Q A H G Y F C Q L I D G L E Y L H S Q G I V H K D I K P G N L L T T	XEEK1
321	D F T V K I C D F G V A E Q I N L F Q R D G R C T K V N G T F K F Q P P E C I Y	PAR-4
187	G G T L K I S D L G V A E A L H P F A A D D T C R T S Q G S P A F Q P P E - I A	STK11
190	D G T L K I S D L G V A E A L H P F A E G D T C R T S Q G S P A F Q P P E - I A	XEEK1
361	G N H D F F D G Y K A D M * * * G K Y P P F E K P V L L K L Y E	PAR-4
226	N G L D T F F S G F K V D I W S A G V T L Y N I T T G L Y P P F E G D N I Y K L F E	STK11
229	N G L D T F S G F K V D I W S A G V T L Y N I T T G L Y P P F E G D N I Y K L F E	XEEK1
401	C I G T E P L Q M P T N V Q L T K D L Q S D L L T K L L E K D F N E R P T C L E T	PAR-4
266	N I G K G S Y A I P G D C - - G P P L S D L L K K G M L E Y E P A K R R F S I R Q I	STK11
269	N I G K G D Y S I P E E C - - G P P L S D L L R G M L E Y D P A K R R F S I Q Q I	XEEK1
441	M I H P W F L S T F P E D Q G L G R I M E R M R T K D R - - P L T M L S S M T A	PAR-4
304	R Q H S W F R K K H P P A E A P V P I I P P S P D T K D R W R S M T V V P Y L E D	STK11
307	R Q H N W F R K K H P P H M D P I V P I I P P S P E T K D R W R S L T V V P Y L E D	XEEK1
479	L Y D G I T P E D E L I - I E D N L G I I Q Q I - L P I N L T S E A V L E R G S	PAR-4
344	L - H G A D E D E D L F D I E D D I I Y T Q D F T V P G Q V P E E A S H N G -	STK11
347	L - H G Y S E E E D L F C D F E D D I I Y T Q D F T V P G Q V A E D Y F A Q T -	XEEK1
517	F P G F K F L E A K P G D G P D G V E G S E D S A A P L G P Q R R P S S R S M P	PAR-4
382	- - - - - - - - - - - - Q R R G L P K A V C M N G T E A A Q L S T K S R A E G R A - -	STK11
385	- - - - - - - - - - - - Q S T A P S K Q L C M N G T E S - Q L K T E R R V - - - S - -	XEEK1
557	T C A P P G P A A G N A Q N S T A E N G A E T D G V A S A S D P P P T A A P G A	PAR-4
411	- -	STK11
410	- -	XEEK1
597	P P R R R K R N F F S C I F R S R T D S A	PAR-4
419	S A S S K I R R L S A C - - - - - K Q Q	STK11
418	T T G S K V R K L S A C - - - - - K Q Q	XEEK1

**Fig. 3.** The sequence of the predicted PAR-4 protein is shown (Genbank Accession no. AF160189), and aligned with human serine-threonine kinase 11 (STK11/LKB1) and *Xenopus* egg and embryo kinase (XEEK1). Identical sequences are shaded. The kinase domain of the proteins is underlined. Amino acids changed by *par-4* missense mutations are marked with asterisks. See Table 1 for identification of mutational changes.

identity between PAR-4 and either of these two proteins (Fig. 3). Mouse LKB1 (not shown) also has 42% amino acid identity with the PAR-4 kinase domain, with nearly the same matches to PAR-4 as the human LKB1/STK11 sequence.

The partial *C. briggsae* cDNA that we analyzed predicts a protein that is 82% identical to the *C. elegans* PAR-4 protein and is likely to encode a PAR-4 homologue, since the sequence similarity extends beyond the kinase domain into the C-terminal region. Both proteins end at the same distance from the kinase domain. However, the *C. briggsae* partial cDNA did not extend into the N-terminal coding region, so we do not know if the similarity continues through the full length of the proteins.

In order to identify residues of the PAR-4 protein that may be important for function, we have determined the sequence changes in six alleles of the *par-4* gene for which stable protein is still detected (see below). Four of these alleles, *it33*, *it83*, *it88* and *it138*, produce a strong phenotype in mutant embryos, and two alleles, *it47* and *it57*, are temperature-sensitive. We also sequenced one allele, *it75*, which, like most *par-4* alleles, produces no detectable protein. We find that all seven mutations are located in the kinase domain of the predicted PAR-4 protein (Fig. 3; Table 1). The mutations *it33* and *it83* are identical, changing glycine-192 to aspartic acid in kinase subdomain I. This glycine is invariant in all known kinases (Hanks et al., 1988) and is thought to play a critical role in



**Table 1. Nucleotide and amino acid changes conferred by *par-4* missense mutations and the nonsense mutation, *it75***

Allele	Codon change	Predicted amino acid change*
<i>it33</i>	GGA→GAA	192 Glycine→Aspartic acid
<i>it83</i>	GGA→GAA	192 Glycine→Aspartic acid
<i>it47ts</i>	ATT→AGT	290 Isoleucine→Serine
<i>it88</i>	GGT→AGT	377 Glycine→Serine
<i>it138</i>	GAT→GAA	372 Glutamic acid→Aspartic acid
<i>it75</i>	TGG→TGA	374 Tryptophan→UGA Stop
<i>it57ts</i>	CCA→TCA	389 Proline→Serine

\*Amino acid position is determined relative to the putative PAR-4 translation initiation site.

nucleotide binding (Knighton et al., 1991). The *it88* and *it138* mutations change invariant residues in kinase subdomain IX. *it138* changes glutamic acid-372 to aspartic acid and *it88* changes the conserved glycine-377 to serine. *it47ts* and *it57ts* change residues within the kinase domain near the catalytic loop. The *it47* allele was also found to carry a second mutation: a 2-nucleotide deletion in the 3'UTR, 81 nucleotides downstream from the predicted stop codon. The *it75* mutation results in a UGA translation termination signal within the kinase domain. The missense mutants *it33*, *it83*, *it88* and *it138* have a maternal effect lethal phenotype as severe as that produced by the *it75* null mutation (Morton et al., 1992 and unpublished observations). Because the missense mutations are predicted to abolish kinase activity, it is likely that the PAR-4 protein functions as a kinase.

#### PAR-4 is present in the cytoplasm and at the cortex of early embryos

We generated polyclonal antibodies in two rabbits to the amino-terminal portion of PAR-4 including amino acids 6-169, preceding the kinase domain. Antibodies from both rabbits recognize an 80 kDa protein in wild-type *C. elegans* extracts that is absent in *par-4(it75)* mutant worms (Fig. 4). The protein is not detected in *par-4* mutants *it72*, *it74*, *it75*, *it84*, *it120*, *it122*, *lw38*, *zu160* or *zu198* but is detected in *it33*, *it83*, *it47ts*, *it57ts*, *it88* and *it138* (not shown). As discussed above, all the alleles for which protein can still be detected carry missense mutations predicted to alter single amino acids in the PAR-4 kinase domain. Our *par-4* nucleotide sequences predict a protein of 70 kDa; the increase in apparent molecular mass of the protein on gels may be due to the overall negative charge of the protein or to post-translational modifications. The XEEK1 protein has also been observed to migrate more slowly during gel electrophoresis than predicted by its primary sequence (Su et al., 1996).

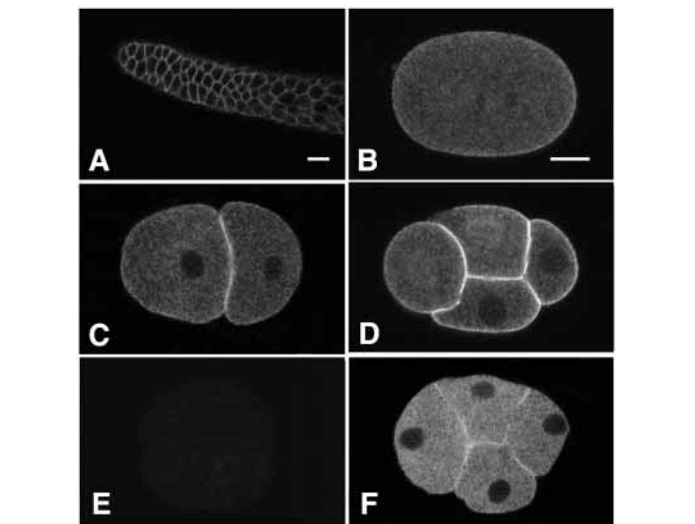
We have used the anti-PAR-4 antibodies to investigate the expression of PAR-4 protein at different stages of the *C. elegans* life cycle. We detect PAR-4 in extracts of adult hermaphrodites, males, embryos and L4 larvae (Fig. 4 and data not shown). We have also examined PAR-4 distribution by indirect immunofluorescence (Fig. 5). PAR-4 protein is apparent in the gonads of adult hermaphrodites, particularly at the actin-rich boundaries between nuclei in the syncytial region. In oocytes, the protein is present in the cytoplasm, is excluded by nuclei and is faintly present at the cell periphery. 1-cell embryos show cytoplasmic staining and faint cortical staining. We do not see any asymmetry in the distribution of PAR-4 protein in the cytoplasm or at the cell periphery. Cortical

staining is stronger at the time of first cleavage, with prominent PAR-4 staining of the cleavage furrow. The cortical staining remains at high levels at the 2-cell and 4-cell stages and continues to be present in all blastomeres through early development. Staining of late stage embryos is reduced, but the germline precursor cells, Z2 and Z3, show somewhat stronger cortical staining than other cells.

We have also examined the distribution of PAR-4 in embryos produced by *par-4* mutant mothers. No signal is detected in gonads or embryos of worms carrying *it72*, *it74*, *it75* (Fig. 5E), *it84* or *it120* mutations ( $n>50$  for each genotype) indicating that the staining that we see in wild type is specific for PAR-4. PAR-4 staining in the missense mutant *it33* appears to be very similar to that seen in wild-type embryos. However, in the other missense mutants, *it88* and *it138*, and in *it57ts* at the nonpermissive temperature, there is reduced staining at the

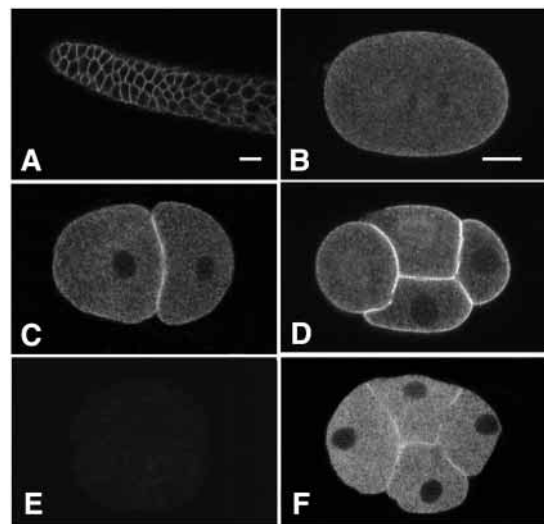
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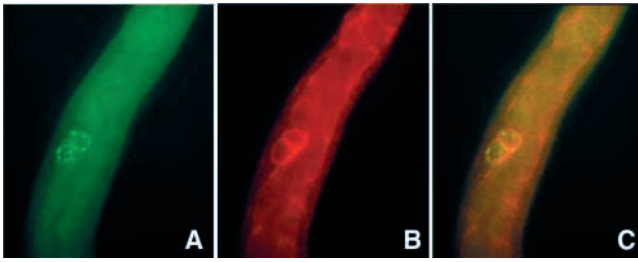
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**Fig. 6.** PAR-4 staining at the cortex of the germline precursor cells, Z2 and Z3, in an L1 larva. Wild-type L1 larvae were fixed and co-stained with antibodies to P granules (A) and PAR-4 (B). P granule distribution was visualized with FITC-labeled anti-mouse secondary antibody. PAR-4 distribution was detected with Texas Red-labeled anti-rabbit secondary antibody. C shows a merged image of A and B.

periphery of the early blastomeres, but high levels in the cytoplasm (see Fig. 5F). After the 16-cell stage, the cortical staining is stronger in these mutants and appears more like that seen in wild-type embryos.

Mutations in the other *par* genes do not affect synthesis or distribution of PAR-4. PAR-4 protein is present in gonads, oocytes and all blastomeres of early embryos from mothers homozygous for *par-1(b274)*, *par-2(lw32)*, *par-3(it71)*, *par-5(it55)* or *par-6(zu222)* ( $n > 50$  for each genotype). There appear to be the same levels of cortical and cytoplasmic PAR-4 immunofluorescence in these *par* embryos as in wild type.

Immunofluorescence with the PAR-4 antibody in *C. elegans* larvae shows staining at the periphery of the germline precursor cells, Z2 and Z3 at the L1 stage (Fig. 6). As the germ cell precursors proliferate during larval growth, PAR-4 protein continues to be present around each germline-derived cell (determined by co-staining with P granule-specific antibodies). PAR-4 is present in the syncytial gonad of males, and is in both the cytoplasm and the cortex of spermatocytes (not shown). After these cells undergo meiosis to form spermatids, PAR-4 is no longer detectable.

To investigate whether the PAR-4 staining in L1 larvae reflects zygotic gene expression, we have examined the staining pattern in larvae produced by mothers heterozygous for the *par-4(it75)* mutation. If PAR-4 staining of germline precursor cells in larvae is due entirely to zygotic expression, then 25% of the larvae (*par-4/par-4*) from a heterozygous *par-4* /+ worm will fail to stain with PAR-4 antibody. All larvae should show PAR-4 staining if any protein is from maternal expression. We found that 18% ( $n=44$ ) of larvae produced by heterozygous mothers fail to show PAR-4 staining of the germline precursor cells (identified by P granule staining). In a control experiment, 100% of wild-type larvae showed co-staining of germline precursor cells with a P granule antibody and PAR-4 antibody ( $n=39$ ). Thus, at least some PAR-4 protein in larvae is derived from expression of the zygotic genome.

To investigate a possible role for PAR-4 in male fertility, we tested whether *par-4* mutant males can be successfully mated. Because of the difficulty of obtaining males homozygous for non-conditional *par-4* alleles, we tested homozygous *par-4(it57ts)* males reared at 25°C for their ability to mate at 25°C. At this temperature, *it57ts* hermaphrodites show a *par-4* maternal effect lethal phenotype as severe as the nonconditional alleles (Morton et al., 1992). We found that

*it57ts* males are fertile at 25°C; approximately 25-100 outcross progeny were produced from each cross. Thus, PAR-4 activity is nonessential in spermatogenesis or there is a higher requirement for PAR-4 activity in oogenesis and early development than in spermatogenesis.

## DISCUSSION

In this paper, we describe the identification of the *par-4* coding sequence and the immunolocalization of the PAR-4 protein. Evidence that the coding sequence is *par-4* comes from the discovery of DNA rearrangements affecting this sequence in five different *par-4* alleles, missense or nonsense mutations in the coding region in seven other alleles and the finding that RNA interference with this sequence produces a Par-4 phenocopy.

The predicted protein product of the *par-4* gene is a serine-threonine kinase. The protein contains the signature serine-threonine substrate recognition sequence as well as all of the conserved amino acids shared by protein kinases. We believe that its function as a kinase is critical for early development, since mutations that change single residues within the kinase domain result in a developmental phenotype as severe as that produced by mutants that fail to make detectable protein. BLAST searches revealed three proteins, human LKB1 (STK11), mouse LKB1 and *Xenopus* XEEK1, with structural similarity to the predicted PAR-4 protein. These proteins are serine-threonine kinases, with much more similarity to each other than to the *C. elegans* PAR-4 protein. Mutations in the gene encoding human LKB1/STK11 are associated with Peutz-Jeghers syndrome, an autosomal dominant disorder characterized by melanocytic macules on the lips, multiple gastrointestinal polyps and predisposition to cancer development in a variety of tissues (Hemminki et al., 1998; Jenne et al., 1998; Mehenni et al., 1998). Thus the STK11/LKB1 protein is thought to act as a tumor suppressor in humans; a role in embryogenesis has not been investigated. *Lkb1* RNA is ubiquitously expressed in early embryos of mice and becomes more restricted in later development to specific tissues including intestine and testis (Luukko et al., 1999). The XEEK1 protein appears to be maternally expressed; it is present in *Xenopus* oocytes, unfertilized eggs and early embryos, but is reduced in gastrulating embryos (Su et al., 1996). This suggests that the XEEK1 kinase, like PAR-4, may play a pivotal role in early embryogenesis.

The PAR-4 sequence is longer than human or mouse LKB1 or XEEK1, with an acidic amino-terminal domain preceding the kinase domain, as well as a unique carboxy-terminal region. The regulation and/or intracellular localization of PAR-4 may be very different from these vertebrate kinases. The mouse LKB1 protein has been reported to be targeted primarily to nuclei, and a putative nuclear localization signal has been identified (Smith et al., 1999). This signal is not present in the PAR-4 sequence, and we see no evidence of nuclear localization of PAR-4. Human LKB1/STK11 expressed in COS-7 cells is detected in both the nucleus and cytoplasm (Nezu et al., 1999). A mutation identified in patients with Peutz-Jeghers Syndrome causes LKB1/STK11 to become restricted to nuclei but does not affect kinase activity in vitro, suggesting that retention of LKB1/STK11 activity in the

cytoplasm is necessary for its normal function (Nezu et al., 1999). It will be of interest to determine whether the cytoplasmic substrates for LKB1 kinases, XEEK1 kinase and PAR-4 share common features.

How might the kinase function of PAR-4 affect asymmetry? Unlike PAR-1, PAR-2, PAR-3 and PAR-6, which are themselves asymmetrically distributed (Boyd et al., 1996; Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995; Hung and Kemphues, 1999), PAR-4 is present uniformly throughout the early embryo, at the cortex and within the cytoplasm. Thus asymmetric localization of PAR-4 cannot be contributing to polarity in the early embryo. Furthermore, neither the ratio of cytoplasmic to cortical PAR-4 nor the stability of PAR-4 in the early embryo is affected by mutations in the other *par* genes. It is possible, however, that asymmetries in the other proteins could lead to asymmetric activation of PAR-4.

Alternatively, the primary role of PAR-4 could be during oogenesis. PAR-4 protein is present throughout the gonad. Our previous studies of the temperature-sensitive period in *par-4(it57ts)* mutants suggested that PAR-4 synthesis or activity before fertilization is critical for embryo viability (Morton et al., 1992). Embryos of *par-4* mutants are generally smaller and rounder than wild type, reflecting differences in oogenesis between *par-4* and wild type. Reduced cytoplasmic volume alone cannot be responsible for the *par-4* defects, because wild-type embryos with reduced cytoplasmic volume show normal early development (Schierenberg, 1988), and *par-4* embryos with wild-type size still exhibit defects (D. G. M. and K. J. K., unpublished observations).

One effect of the absence of PAR-4 is a subtle alteration in the distribution of PAR-3 and PAR-6 proteins in the zygote (Hung and Kemphues, 1999). PAR-4 may be contributing to embryonic polarity by mediating the distribution or activities of these proteins and other asymmetry-determining components. Since *par-4* mutations have more severe effects on P granule distribution, intestinal development and muscle differentiation than mutations in *par-3* and *par-6*, PAR-4 cannot be acting only through its effects on these two proteins. Furthermore, *par-2* mutations have similar effects on the distributions of PAR-3 and PAR-6, but produce developmental defects different from *par-4* mutations (Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Kemphues et al., 1988). Identifying substrates and other molecules that interact with the PAR-4 kinase may help us to determine additional components involved in establishing intracellular asymmetry in the *C. elegans* embryo.

The *par-1* gene also encodes a serine-threonine kinase (Guo and Kemphues, 1995) and is more similar in phenotype to *par-4* than to the other *par* genes. The two genes are not likely to act upon the same substrates, however; PAR-4 and PAR-1 kinases differ substantially at the amino acid sequence level, having only 29% identity and some gaps between kinase subdomains. One hypothesis to explain the relationship between the two proteins is that PAR-4 and PAR-1 are components of the same kinase cascade signaling pathway in the zygote. However, although *par-4* and *par-1* mutations have similar effects upon the distributions of P-granules, GLP-1, MEX-3 and PAL-1 and mutations in either gene completely prevent formation of intestine (Bowerman et al., 1997; Crittenden et al., 1997; Draper et al., 1996; Hunter and Kenyon, 1996; Kemphues et al., 1988; Morton et al., 1992), mutations

in the two genes have different effects upon spindle placement at the 1-cell stage, muscle differentiation, and PAR-3, PAR-6 and SKN-1 distribution (Bowerman et al., 1993, 1997; Etemad-Moghadam et al., 1995; Hung and Kemphues, 1999; Kemphues et al., 1988; Morton et al., 1992). PAR-1 and PAR-4 may act in independent processes that only partially overlap.

The presence of PAR-4 protein beyond the early embryonic stages suggests that PAR-4 may function in processes other than polarity establishment in the 1-cell embryo. All of the identified *par-4* mutations result in a strict maternal-effect lethal phenotype; homozygous mutant worms are viable, but do not produce viable offspring. Thus roles for PAR-4 outside of embryogenesis are likely to be redundant activities or functions that are not essential for viability. The presence of high levels of PAR-4 in male gonads raises the possibility of a role for the protein in male fertility, but this function may be redundant since *it57ts* males are fertile at 25°C, a temperature at which *it57ts* hermaphrodites produce no hatching embryos. PAR-2 protein is also present at high levels in male gonads and shows a distribution identical to PAR-4, and *par-2* males are also fertile (Boyd et al., 1996; Cheng, 1991).

Our previous studies of the *par-4(it57ts)* allele indicated that the period of required *par-4* gene function for embryo viability occurs during oogenesis and the first cell cycle of embryogenesis. However, temperature shifts of *par-4(it57ts)* embryos at later embryonic stages result in incompletely penetrant phenotypes of larval lethality and adults lacking oocytes, suggesting additional functions in germline and somatic development (Morton et al., 1992). In particular, the higher levels of PAR-4 protein in the germline precursor cells relative to other cell types during late embryonic and larval development is consistent with the temperature-shift results and suggests that PAR-4 plays a role in, germ cell development.

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