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PAR-2 is asymmetrically distributed and promotes association of P granules and PAR-1 with the cortex in *C. elegans* embryos

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SUMMARY

The *par* genes participate in the process of establishing cellular asymmetries during the first cell cycle of *Caenorhabditis elegans* development. The *par-2* gene is required for the unequal first cleavage and for asymmetries in cell cycle length and spindle orientation in the two resulting daughter cells. We have found that the PAR-2 protein is present in adult gonads and early embryos. In gonads, the protein is uniformly distributed at the cell cortex, and this subcellular localization depends on microfilaments. In the one-cell embryo, PAR-2 is localized to the posterior cortex and is partitioned into the posterior daughter, P_1, at the first cleavage. PAR-2 exhibits a similar asymmetric cortical localization in P_1, P_2, and P_3, the asymmetrically dividing blastomeres of germ line lineage. This distribution in embryos is very similar to that of PAR-1 protein.

By analyzing the distribution of the PAR-2 protein in various *par* mutant backgrounds we found that proper asymmetric distribution of PAR-2 depends upon *par-3* activity but not upon *par-1* or *par-4*. *par-2* activity is required for proper cortical localization of PAR-1 and this effect requires wild-type *par-3* gene activity. We also find that, although *par-2* activity is not required for posterior localization of P granules at the one-cell stage, it is required for proper cortical association of P granules in P_1.

Key words: cell polarity, *par-1*, *par-2*, *par-3*, P granule, microfilament

INTRODUCTION

Polarity is a feature of many cell types and can serve two general purposes. First, a cell may be polarized in order to accomplish vectorial functions such as selective secretion. Cell types that have polarity as an essential element of their function include neurons and epithelial cells. Second, cell polarity may be used to generate different cell types via the asymmetric distribution of factors within a cell and their unequal inheritance by the daughter cells. This strategy is often employed during early development. For example, *Xenopus laevis* and *Drosophila melanogaster* oocytes are highly polarized cells: the basic body plan for the organism is dependent upon the activities of determinants that are asymmetrically distributed in the eggs (St Johnston and Nusslein-Volhard, 1992; Klymkowsky and Karnovsky, 1994). During *Caenorhabditis elegans* embryogenesis, cell fate specification also occurs via segregation of factors during the early cleavages (Priess, 1994). During cleavage of the early mouse embryo, external cells become polarized at the time of compaction, leading to the formation of two cell types that give rise to two distinct lineages, the trophectoderm and the inner cell mass (Gueth-Hallonet and Maro, 1992).

Exploration of the mechanisms for establishing cellular polarities has revealed some of the strategies used in the polarization process. In all the examples listed there is evidence that microtubule and microfilament structures play a critical role. The cytoskeleton is important for the transport of molecules and vesicles as well as the maintenance of asymmetries. Inhibitor studies have shown that in *Drosophila* and *Xenopus* embryos, the asymmetric distribution of mRNAs or proteins depends upon microtubules and microfilaments (Forristall et al., 1995; Pokrywka and Stephenson, 1991; Theurkauf, 1994; Yisraeli et al., 1990). For the polarization of epithelial cells and early mouse blastomeres membrane components are directed to different domains via vesicle trafficking (Gueth-Hallonet and Maro, 1992; Mays et al., 1994). Neuronal cells also utilize organelle transport along both the microtubule and microfilament cytoskeleton as well as subcellular localization of specific mRNAs (Kelly and Grote, 1993). We have undertaken studies aimed at understanding the polarization process in the early cell divisions of *C. elegans* embryos.

Relevant features of *C. elegans* early development are shown schematically in Fig. 1. The germ cell lineage (P_0-P_4) is characterized by a series of polarized cell divisions (Laufer et al., 1980; Schierenberg, 1987) and is indicated with a bold line in Fig. 1A. The fertilized egg, P_0, becomes polarized along its long axis which corresponds to the anterior-posterior axis of the animal. It has recently been demonstrated that the signal determining the orientation of this axis is the position of the sperm which specifies the posterior end (Goldstein and Hird, 1996). Dramatic cytoplasmic rearrangements occur after com-

pletion of meiosis and formation of the maternal and paternal nuclei. Cytoplasmic material flows anteriorly along the cortex, accompanied by internal streaming of cytoplasm towards the posterior (Hird and White, 1993). During streaming, the maternal pronucleus migrates to the posterior in a microtubule-dependent movement (Strome and Wood, 1983). After meeting in the posterior the two pronuclei move toward the center as the spindle is forming. The spindle is eventually positioned closer to the posterior end of the cell (Albertson, 1984; Kemphues et al., 1988). Due to this asymmetric positioning of the spindle, the resulting daughter cells are unequal in size.

The two resulting blastomeres, AB and P_1, differ not only in size, but also in cell cycle length, spindle orientation, and developmental potential. Isolated AB and P_1 express tissue markers appropriate for their respective lineages (Laufer et al., 1980). Experiments involving the introduction of cytoplasm from one blastomere to another indicate that differences in cell cycle length and gut potential between AB and P_1 can be attributed to differences in cytoplasmic components (Schierenberg, 1985). Therefore, the asymmetric inheritance of factors from the P_0 cell is critical for the appropriate cell lineage and tissue diversification.

Several components are asymmetrically distributed after the first cleavage. P granules are a molecular aggregate that can be detected with antibodies (Strome and Wood, 1983). P granules segregate with germ line precursor cells but it is not known whether they have a role in specification of the germ line. One known determinant that is asymmetrically distributed after the first cleavage is the SKN-1 protein (Bowerman et al., 1993). SKN-1 is a putative transcription factor that is required to specify the fate of the EMS blastomere. Another such component is GLP-1, a cell signaling molecule that is restricted to the anterior blastomere, AB, and its daughters (Evans et al., 1994). The PAR-1 and PAR-3 proteins are involved in establishing asymmetries in the one-cell embryo and they are both asymmetrically localized with approximately reciprocal distributions (Etemad-Moghadam et al., 1995; Guo and Kemphues, 1995).

Efforts to understand the mechanisms responsible for the generation of polarity in the early *C. elegans* embryo have identified two important elements. First, microfilaments appear to play a critical role in the polarization since cytochalasin treatment prevents establishment of the normal asymmetries (Hill and Strome, 1990). Second, the *par* genes are required. Mutations in the *par* genes cause failures in the generation of asymmetries in the zygote (Kemphues et al., 1988). In *par-1*, *par-2*, and *par-3* mutant embryos the first cleavage spindle is not asymmetrically positioned and the AB and P_1 blastomeres are equal in size. In *par-1*, *par-3*, and *par-4* mutant embryos the P granules do not become asymmetrically distributed. The asymmetric distributions of other components such as the SKN-1 protein, cell cycle length and developmental potential are also disrupted in *par* mutant embryos (Bowerman et al., 1993; Kemphues et al., 1988; K. J. Kemphues, unpublished data). The phenotypic analysis of individual *par* mutants and of various double mutant combinations suggests that the process of polarizing the zygote is complex and does not simply involve a single event that is responsible for all aspects of asymmetry (Cheng et al., 1995).

A molecular analysis of the *par* genes is contributing to an understanding of the biochemical nature of *par* gene function. The *par-1* gene encodes a putative ser/thr kinase that is localized to the posterior cortex in the zygote and in subsequent P cells (Guo and Kemphues, 1995). The *par-3* gene encodes a large protein with three DHR motifs (Etemad-Moghadam et al., 1995; Pointing and Phillips, 1995). PAR-3 localization is complementary to that of the PAR-1 protein: it is at the anterior cortex in the zygote. The previously reported sequence of a *par-2* cDNA showed that the predicted PAR-2 protein has two regions of similarity to other proteins (Levitan et al., 1994). One is a cysteine rich region in the amino-terminal half of the protein that is a putative zinc binding site but whose biochemical function is not understood (sometimes referred to as a RING finger motif; Lovering et al., 1993), and the other is a motif in the carboxy terminus that appears to be an ATP-binding site.

In the analysis reported in this paper we address the role of the *par-2* gene by determining the distribution of the PAR-2 protein in wild-type and various mutant backgrounds and by further examination of *par-2* mutant defects. The immunolocalization of the protein and an analysis of the distribution of P granules, PAR-1, and PAR-3 in *par-2* mutants provide evidence that the function of PAR-2 is to regulate the cortical association of P granules and the PAR-1 protein, and to promote the anterior restriction of PAR-3. These data along with other results regarding *par-1* and *par-3* are used to formulate a model for how the PAR proteins are involved in the polarization events of the first cell cycle.

MATERIALS AND METHODS

Worm strains

The Bristol N2 strain was used as the wild type. Other strains used in

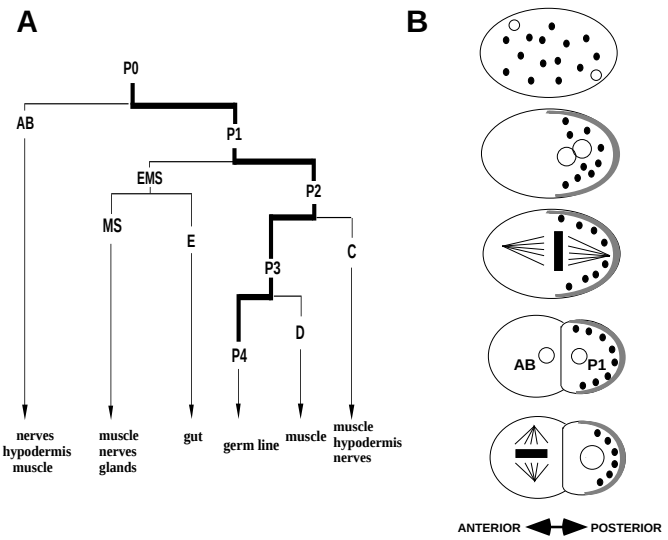

Fig. 1. Early events in *C. elegans* embryogenesis. (A) This diagram shows an abridged cell lineage (Sulston et al., 1983; Schierenberg, 1987). The six founder cells that originate from asymmetric cell divisions are shown along with the cell types they produce. The P lineage is indicated with a bold line. (B) This schematic drawing depicts relevant events leading up to and just after the first cleavage. After fertilization, the maternal nucleus migrates to the posterior to join the paternal nucleus. The first cleavage is asymmetric and gives rise to the AB and P_1 blastomeres. These two blastomeres are unequal in size and also have differing cell cycle lengths with AB dividing prior to P_1. P granules are indicated as black circles and PAR-1 protein is depicted as a stippled line.

this analysis include: *unc-45(e286ts) par-2(lw32)/sC1 [dpy-1(s2171) let]* (Edgley et al., 1995), *par-2(it5ts), lon-1(e185) par-3(it71)/qC1, rol-4(sc8) par-1(b274)/DnT1, daf-7(e1372ts) par-2(it5ts); lon-1(e185) par-3(it71)/+*.

Antibodies and immunolocalization

Antibodies to the carboxy terminus of PAR-2 were derived from antiserum 343. This antiserum was generated by injecting a rabbit with a trplE-PAR-2 fusion protein. This serum was affinity purified by using a GST-PAR-2 fusion protein which consisted of the Pharmacia pGEX-3X vector and the carboxy-terminal 271 amino acids of PAR-2. The amino-terminal antibody, 3700, was generated by injecting a rabbit with a β-galactosidase-PAR-2 fusion which consisted of the β-galactosidase coding region in the PWR vector fused to amino acids 49-628 of PAR-2. This serum was affinity purified using a GST-PAR-2 fusion containing the first 270 amino acids of PAR-2. Both affinity-purified antibodies were used to stain gonads and embryos. Similar results were obtained with both affinity-purified antisera, with the exception that the 343 affinity-purified antibody also detected P granules in embryos. The P granules staining was not specific for PAR-2 as *par-2(lw32)* also stained for P granules.

P granules were detected with the $OICD_14$ mouse monoclonal antibody supernatant provided by S. Strome. P granule distribution was examined in wild type, *lw32*, and *it5ts* mutants. In *it5ts* mutant embryos at the two and four-cell stage, P granule distribution was normal at the permissive temperature (16°C), but abnormal at the non-permissive temperature (25°C).

Actin was detected using monoclonal antibody C4 purchased from ICN and diluted 1:100.

Antibody staining of gonads and embryos were essentially the same except for the initial step. For gonads, adult hermaphrodites were washed in M9 and then put in a solution recommended by Lois Edgar, University of Colorado (60 mM NaCl, 32 mM KCl, 3 mM Na_2HPO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM Hepes, pH 7.2, 0.2% glucose) plus 4 mM levamisole. The worms were placed with a drop of this solution onto a poly-lysine/gelatin coated slide, cut open near the pharynx using a 22.5 gauge needle, covered with a coverslip and immediately immersed in liquid N_2. Embryo staining followed Method II from Waddle et al. (1994). Adult hermaphrodites were placed in a drop of water on a poly-lysine/gelatin coated slide. A coverslip was placed on the worms and water was wicked away until the embryos were extruded and then the slides were immersed in liquid nitrogen. For both procedures, the coverslips were then popped off and the slides were placed in fix (75% methanol, 3.7% formaldehyde, 0.5× PBS) for 15-20 minutes followed by 5 minutes in 100% methanol. The slides were washed in PBS with 0.5% Tween-20 several times. The PAR-2 antibody was diluted 1:5 in a solution of 7:3 (PBS, 0.5% Tween-20: fetal calf serum) and incubated at 4°C overnight. Secondary antibodies (Jackson ImmunoResearch) were diluted 1:100 or 1:200 in the same solution and incubated for 2 hours at room temperature.

PAR-1 staining and western blotting was done as previously described (Guo and Kemphues, 1995). *par-2(it5ts)* embryos were taken from hermaphrodites grown at 25°C.

The extent of PAR-2 distribution in embryos was measured from color slides. Percentage egg length is defined as the distance from the posterior end divided by the total length of the egg.

Microfilament disruption

In order to disrupt microfilaments in the gonad, cytochalasin D (Sigma) was injected into the gonads of adult hermaphrodites. Several concentrations were tested and an injection solution of 40 μg/ml of cytochalasin D in water was found to reproducibly disrupt the actin localization as judged by immunostaining. The gonads of worms were injected as described (Mello and Fire, 1995). Immediately after injection, recovery buffer (Mello and Fire, 1995) was added. Control worms were injected with water. After 30 minutes a drop of M9 was added and 10 minutes later the gonads were stained as described above.

RESULTS

PAR-2 is localized to the cell periphery in the gonad

We used affinity-purified, polyclonal antibodies to the PAR-2 protein to stain the gonads of adult hermaphrodites (Fig. 2). The same pattern of staining is observed with two separate antisera directed against either the amino or the carboxy-

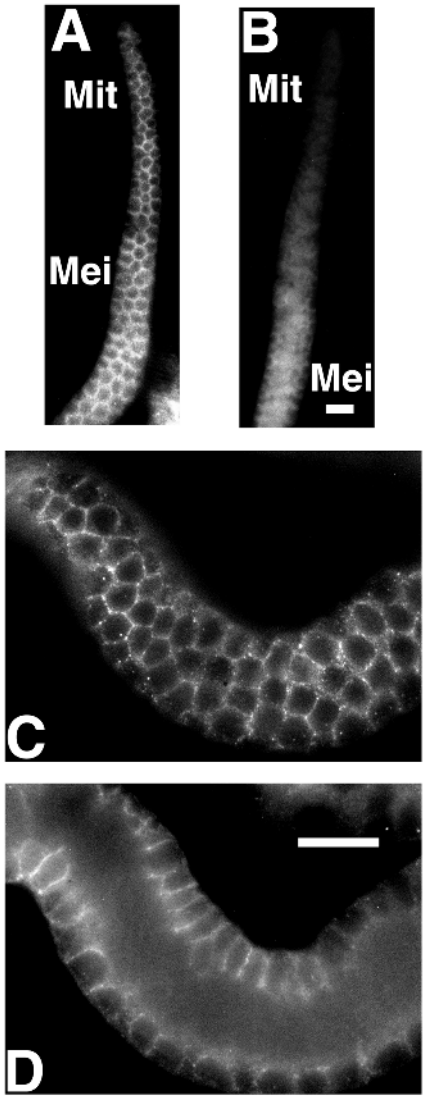

Fig. 2. Distribution of PAR-2 in gonads. Gonads were dissected from adult hermaphrodites and stained with anti-PAR-2 antibody affinity purified from serum 343. Bars, 10 μm. (A) PAR-2 protein is detected in a honeycomb pattern throughout the length of the distal arm. Mitotic (Mit) and meiotic (Mei) regions of the gonad are indicated. (B) Distal arm of a gonad from a *par-2(lw32)* adult hermaphrodite. (C) Higher magnification of a gonad from a wild-type adult hermaphrodite. This tangential view shows the honeycomb nature of the PAR-2 distribution. (D) A different focal plane of the same gonad shown in C. PAR-2 staining is strong between the nuclei (DAPI not shown) and less intense at the external surface.

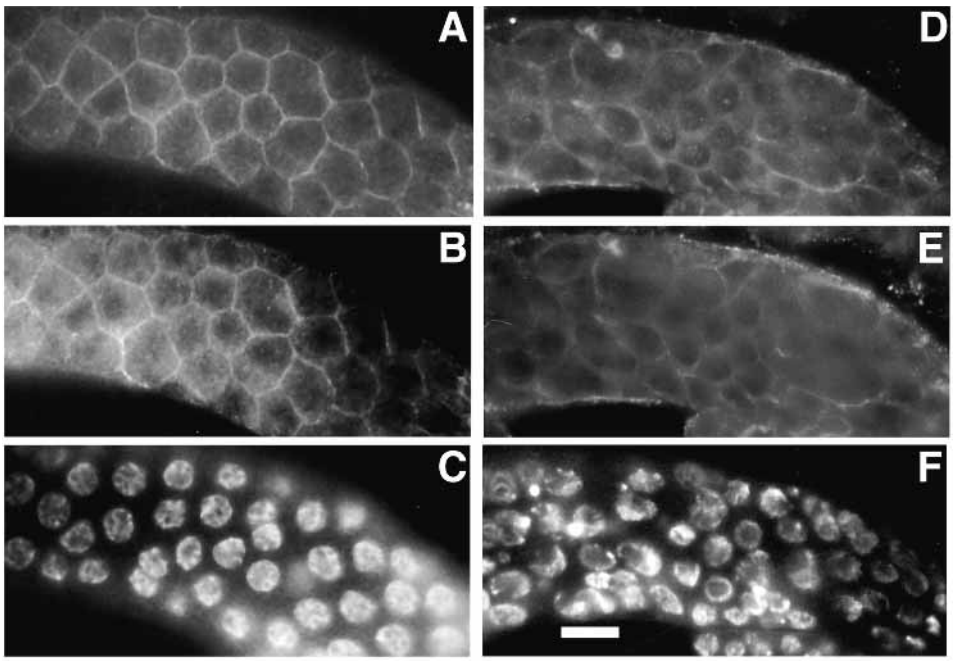

Fig. 3. Immunofluorescence of gonads injected with cytochalasin D. The gonad on the right (D-F) was injected with a solution of 40 μg/ml cytochalasin D. In the same experiment the gonad on the left (A-C) was injected with H_2O and treated in the same way as the one on the right. (A and B) Immunofluorescence using the affinity purified 343 antibody. (B and E) Immunofluorescence using an anti-actin monoclonal antibody. (C and F) Nuclei stained with DAPI. Bar, 5 μm.

terminal portions of the PAR-2 protein. The staining is absent in gonads from hermaphrodites homozygous for the *par-2(lw32)* allele (Fig. 2B), a nonsense mutation at amino acid 234 (Levitan et al., 1994). Most of the signal is associated with the cell periphery. Since there are no predicted transmembrane domains in the PAR-2 protein and peripheral localization is cytochalasin sensitive (see below), we interpret this pattern as staining at the cell cortex.

The hermaphrodite gonad is an elongated, U-shaped organ that is positioned longitudinally inside the body. Oogenesis proceeds from newly formed oogonia in the distal region to mature oocytes directly adjacent to the spermatheca in the proximal gonad (Hirsh et al., 1976). The distal portion of the gonad is a syncytium with the most distal nuclei dividing mitotically. Mature oocytes separate from the syncytium at the proximal end of the gonad. PAR-2 protein is found throughout the entire length of the gonad. Tangential optical sections reveal a honeycomb pattern in the gonad due to PAR-2 localization to the periphery of the syncitial germ cells in the distal gonad (Fig. 2A). An optical cross section shows that staining is strongest at the septa between the nuclei and less intense along the exterior surface (Fig. 2D). Maturing oocytes show uniform cortical staining that diminishes as oocytes mature (not shown).

Male gonads showed a similar honeycomb pattern when stained with either of the PAR-2 antibody preparations (data not shown). The significance of PAR-2 protein in males is not clear since *par-2* mutant males are fertile and *par-2* mutations show neither paternal effects nor paternal rescue (Cheng, 1991).

PAR-2 protein distribution appears very similar to that reported for actin in the gonad (Strome, 1986). Gonads doubly stained with antibodies to PAR-2 and actin confirm that the distribution of the two proteins is similar (Fig. 3). There are two differences in the distributions of the two proteins: (1) actin is found in the sheath cells surrounding the gonad where PAR-2 is not detected; and (2) cortical actin staining in the maturing oocytes of the proximal region is stronger than PAR-2 staining (data not shown).

PAR-2 localization in the gonad depends upon microfilaments

The localization of the PAR-2 protein to the peripheral regions in the gonad suggests that PAR-2 is either associated with the membrane or with the cortical cytoskeleton. To test whether PAR-2 localization in the gonad might depend upon the microfilament cytoskeleton, we disrupted microfilaments by injection of cytochalasin D into gonads. Antibody staining showed that the actin distribution was affected by this procedure in many of the worms. Table 1 shows the results from the injection experiments and examples of injected gonads are shown in Fig. 3. In 76% of worms injected with cytochalasin D, the actin staining was diffuse in the cytoplasm around the nuclei in contrast to the distinct honeycomb pattern seen in most control injected worms (Fig. 3B). As seen in Fig. 3E, this treatment had a local effect on the actin distribution. The PAR-2 distribution showed a strong correlation with the actin distribution: in most gonads with a disrupted actin honeycomb, the PAR-2 honeycomb was also abnormal. In fact, the abnormalities in the two protein distributions were virtually identical (compare Fig. 3D and E). Therefore, we conclude that the PAR-2 distribution at the cell periphery in the gonad is dependent upon the microfilament cytoskeleton.

We tested to see if actin distribution in the gonad is dependent upon PAR-2 by anti-actin antibody staining of gonads from adult *par-2(lw32)* hermaphrodites. Actin distribution appeared normal in these mutant worms (data not shown). In addition, the overall organization of the germ cell nuclei in these gonads is normal. Thus, PAR-2 is dependent upon the actin cytoskeleton for its cortical localization, but it is not required for normal gonad structure.

PAR-2 is asymmetrically distributed at the cortex in blastomeres of the germ-line lineage

As in the gonad, most PAR-2 staining is found at the cortex in embryonic blastomeres. In contrast to its broad distribution in gonads, however, cortical PAR-2 is found only in P_0, P_1, P_2,

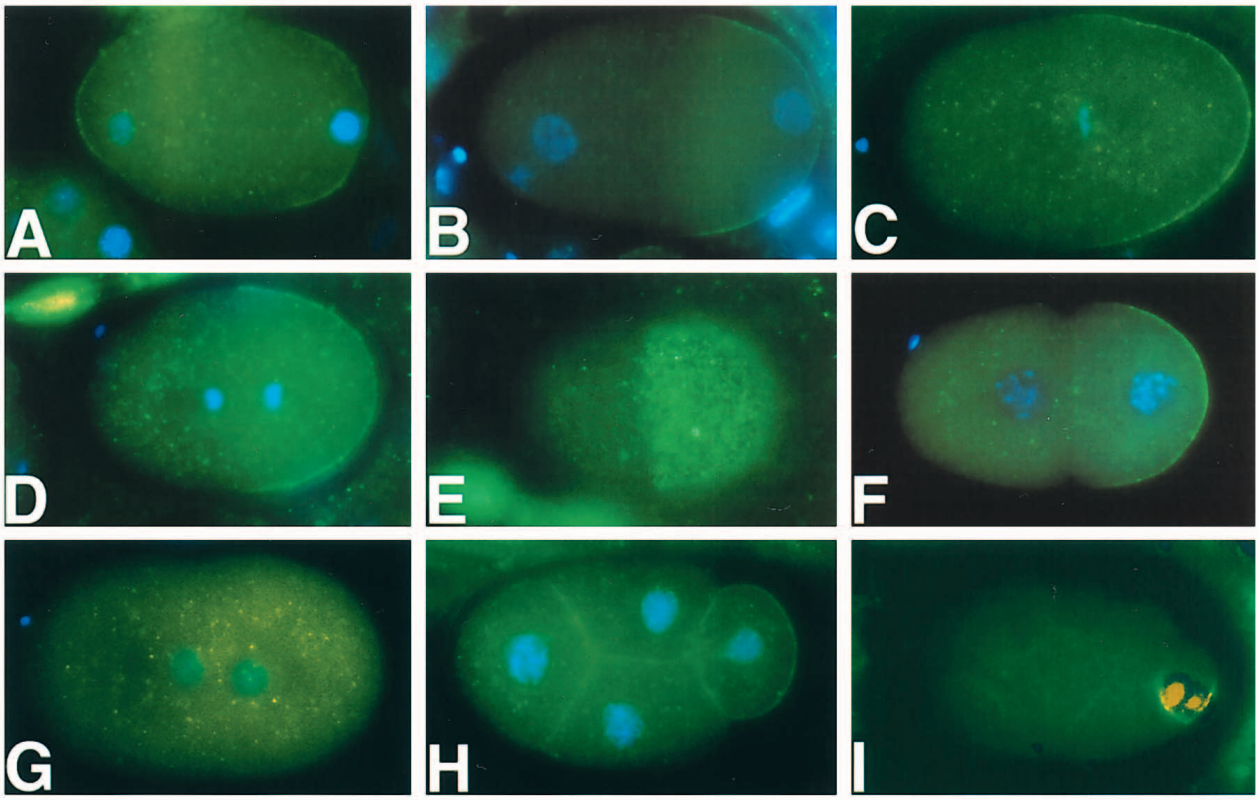

Fig. 4. Distribution of PAR-2 in early embryos. These embryos were stained with affinity purified antibody 3700 which is directed against the amino terminus of PAR-2. A fluorescein labeled secondary antibody was used. The DNA is indicated in blue via staining with DAPI. In most images the polar body is evident as a blue dot at the anterior (left) end of the egg shell. *C. elegans* embryos are approximately 50 μm in length. (A) Pronuclear stage embryo. The pronuclei are not completely decondensed. PAR-2 is present at the anterior and posterior poles of this embryo and absent from the lateral regions. (B) Pronuclear stage embryo. PAR-2 is restricted to the posterior cortex. (C) One-cell embryo in metaphase. The endpoints of the PAR-2 distribution have extended towards the anterior. (D) One-cell embryo in anaphase. The PAR-2 distribution is similar to that of the previous embryo. (E) The same embryo as in D. The glancing focal plane of this view shows the punctate nature of PAR-2 distribution. (F) Two-cell embryo in prophase. PAR-2 protein is restricted to the posterior cortex of P_1. (G) One-cell embryo from a hermaphrodite homozygous for the *par-2(lw32)* mutation. Some background staining in the cytoplasm is evident, but there is no cortical staining. (H) Four-cell embryo. PAR-2 is found all along the cortex of the P_2 cell. PAR-2 is also detected in the blastomere adjoining regions of embryos at this stage. (I) 15-cell embryo doubly stained for PAR-2 and P granules. PAR-2 is seen in green and P granules are in red. The P_3 cell is preparing to divide and has polarized. The P granules and PAR-2 are localized to the ventral half of the cell.

P_3 and P_4, the cells of the germ-line lineage. Furthermore, in all but P_4, PAR-2 exhibits an asymmetric cortical distribution.

PAR-2 protein is first detected at the cortex of some fertilized eggs during the meiotic divisions. In three of 14 meiosis I embryos, and eight of 19 meiosis II embryos examined, faint uniform cortical staining was observed (not shown). Later in the cell cycle, after pronuclei had begun to decondense but before migration, two distributions of PAR-2 were observed. In six of 19 embryos, PAR-2 was localized to the periphery at both the anterior and posterior poles (Fig. 4A). In the remaining 13 embryos PAR-2 was restricted to the posterior cortex (Fig. 4B). The anterior boundary of the staining changes during the cell cycle, beginning at 40% egg length during pronuclear migration (n=6), extending to 48% during metaphase (n=4; Fig. 4C) and retracting to 41% at anaphase and telophase (n=5; Fig. 4D). A tangential optical section shows that PAR-2 is distributed in variously sized spots at the cortex (Fig. 4E). When embryos from *lw32* mutant mothers are stained with PAR-2 antibodies, this asymmetric, cortical staining is not detected (Fig. 4G), indicating that the cortical staining is due to the PAR-2 protein.

When the zygote cleaves, cortical PAR-2 is partitioned to the posterior blastomere, P_1. Initially, PAR-2 is uniformly distributed at the cortex of P_1, but as the cell cycle proceeds, the protein becomes restricted to the posterior (Fig. 4F). PAR-2 is segregated to P_2 (Fig. 4H) and becomes localized to the ventral cortex as the cell cycle proceeds (not shown). In early four-cell embryos, PAR-2 staining is also seen between all four blas-

Table 1. Actin and PAR-2 distribution in gonads injected with cytochalasin D

	H_2O	40 μg/ml cytochalasin D
Actin normal PAR-2 normal	25/35* (71%)†	11/45 (24%)
Actin abnormal PAR-2 normal	4/35 (11%)	3/45 (7%)
Actin normal PAR-2 abnormal	0/35 (0%)	0/45 (0%)
Actin abnormal PAR-2 abnormal	6/35 (17%)	31/45 (69%)

*Number of gonads/number of total gonads injected.
†Numbers in parentheses indicate percentage of total gonads.

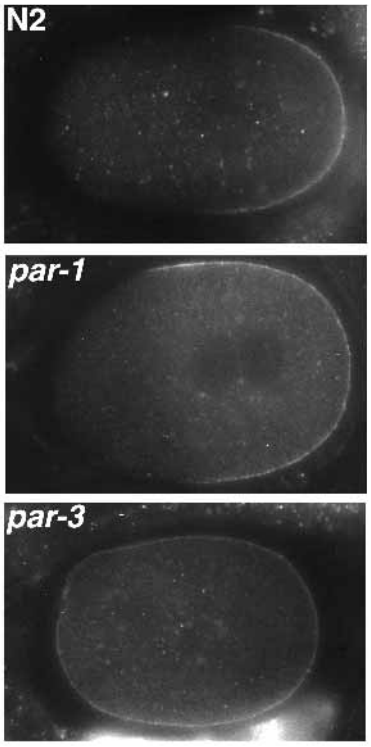

Fig. 5. PAR-2 distribution in *par-1* and *par-3* mutant embryos. Embryos were stained with affinity purified antibody 3700 which is directed against the amino terminus of PAR-2. Shown are one-cell embryos from wild-type (N2), *par-1(b274)*, and *par-3(it71)* hermaphrodites.

tomeres (Fig. 4H). When P_2 divides, PAR-2 is segregated to P_3 where it becomes localized to the ventral side of the cell. The localization of PAR-2 coincides with the localization of P granules in the germ line, as is shown for P_3 in Fig. 4I. In the germ-line founder P_4, which divides symmetrically and partitions the P granules equally to both daughters, PAR-2 shows uniform cortical staining that fades as the cell cycle proceeds (not shown).

PAR-2 is mislocalized in *par-3* mutant embryos

To determine whether PAR-2 localization is dependent upon activities of the other *par* genes, we examined PAR-2 distribution in *par-1*, *par-3* and *par-4* mutant embryos. As shown in Fig. 5, PAR-2 protein is cortical in one-cell embryos in all mutant backgrounds examined. In *par-1* (n=24) and *par-4* (not shown; n=13), PAR-2 distribution is like wild type. In 21/26 *par-3* mutants, however, PAR-2 was no longer restricted to the posterior cortex, but showed a uniform cortical distribution. Three embryos showed no staining and two showed asymmetric staining. PAR-2 distribution is also like wild type in two- and four-cell *par-1* and *par-4* embryos but staining is not restricted to a single cell in later embryos. In two- and four-cell *par-3* embryos, PAR-2 protein is found uniformly at the cortex of all blastomeres (not shown), but is rarely detected in later stage embryos.

par-2 acts with *par-3* to localize the PAR-1 protein

PAR-2 protein distribution in wild type is very similar to that of PAR-1. (Two differences are an earlier onset of PAR-2 staining and persistence of staining of PAR-1 in Z2 and Z3 cells.) Because of this similarity we tested whether *par-2* activity was required to localize PAR-1. In wild type, PAR-1 is restricted to the posterior cortex of the zygote and to the posterior cortex of P_1 in two-cell embryos. We found that PAR-1 is not present at the cortex of *par-2* embryos (Fig. 6A) although it is present at wild-type levels in these embryos (Fig. 6B). Identical results were obtained with two *par-2* alleles, *it5* and *lw32*.

It has been previously shown that *par-3* is required for proper asymmetric localization of the PAR-1 protein (Etemad-Moghadam et al., 1995). In *par-3* mutants, PAR-1 is present uniformly at the cortex of the zygote as well as the cortex of both two-cell stage blastomeres (Fig. 6A). To better understand the relationship between *par-2* and *par-3* in achieving proper localization of PAR-1, we examined PAR-1 distribution in *par-2 par-3* double mutants. As shown in Fig. 6A, PAR-1 distribution in *par-2 par-3* two-cell embryos (n=15) is similar to that seen in *par-3* single mutants (n=17). In particular, it can be seen that PAR-1 protein binds to the cortex in the absence of both gene activities. In one-cell double mutant embryos (n=16), however, the PAR-1 distribution is intermediate between the distributions in *par-2* (n=17) and *par-3* (n=30) single mutants; PAR-1 asymmetry is lost, and some PAR-1 is seen at the cortex, but the amount of PAR-1 protein at the cortex of *par-2 par-3* one-cell embryos is reduced relative to *par-3* single mutants. Thus, it appears that *par-2* activity is not absolutely required for PAR-1 to be localized at the cortex, but, at least in the one-cell embryo, it plays some role.

par-2 activity is not required for the posterior localization of P granules in the zygote, but is required for cortical localization of P granules in P_1

The behavior of P granules in *par-2* mutant embryos is different from that of the other *par* mutants (Cheng, 1991; this report). In embryos mutant for the other *par* genes, P granules fail to localize to the P_1 blastomere at the first cleavage (Kemphues et al., 1988; Guo and Kemphues, 1995). In *par-2(lw32)* mutants, P granules are segregated to P_1 during the first cell cycle (Fig. 7) although they fail to segregate to a single cell in subsequent divisions (Kemphues et al., 1988). The distribution of posteriorly localized P granules in *par-2* differs from wild type. In wild-type embryos, the P granules become associated with the cortex in the posterior of P_0 after prophase (Strome and Wood, 1983; L. Boyd, unpublished observations). They remain cortical in P_1. In *par-2* mutants P granules are found in the posterior of P_0. In most cases it was difficult to discern whether the P granules were cortical in P_0 and often they seemed to be positioned further towards the posterior than in wild-type embryos. However, P granules were clearly not cortical in *par-2* mutant two-cell embryos. P granules tend to be found in the center of P_1 rather than at the cortex (Fig. 7) and are often larger than normal (21 of 26 embryos showed abnormal P granule localization in P_1 as compared to 0 out of 41 for N2). In addition, 7 of 9 embryos of the temperature sensitive *par-2(it5)ts* allele showed a P-granule distribution similar to that shown and described for *lw32*. Therefore, *par-2* activity is required for the cortical localization of P granules in P_1.

DISCUSSION

The *par-2* gene plays an essential role in establishing polarity

in the early embryo. The results reported here provide insight into the nature of this role. PAR-2 protein is synthesized during oogenesis and is present at the cell periphery of oogonia, developing oocytes and early embryos. In embryos, the protein is found only at the cortex of cells of the germ-line lineage and is asymmetrically distributed in P_0, P_1, P_2 and P_3. We show that cortical localization of PAR-2 in the gonad requires intact microfilaments and that asymmetric distribution of PAR-2 requires *par-3* but not *par-1* or *par-4* gene activities. We also show that *par-2* acts with *par-3* to localize PAR-1. Finally our results indicate that PAR-2 plays a role in promoting the association of the P granules with the cortex and that PAR-1 need not be localized to contribute to localization of P granules to the posterior of the zygote.

PAR-2 is asymmetrically distributed in the cells of the P lineage

The *C. elegans* zygote cleaves unequally and generates two daughter cells with different characteristics. *par-2* is required for unequal cleavage as well as differences in cell cycle length and spindle orientation (Cheng et al., 1995; Kirby et al., 1990). The asymmetric distribution of PAR-2 in the zygote (P_0) is consistent with the requirement of *par-2* activity for the polarization of this cell. Since the divisions of P_1, P_2, and P_3 are also asymmetric (Schierenberg, 1987), it is reasonable to propose that some of the factors that effect asymmetric division of P_0 might also function in later P cell divisions. Since PAR-2 is present in the P lineage throughout early embryogenesis it may have a role in the asymmetric divisions of these cells. The aberrant, non-cortical localization of P granules in P_1 of *par-2* mutant embryos supports the hypothesis that PAR-2 is functioning in this cell.

Further evidence that PAR-2 has a role in later P cell divisions comes from the maternal effect sterile phenotype of *par-2* mutations (Cheng et al., 1995; Kemphues et al., 1988). With all *par-2* alleles, some embryos escape the maternal effect lethality and mature to become viable adults. Most of these escapers completely lack a germ line. Since the P cells are germ line precursor cells, the absence of a germ line in *par-2* embryos is consistent with a role for PAR-2 in the asymmetric P cell divisions.

PAR-2 contributes to the localization of P granules, PAR-1 and PAR-3

In wild-type zygotes, PAR-3 protein is restricted to the anterior cortex, PAR-1 and PAR-2 proteins are restricted to the posterior cortex in a pattern reciprocal to PAR-3, and P granules form a loose association with the posterior cortex (see Fig. 8). Since the cortical domain of PAR-3 protein is extended into the posterior of P_0 and P_1 in *par-2* mutant embryos (Etemad-Moghadam et al., 1995), PAR-2 somehow excludes PAR-3 from the posterior. PAR-2 seems to have the opposite effect on the P granules and PAR-1: PAR-2 is promoting the cortical association of PAR-1 and P granules in the posterior because these are both displaced from the posterior cortex in *par-2* mutant embryos.

The results from PAR-1 localization in *par-2* and *par-3* single mutants and the *par-2 par-3* double mutant suggest that there are two different mechanisms by which PAR-2 contributes to proper localization of PAR-1. First, PAR-2 excludes PAR-3 from the posterior and second, PAR-2 promotes the binding of PAR-1 to the cortex. In *par-2* mutant zygotes PAR-3 distribution extends into the posterior and in two-cell embryos PAR-3 is present uniformly at the periphery of both AB and P1; in *par-2* mutants PAR-1 does not bind to the cortex. In *par-3* mutants, PAR-1 does bind to the cortex but its distribution extends into the anterior (Etemad-Moghadam et al., 1995). Because PAR-2 also extends into the anterior in *par-3* mutants these observations could be interpreted to mean either that PAR-1 is localized to the posterior cortex because PAR-3 excludes it or that PAR-1 is localized to the posterior because PAR-2 promotes its binding. In the former case, *par-3* should be epistatic to *par-2* in the double mutant, but in the latter, *par-2* should be epistatic. In fact, neither gene shows clear epistasis; the double mutant shows an intermediate phenotype. Thus it appears that PAR-2 contributes to PAR-1 localization both by excluding PAR-3 from the posterior and by promoting the cortical localization of PAR-1.

PAR-1 can function when not localized normally

One important and unanticipated implication of our results is that the PAR-1 protein can function without being asymmetric or cortical. *par-1* activity is required for P granule segregation to the P_1 blastomere (Guo and Kemphues, 1995; Kemphues et al., 1988). *par-2* activity is not required for this segregation. Our finding that PAR-1 is neither cortical nor asymmetric in *par-2* mutants shows that PAR-1 can function when it is not visibly associated with the cortex. What is the purpose of the asymmetric cortical localization of PAR-1? One possibility is that cortical PAR-1 is required for the cortical association of the P granules which fails to occur in *par-2* mutants. Another possibility is that the cortical association of PAR-1 is a mechanism to assure that PAR-1 is itself segregated to the cells of the P lineage. Cortical attachment of embryonic determinants also occurs in *Drosophila* and *Xenopus* embryos (Klymkowsky and Karnovsky, 1994; Pokrywka and Stephenson, 1991). The attachment of important fate determinants to the cortical cytoskeleton could prevent their diffusion. Cortical localization may facilitate the partitioning of the numb and prospero proteins, cell fate determinants in *Drosophila* CNS development which are cortically localized in the mother cell and asymmetrically partitioned to one of the daughter cells (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995).

How do the *par* genes control embryonic polarity?

The activities of all of the *par* genes are required for establishing the appropriate asymmetries during the first cell cycle (Kemphues et al., 1988; Kirby et al., 1990). The results presented here taken together with similar analyses of the *par-1* and *par-3* genes indicate that the establishment of polarity in the zygote is accomplished by an interplay of the PAR proteins at the cortex. A general model summarizing the relationships between the PAR proteins is presented in Fig. 8. The sperm enters the egg and defines the posterior pole (Goldstein and Hird, 1996). PAR-2 and PAR-3 respond to this cue by becoming asymmetrically distributed. We place PAR-1 localization downstream of PAR-2 and PAR-3 because *par-1* activity is not required for the localization of either PAR-2 or PAR-3, whereas the activities of those two genes are required for the localization of PAR-1. Also, PAR-1 localization to the cortex occurs slightly later than that for PAR-2 and PAR-3. The relationship between PAR-2 and PAR-3 in establishing the

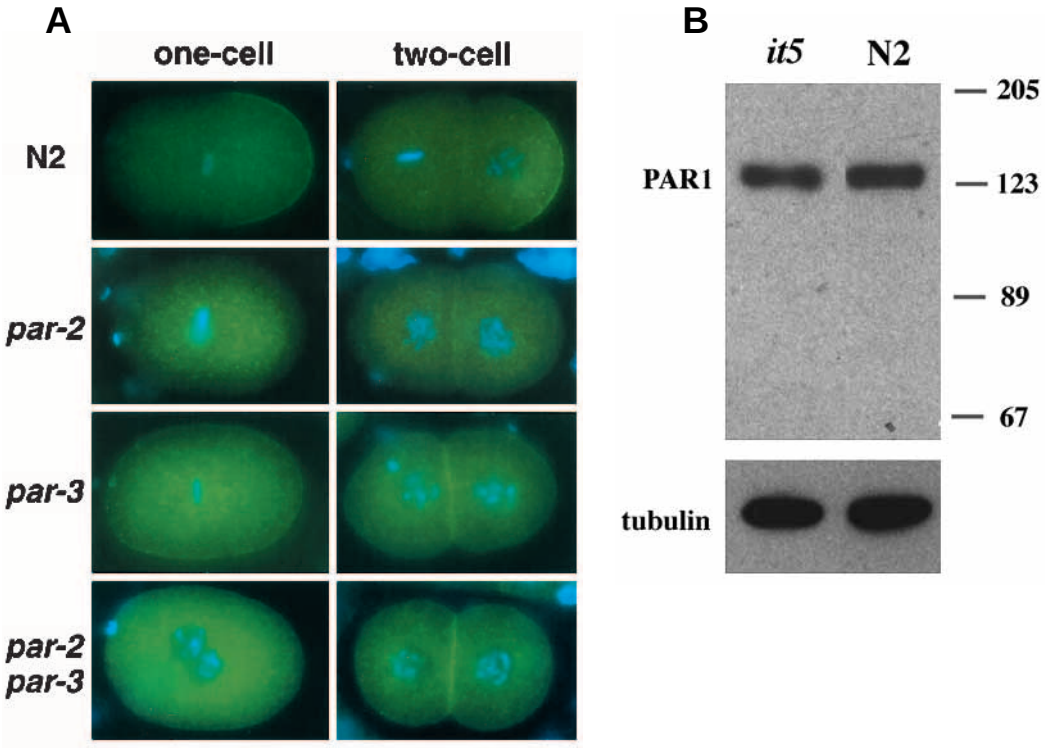

Fig. 6. PAR-1 distribution in *par-2*, *par-3* and *par-2 par-3* mutant embryos. (A) Embryos were stained with an affinity-purified polyclonal antibody to the PAR-1 protein. PAR-1 appears as green and DNA is blue due to staining with DAPI. One-cell embryos are in the left column and two-cell embryos are in the right column. Embryos are oriented with their anterior pole to the left. Genotypes are indicated at the extreme left. N2: in these standard micrographs, which include light from above and below the focal plane, strong cortical staining can be seen at the posterior of the N2 one-cell as indicated not only by the stronger peripheral signal but also by a sharp staining boundary. Where cortical staining is absent, as in the anterior of the N2 one-cell and in the N2 AB cell, the signal falls off as the thickness of cytoplasm decreases toward the periphery; as a result the edge of the cell has an indistinct boundary. *par-2*: *par-2* mutant embryos show indistinct staining at the boundary of the cell indicating that cortical staining is absent. *par-3*: in the *par-3* embryos, the PAR-1 protein is uniformly present at the periphery (note the distinct boundary and weak peripheral signal), although the level is reduced. *par-2 par-3*: in the *par-2 par-3* one-cell embryos, although the peripheral signal is not noticably higher than the cytoplasm in this micrograph, a distinct cell boundary is seen; compare to the staining at the posterior of the N2 one-cell embryo. Based on observations of numerous embryos in multiple focal planes we are able to interpret this staining pattern as weak cortical staining. The PAR-1 signal in the *par-2 par-3* mutant two-cell embryo is not discernibly different from that seen in the *par-3* mutants. (B) Western blot of protein extracts made from N2 and *par-2(it5ts)* embryos. This western blot was probed with the same antibody as used in the immunofluorescence images in A.

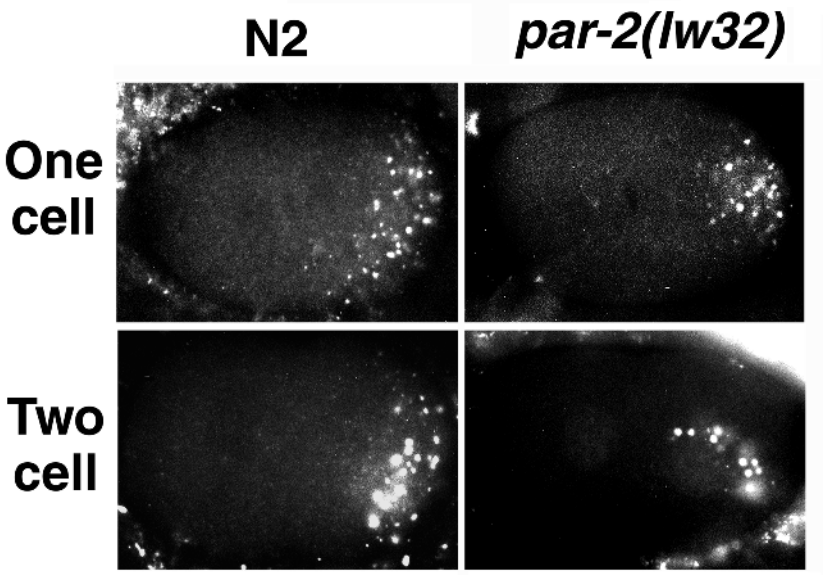

Fig. 7. P granule distribution in wild-type and *par-2* mutant embryos. Embryos in the left column are wild type and in the right column are from hermaphrodites homozygous for the *par-2(lw32)* mutation. In *par-2* mutant one-cell embryos, P granules are segregated to the posterior end of the cell as they are in wild-type embryos. In *par-2* mutant two-cell embryos, the P granules fail to associate with the cortex.

appropriate polarity is less obvious. It has been shown that *par-2* is required to restrict PAR-3 to the anterior and that *par-3* is needed for posterior PAR-2 localization. Whether these restrictions are due to competition, direct inhibition, or some other, indirect mechanism is not clear at this point.

The relationship of the asymmetric distributions of the PAR proteins to the asymmetric distributions of P granules and putative cytoplasmic determinants like SKN-1 is unclear. Although it is reasonable to propose that localized activity of the PAR genes contributes directly to intracellular asymmetry,

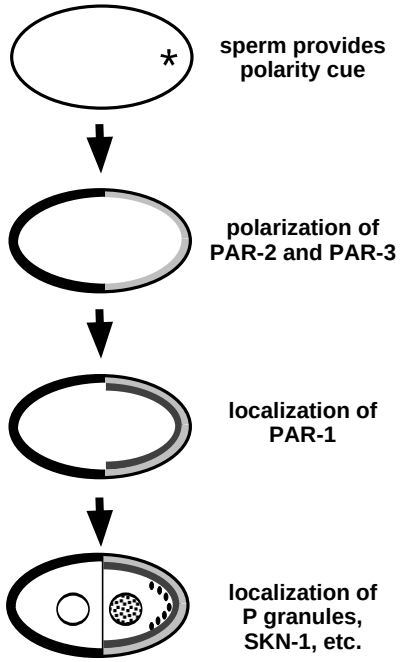

Fig. 8. A model for PAR proteins and polarization of the zygote. The position of the sperm defines the posterior end of the zygote. In response to this cue, PAR-2 (light grey) becomes localized to the posterior cortex and PAR-3 (black) becomes localized to the anterior cortex by a process of mutual exclusion. PAR-1 (dark grey) becomes localized to the posterior in two ways. PAR-2 promotes PAR-1 binding at the cortex while PAR-3 excludes PAR-1 from the anterior. By means yet to be determined, the activities of the PAR proteins lead to the asymmetric partitioning of P granules and cell fate determinants such as SKN-1.

the residual activity of unlocalized PAR-1 reveals unexpected complexity. A more complete understanding of *par* gene function will require identifying molecular links between the PAR proteins and the localized molecules.

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