RNA localization along the anteroposterior axis of the Drosophila oocyte requires PKA-mediated signal transduction to direct normal microtubule organization

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Microtubule polarity has been implicated as the basis for polarized localization of morphogenetic determinants that specify the anteroposterior axis in Drosophila oocytes. We describe mutations affecting Protein Kinase A (PKA) that act in the germ line to disrupt both microtubule distribution and RNA localization along this axis. In normal oocytes, the site of microtubule nucleation shifts from posterior to anterior immediately prior to polarized localization of bicoid and oskar RNAs. In PKA-deficient oocytes, posterior microtubules are present during this transition, oskar RNA fails to accumulate at the posterior, and bicoid RNA accumulates at both ends of the oocyte. Similar RNA mislocalization patterns previously reported for Notch and Delta mutants suggest that PKA transduces a signal for microtubule reorganization that is sent by posteriorly located follicle cells.

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pearance of the posteriorly localized MTOC and the nucleation of microtubules at the anterior margin of the oocyte, which subsequently extend to the extreme posterior. Because microtubules are nucleated at the minus end (Mitchison 1992), this provides a microtubule cytoskeleton of uniform anteroposterior polarity that could accommodate transport of bcd RNA to the anterior by minus-end-directed motor proteins, whereas osk RNA moves to the posterior in association with plus-end-directed motor proteins.

A small number of mutations that disrupt bcd or osk RNA localization during oogenesis have been identified on the basis of an embryonic phenotype similar to that of bcd or osk. Thus, mutations in the exuperantia (exu) or swallow (swa) genes result in embryos that lack some anterior structures; these mutations also result in the failure to initiate (exu) or maintain (swa) anterior localization of bcd RNA in the developing oocyte (St. Johnston et al. 1989). Similarly, mutations in cappuccino (capu), spire (spir), or staufen (stau), which result in embryos lacking germ cells and with posterior structures deleted, also cause mislocalization of osk RNA in oocytes (Ephrussi et al. 1991; Kim-Ha et al. 1991; St. Johnston et al. 1991). Because exu, swa, capu, stau, and spir activities are required in the germ cells, most models for the generation of anteroposterior polarity involve a germ-line autonomous mechanism.

Conditional alleles of the neurogenic genes, Notch (N) and Delta (Dl), can also disrupt the localization of RNAs in the oocyte (Ruohola et al. 1991). Although these mutations have pleiotropic effects on oogenesis, carefully controlled temperature shift experiments indicate that they have a specific role in the determination of oocyte polarity. Furthermore, N and Dl mutations affect the localization patterns of both bcd and osk, making them unique among genes so far identified. In addition, N and Dl functions appear to be required in somatic follicle cells rather than germine cells for normal oocyte polarity.

We now report that germ-line protein kinase A (PKA) mutations cause similar mislocalizations of bcd and osk RNAs to those observed previously to result from N mutations. This supports the hypothesis that cell communication between follicle cells and the oocyte is essential for the machinery that localizes RNAs along the anteroposterior axis (Ruohola et al. 1991). We also show that PKA mutations selectively disrupt the organization of microtubules at the posterior of the oocyte at the time of bcd and osk RNA localization. This can account for the observed mislocalization of bcd and osk RNAs at ectopic microtubule minus and plus ends, respectively, and therefore provides key evidence in favor of a direct role for microtubule-based transport in RNA localization. Therefore, we suggest that PKA normally acts in the oocyte in response to a temporal signal from posteriorly located follicle cells to mediate the disassembly of microtubules at the posterior and thereby permit a uniform gradient of microtubule polarity that is essential for the correct localization of RNA molecules along the anteroposterior axis of the oocyte.

Results

PKA mutations disrupt bcd and osk RNA localization

We have previously described female sterile alleles of the catalytic subunit gene, DCO, of Drosophila PKA (Lane and Kalderon 1993). Females that carry the deficiency Df(2L)Tw2, which deletes the 3'-untranslated region of the DCO gene in trans to null alleles do not lay eggs, although mature oocytes are produced. To examine more carefully the extent to which oocyte development is affected by PKA mutations, we stained oocytes with probes against RNAs that become localized to specific regions of the oocyte during oogenesis.

In wild-type ovaries, RNA for the osk gene accumulates in one cell of the developing cyst in the gerarium and throughout stages 1–6 of oogenesis (Ephrussi et al. 1991; Kim-Ha et al. 1991). Between stages 8 and 9, osk RNA becomes localized to the posterior of the oocyte, with some of the RNA transiently accumulating at the oocyte anterior early in stage 8 (Fig. 1B). By stage 9, all of the osk RNA in the oocyte is tightly localized to the posterior pole, where it remains detectable until early embryogenesis. This localization has been shown to be required for the subsequent posterior localization of many RNAs and proteins that are required for germ cell specification and abdominal patterning, including vasa protein and nanos RNA (Hay et al. 1990; Lasko andAshburner 1990; Gavis and Lehmann 1992).

We observed oocyte-specific accumulation of osk RNA in stage 1–6 egg chambers from PKA-deficient females [Fig. 1A]. However, in 42% (80/190) of all stage 8 and 9 egg chambers, osk RNA failed to accumulate at the posterior pole and, instead, appeared concentrated in the middle of the oocyte [Fig. 1C]. This ectopic localization persisted until stage 10. After this time we saw only oocytes with normal osk localization and oocytes in which no osk RNA was detectable. Staining PKA mutant egg chambers with antibodies against the vasa protein showed that vasa failed to localize to the posterior in 48% (111/228) of stage 10 egg chambers [data not shown], as would be predicted from the absence of posteriorly localized osk RNA.

RNA for the anterior morphogen bcd also becomes localized in a stepwise manner [St. Johnston et al. 1989], first accumulating in the oocyte during stage 5, and becoming localized to the anterior margin at stage 8 [Fig. 1D], where it remains until early embryogenesis. In 28% (47/167) of all stage 8 egg chambers from Df(2L)Tw2/DCO females, we saw transient accumulation of bcd RNA at the posterior as well as the anterior end [Fig. 1E]. The anterior localization appeared indistinguishable from wild type and persisted throughout oogenesis. By mid-stage 9, bcd was no longer detected at the oocyte posterior.

Specification of the dorsoventral axis can also be detected by RNA localization patterns in the oocyte. Newman-Silberberg and Schüpbach (1993) have shown that RNA for the gurken (grk) gene, which is involved in the communication of positional information between the oocyte and the surrounding follicle cells, is localized first
PKA mutations disrupt the localization of a kinesin fusion protein

To investigate the role of microtubules in polarized localization of morphogenetic determinants in oogenesis, Clark et al. (1994) have constructed a kinesin:lacZ fusion gene (khc:lacZ) to act as a reporter of microtubule polarity. Kinesin has been shown to act as a plus-end-directed motor in vitro (Vale et al. 1985). In otherwise wild-type flies that express the khc:lacZ gene under the control of a promoter that is active in oogenesis, Kinesin:β-gal is first detected in the oocyte early in stage 8 (Clark et al. 1994). The fusion protein is detected at progressively more posterior positions throughout stage 8, and by early stage 9 it is tightly localized to the extreme posterior (Clark et al. 1994; Fig 2A). This is consistent with the expectation that the fusion protein would move from minus ends of microtubules, nucleated at the anterior, toward the plus ends extending to the posterior (Theurkauf et al. 1992).

We used this reporter construct as an indirect assay of the effect of PKA mutations on microtubule polarity. In oocytes from sibling control females, Kinesin:β-gal could be detected in the oocyte at stage 8, and all stage 9 oocytes showed localization to the posterior cortex, as was reported by Clark et al. (1994) for wild-type oocytes. However, in egg chambers from PKA-deficient females, we saw a failure to localize Kinesin:β-gal to the extreme posterior in 52% (25/48) of all stage 9 egg chambers. Instead, we saw Kinesin:β-gal protein, somewhat diffusely distributed, confined to an area that is ~25% of the oocyte’s length from the posterior [Fig. 2B]. No obvious differences between mutant and wild-type egg chambers were detected before this stage.

We stained egg chambers with rhodamine-phalloidin to determine whether or not there was a correlation between the localization phenotype and a previously described defect in the integrity of nurse cell junctions associated with female sterile alleles of PKA (Lane and Kalderon 1993). The egg chamber in Figure 2B appears to have undergone a number of nurse cell fusions, as evidenced by the large area outlined by phalloidin in the top of the picture and the actin inclusions in the center of this area. We believe the actin inclusions are ring canals that have become detached from cell membranes and subsequently degenerated. Figure 2C shows an egg chamber in which nurse cell junctions appear relatively intact, yet Kinesin:β-gal failed to reach the posterior.

Figure 1. PKA mutations disrupt bcd and osk RNA localization. In situ hybridization for osk (A–C), bcd (D,E), and grk (F) RNA in ovaries from Df(2L)Tw2/DC0b3 (A,C,E,F) or sibling (B,D) females. The oocyte is at the posterior (right). (A) Early stages of osk RNA localization are not disrupted by PKA mutations. osk RNA accumulates in the oocyte, beginning in the germarium (Ephrussi et al. 1991; Kim-Ha et al. 1991). (B) osk RNA is localized to the posterior of the oocyte in stage 9 egg chambers from control females. (C) At stage 9, osk has failed to reach the posterior in half of all PKA mutant oocytes. In most cases, accumulation near the middle of the oocyte is observed. (D) bcd RNA is localized to the anterior margin in control stage 8 oocytes. (E) In oocytes from PKA mutant females, we see anterior localization of bcd RNA as well as ectopic bcd RNA in the posterior of many stage 8 oocytes. Posteriorly localized bcd RNA can no longer be detected after stage 9 (not shown). (F) grk is localized normally in the anterior dorsal region of stage 8 oocytes from PKA mutant females.
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that had undergone many nurse cell fusions [data not shown]. Therefore, we do not think that the localization defects result form defects in nurse cell integrity but, rather, that these two phenotypes reflect two separate requirements for PKA in oogenesis.

PKA activity is required in the germ line for Kinesin: β-gal localization

Conditional alleles of the neurogenic genes N and Dl result in patterns of localization for Kinesin: β-gal protein, bcd RNA, and osk RNA similar to those in oocytes from females carrying PKA mutations (Ruohola et al. 1991; Clark et al. 1994). N and Dl appear to play several roles in oogenesis, particularly in the specification of follicle cell fate. Normally, the posterior polar follicle cells, a pair of follicle cells that are adjacent to the posterior of the oocyte, express the adhesion protein fasciclin III [Fas III] throughout most of oogenesis. In N and Dl mutations, the number of posteriorly located follicle cells that express Fas III is between 5 and 20 [Ruohola et al. 1991]. As both N and Dl activities are required somatically for proper bcd and osk localization, it is thought that the mislocalization phenotypes result from the failure to specify correctly the fate of either the posterior polar follicle cells or their immediate neighbors. These cells have therefore been implicated in a signal transduction process by which positional information is communicated to the oocyte.

We asked whether PKA was required in the follicle cells, where it might play a role in the generation of a signal, or in the oocyte, where it might be activated in response to a signal. In PKA-deficient egg chambers, we observed normal Fas III staining in follicle cells, indicating that hypomorphic PKA mutations disrupt RNA localization without detectable alterations in follicle cell fates [data not shown]. We also made use of an autosomal insertion of a P element carrying the ovoD1 mutation (Chou et al. 1993), a dominant female sterile mutation, to generate germ-line clones homozygous for PKA null mutations. Germ-line recombinants were easily identified by the presence of a large ovary with vitellogenic eggs. Clones of the null alleles, DCO^B^ and DCO^H^, were recovered at similar frequencies to the parent nonmutagenized chromosome (4–8%). These ovaries were dissected and stained with phalloidin and anti-β-galactosidase antibody. Many of these egg chambers showed numerous nurse cell fusions, but the geometry of the egg chamber was largely unaffected so that the localization of Kinesin: β-gal could be accurately scored. We observed a failure of Kinesin: β-gal to localize to the posterior of all 40 stage 9 oocytes from 26 females [Fig. 3], indicating that PKA activity is required in the germ line and that it is essential for the posterior localization of Kinesin: β-gal. Furthermore, normal Fas III staining was observed in polar follicle cells of these egg chambers, demonstrating that polar follicle cell specification does not depend on PKA activity in the germ line.

Conversely, we saw egg chambers from mutant females that showed normal localization of Kinesin: β-gal but
PKA mutations affect the distribution of microtubules in the oocyte

For a more direct investigation of the relationship between microtubule distribution and RNA localization, we compared the distribution of microtubules, revealed by anti-tubulin immunofluorescence, between wild-type and PKA-deficient oocytes. Theurkauf et al. (1992) have shown that in early egg chambers, the highest density of microtubules is seen in the oocyte. These microtubules originate from an MTOC that can be found in the posterior of a stage 6 oocyte. By stage 7, microtubules can no longer be seen at the posterior; instead the anterior margin of the oocyte acts as a nucleating region, and microtubules nucleated here continue to extend more posteriorly throughout stages 8-10a. During these stages, an anterior to posterior gradient of decreasing microtubule density can be seen.

In early egg chambers (stages 1-6), our observations are consistent with those of Theurkauf et al. (1992, 1993) and we did not detect any differences in microtubule distribution between wild-type and oocytes from PKA hypomorphs (Fig. 4A, D). However, at the time when bcd and osk RNAs are normally in the process of being localized to the anterior and posterior poles (stages 7 and 8), clear differences were apparent. In 46% of mutant oocytes (21/45) (Fig. 4E, F), but never in wild-type oocytes (Fig. 4B), microtubules were present at the posterior, in addition to the normal microtubule distribution at the anterior. In many cases (11/21), the resolution was sufficient to show these as distinct populations of microtubules, consistent with their being nucleated from separate sites (Fig. 4F). Later in stage 9, microtubules extend
farther along the cortex toward the posterior in wild-type oocytes. Thus, although posterior microtubules were present in many mutant oocytes at this stage, they were not obviously discontinuous with microtubules extending from the anterior. We cannot, therefore, determine how long the microtubules found at the posterior of stage 7 and 8 mutant oocytes persist. We do conclude, however, that an abnormal microtubule distribution is present in mutant oocytes, at the time when bcd and osk RNAs are normally transported to the anterior and posterior of the oocyte [Fig. 5].

Discussion

In this study we have shown that PKA mutations affect the localization of RNAs along the anteroposterior axis of the Drosophila oocyte. Direct visualization of microtubules has shown that PKA mutations also affect the reorganization of the microtubule cytoskeleton that normally occurs immediately prior to polarized RNA localization. We argue that these two effects are causally related and that PKA is absolutely required in the oocyte to transduce a temporal signal from posterior follicle cells that initiates microtubule reorganization and, hence, the correct polar localization of RNAs.

**Figure 5.** Diagrammatic representation of microtubule distribution in wild-type and PKA mutant oocytes. In early egg chambers, high microtubule density is observed in the oocyte, and partial depolymerization experiments suggest that an MTOC is present at the posterior of stage 6 oocytes. This population is no longer detected at stage 7, and new microtubules are present at the anterior margin of the oocyte, which appears to act as a nucleating region [Theurkauf et al. 1992]. In oocytes from PKA mutant females, which have apparently normal microtubule distribution up to stage 6, the population of microtubules at the posterior of the oocyte persists until stages 7 and 8, while microtubules are being nucleated at the anterior. According to the current models for microtubule polarity in oocytes, this would result in microtubule minus ends being located at the anterior and posterior poles (as opposed to only the anterior in wild type), whereas plus ends, found at the posterior of wild-type oocytes, would be found near the middle of the oocyte.

**Specificity of PKA action in oogenesis**

Intensive investigation of the effects of PKA mutations on oogenesis has revealed two specific and distinct phenotypes, the concomitant mislocalization of RNAs and microtubules at stages 7 and 8 and the progressive fusion of nurse cells during stages 5–10. Even germ-line null mutations of the catalytic subunit gene have no additional effects, allowing the formation of a mature oocyte and normal nurse cell–oocyte transport [Lane and Kalderon 1994], a process that requires an intact filamentous actin cytoskeleton [Knowles and Cooley 1994]. We believe that the requirement for PKA in oocyte polarity is distinct from its role in nurse cell junctional integrity for several reasons. First, we did not see a correlation between the severity of these two incompletely penetrant phenotypes in individual egg chambers of PKA heteromorphs. Second, germ-line armadillo mutations cause extensive fusion of nurse cells without affecting RNA localization [Peifer et al. 1993]. Third, the association of catalytic subunit protein with nurse cell membranes [Lane and Kalderon 1994] suggests that it has access to proteins associated with the membrane skeleton and therefore is likely to play a very direct role in the stabilization of cell junctions. In contrast, the disruptions of microtubules, RNA, and Kinesin:β-gal localization that we observed were confined to the posterior of the oocyte, far removed from nurse cell junctions. The temporal and spatial selectivity of PKA mutations on RNA localization further emphasizes the specificity of its action in the oocyte. Thus, the initial oocyte-specific accumulation of osk RNA and the concentration of microtubules at the oocyte posterior prior to stage 7 were unaffected by PKA mutations, as were the anterior accumulation of bcd RNA and the dorsal anterior localization of grk RNA, which normally accompany the posterior localization of osk RNA at stage 8.

**PKA and microtubule reorganization**

The distribution of microtubules in egg chambers has been shown to be dynamic, with distinct phases that correlate with two phases of RNA accumulation in the oocyte. In the germlarial stages, a single microtubule cytoskeleton forms within the 16-cell cyst originating from an MTOC in the oocyte [Theurkauf et al. 1993]. Partial depolymerization experiments demonstrate the presence of this MTOC until stage 6, when it is found at the posterior of the oocyte. The loss of this MTOC and the appearance of microtubule nucleation sites at the anterior is seen during and after stage 7 [Theurkauf et al. 1992]. Thus, in wild-type egg chambers microtubules nucleated at the posterior are lost prior to the appearance of microtubules nucleated at the anterior. In PKA mutants a high density of microtubules was observed at the posterior of stage 7 and 8 oocytes, well after microtubules had formed at the anterior. The logical assumption that these ectopic microtubules were persisting from previous stages and, therefore, nucleated at the posterior is supported by three observations. First, these microtubu-
Microtubules were often seen to be clearly discontinuous with microtubules extending from the anterior. Second, preliminary evidence from partial depolymerization experiments shows the abnormal persistence of an MTOC at the extreme posterior of stage 8 oocytes from PKA hypomorphs (see legend to Fig. 4). Third, as discussed below, the localization of bcd and osk RNAs, as well as the kinesin fusion protein, was consistent with the inferred polarity of microtubules that would result from persistence of microtubules nucleated at the posterior.

The appearance of microtubules at the anterior margin proceeded normally in PKA mutants and, therefore, appears to be independent of the depolymerization of microtubules at the posterior. It is possible that molecules that are capable of nucleating microtubules are supplied by the nurse cells during stage 7. The location of the anterior margin of the oocyte adjacent to the nurse cells could lead to the preferential accumulation of microtubule-nucleating molecules at the point of entry. The distribution of molecules such as γ-tubulin [Stearns and Kirchner 1994] and pericentrin [Doxsey et al. 1994] will be particularly informative in addressing this issue.

**RNA localization and microtubule organization**

A number of recent studies have provided evidence of a link between microtubules and localization of bcd and osk RNAs. The use of drugs that depolymerize microtubules can disrupt both the initial oocyte specific and later polarized accumulation of osk and bcd RNAs [Pokrywka and Stephenson 1991; Theurkauf et al. 1993; Clark et al. 1994]. Also, exu protein, which is required for bcd RNA localization, has been found in large particles that colocalize with microtubules [Wang and Hazelrigg 1994].

Directional microtubule-based transport requires an appropriate geometry of microtubules, signals on the cargo to specify destination and transporters that both recognize destination tags and actively translocate along microtubules. PKA mutations cannot be affecting the cargo itself, as transport of several macromolecules was affected, each of which was present in its normal location prior to transport. It is possible that PKA regulates the activity of motor proteins or their engagement with cargo and microtubules, as has been suggested for the movement of organelles in neurons [Azhdarian et al. 1994]. However, this would not easily explain the normal accumulation of bcd and grk RNA at the anterior nor the ectopic accumulation of bcd RNA at the posterior. In contrast, the third possibility, that PKA affects RNA localization by regulating the distribution of microtubules, is supported by several observations. Most important, we saw clear defects in microtubule distribution in PKA hypomorphs at the time when RNA localization and active microtubule reorganization were in progress. Second, the penetrance of microtubule and RNA localization defects in PKA hypomorphs were very similar. Finally, the aberrant localization of Kinesin:β-gal, which was designed to report the distribution of microtubules and has been found empirically to colocalize with osk RNA in a variety of mutant backgrounds [this paper, Clark et al. 1994], was consistent with the penetrance of both phenotypes and with a coherent explanation of how microtubule polarity prefigures RNA localization in the oocyte.

In the absence of either formal demonstrations of microtubule polarity or the immunolocalization of molecules known to be required for microtubule nucleation, it is not possible to establish indisputably the relationship between microtubule polarity and the anteroposterior axis of the oocyte. However, the implication of the observations by Theurkauf et al. (1992) is that the position of minus ends of microtubules shifts from posterior to anterior between stages 6 and 7, prior to the time when bcd and osk RNAs are first seen to acquire polarized distribution (Fig. 5). This would allow for the initial accumulation of RNAs in the oocyte by association with minus-end-directed motors. Subsequent localization of bcd to the anterior would also be facilitated by association with minus-end-directed motors, whereas localization of osk to the posterior of stage 8–9 oocytes would require plus-end-directed motors [Theurkauf et al. 1992]. The similarity between the distribution of Kinesin:β-gal and osk RNA in wild-type oocytes, as well as oocytes form N, Dl, capu, and spir mutant females, is highly suggestive of plus-end-directed transport of osk RNA [Clark et al. 1994].

On the basis of these models, the transient ectopic posterior localization of bcd RNA in PKA-deficient oocytes can be explained by the persistence of microtubule minus ends at the posterior of oocytes into stages 7 and 8 (Fig. 5). Posterior bcd RNA may be transient either if minus ends are lost from the posterior after stage 8 or if factors required to anchor bcd RNA to the cytoskeleton are absent from the posterior of the oocyte. Similarly, these microtubules may serve to direct osk and Kinesin:β-gal away from their minus ends at the posterior toward the center of the oocyte. Colocalization of stau protein with osk RNA has been observed in all mutant cases examined [St. Johnston et al. 1991; Clark et al. 1994] and would account for the persistence of ectopic osk RNA in PKA mutant oocytes at stages 9 and 10.

**Signal transduction and oocyte polarity**

Most genetic studies on the specification of the anteroposterior axis have led to the proposal of models for a germ-line autonomous mechanism for the generation of polarity, in contrast with the acknowledged role for communication between germ-line and somatic cells in the generation of dorsoventral polarity and in terminal specification [Stein and Stevens 1991]. However, Ruohola et al. (1991) showed that genes required for follicle cell specification can have an effect on anteroposterior axis formation and proposed a model whereby follicle cells situated adjacent to the oocyte posterior communicate information to the oocyte. Because N and Dl mutations affect the specification of posterior follicle cells, leading to hyperproliferation of cells expressing Fas III, it is possible that the observed phenotype reflects a neomorphic con-
dition, brought about by the generation of a signaling pathway that is never used in normal oogenesis. We have now observed a similar RNA localization phenotype resulting from mutations in the catalytic subunit of PKA that we have shown to be recessive loss-of-function alleles by biochemical and phenotypic assays (Lane and Kalderon 1993). Furthermore, we have shown that PKA activity is required in the germ line, whereas large follicle cell clones that are null for catalytic subunit have no effect on localization of Kinesin-β-gal [M.E. Lane, unpubl.]. These observations support the model of Ruohola et al. (1991) [see also Clark et al. 1994; Ruohola-Baker et al. 1994] and suggest a molecular mechanism for the generation of anteroposterior polarity in the oocyte.

A polarized microtubule cytoskeleton exists in egg chambers from stages 1–10a. However, the polarity of the microtubule cytoskeleton changes between stage 6 and 7. In early previtellogenic stages, the microtubule organization facilitates unidirectional transport of materials into the transcriptionally latent oocyte. In later stages as the oocyte grows by yolk uptake, cytoplasmic determinants must be distributed asymmetrically for the specification of the embryonic axes. We have presented evidence that the reorganization of microtubules between stages 6 and 7 is essential for localization of macromolecules at the posterior of the oocyte. We suggest that the redistribution of microtubules is governed by a signal, emanating from either the posterior polar follicle cells or their immediate neighbors, the flanking cells, which results in the activation of PKA, presumably via an elevation in cAMP concentration. In PKA hypomorphs, approximately half of stage 7–8 egg chambers showed abnormal persistence of microtubules at the posterior, ectopic bcd RNA at the posterior, and a failure to localize osk RNA or Kinesin-β-gal to the posterior. In contrast, null mutations of the PKA catalytic subunit, DCO, in the germ line prevented posterior localization of Kinesin-β-gal in all egg chambers, implying that PKA is essential for signal transduction and that the partially penetrant phenotypes of hypomorphs were attributable to residual PKA activity rather than the use of an alternative signal transduction pathway.

We are interested in determining the identity of targets of PKA phosphorylation in this process, as we believe that they will provide information about the control of microtubule dynamics. The microtubule-associated protein [MAP2] has been shown to be a major target of PKA phosphorylation in extracts from vertebrate brain tissue (Sloboda et al. 1975; Theurkauf and Vallee 1983), and type-II PKA has been found to be associated with MAP2 in these extracts [Theurkauf and Vallee 1982]. Association of MAP2 with microtubules can inhibit depolymerization of microtubules, and PKA phosphorylation of MAP2 can release this inhibition [Jameson and Caplow 1981]. A mechanism involving MAP2 or a MAP2-like factor for microtubule redistribution in the oocyte would be consistent with our observations. Alternatively, PKA targets might include centrosomal proteins that regulate the nucleation activity of the posterior MTOC. The type-II regulatory subunit of PKA localizes to centrosomes in cultured neuronal, glial, epithelial, and fibroblastic cells [Nigg et al. 1985; De Camilli et al. 1986]. In addition, a number of centrosomal antigens have been identified that show cell cycle-specific phosphorylation states, suggesting that changes in the nucleating activity of centrosomes are regulated by phosphorylation [Centonze and Borisy 1990; Ohta et al. 1993]. It is possible, therefore, that the activation of PKA in the oocyte is required to inhibit nucleation activity of the posterior MTOC during and after stage 6, and that the presence of active nucleation sites at the anterior results in net depolymerization of microtubules associated with the inactivated posterior MTOC. In this mode of action the effects of PKA could be spatially limited both by the local generation of a cAMP signal at sites of contact with posterior follicle cells and by the anchoring of PKA in the region of the MTOC by cytoskeleton-associated proteins that bind to the regulatory subunit of PKA.

Materials and methods

Drosophila stocks

For in situ hybridizations and tubulin immunofluorescence, ovaries from DCP3/Df(2L)Tw2; ry500 were analyzed. Sibling females from the same cross, which were either DCP3/Cyo or Df(2L)Tw2/Cyo, were pooled and served as internal controls. In all cases, control ovaries reproducibly showed wild-type patterns of RNA localization and microtubule distribution.

Flies bearing the khc: lacZ fusion gene on an enhancer trap transposon inserted on the third chromosome, designated KZ503, were provided by Ira Clark and described in Clark et al. (1994). DCP3/Df(2L)Tw2 females, which were heterozygous for KZ503, were analyzed for Kinesin-β-gal localization by antibody staining [described below], with siblings as controls.

Isolation of ovaries

Ovaries from 50–500 females were isolated and dissociated into individual egg chambers as described by Theurkauf et al. (1992). Alternatively, ovaries were dissected by hand in cold modified Robb’s medium.

In situ hybridization

Hybridization was performed essentially according to Tautz and Pfeifle (1989) with modifications suggested by Liz Gavis and Chris Rongo (Whitehead Institute, Cambridge, MA). Approximately 20 µl of ovaries [mass isolated, or dissected from 10 females] was fixed in 4% paraformaldehyde, 10% DMSO, and 10% bleach for 30 min with gentle rotation, followed by 5×5 min washes in PBS, 1% Tween 20 (PBT). Proteinase K conditions varied for each probe generally, best results were obtained with a 15-min treatment and proteinase concentration between 20–100 µg/ml in PBT when riboprobes were used [grk]. One-hour treatments with 100 µg/ml of proteinase K gave best results when DNA probes were used [bcd, osk]. Ovaries were rinsed twice and then washed 2×5 min in PBT. After postfixation for 20 min in 4% paraformaldehyde, ovaries were washed five times in PBT and stored frozen in 9:1 methanol/DMSO or hybridized according to Tautz and Pfeifle (1989). All subsequent steps were according to Tautz and Pfeifle [1989]. Ovaries were mounted in Aquapolymount [Polysciences]. Riboprobes were synthesized from cDNAs subcloned into
Bluescript Vectors using the Boehringer RNA labeling kit and instructions provided. Following the transcription reaction, probes were hydrolyzed in carbonate buffer (2×: 120 mM Na₂CO₃, 80 mM NaHCO₃ at pH 10.2) for 40 min at 65°C. Following addition of 100 μl of 0.2 M NaOAc (pH 6) to stop the reaction, the probe was precipitated by adding LiCl to 0.4 M, 20 μg of tRNA and 600 μl of ethanol. Following precipitation at −20°C, the pellet was resuspended in 150 μl of hybridization buffer (Tautz and Peifle 1989), and 0.5 μl of this stock was added to a 500-μl hybridization reaction. DNA probes were synthesized according to Tautz and Peifle (1989).

Antibody staining

Ovaries were either dissected or mass isolated. In general, ovaries were fixed in 4% paraformaldehyde for 30 min, washed in PBT, and blocked for 1 hr in PBSTT (PBS, 1% BSA, 0.1% Triton, 0.1% Tween). Primary antibodies were diluted in PBSTT and incubated overnight at 4°C. Rabbit anti-β-galactosidase antibody (Cappel) was diluted 1:5000.

Ovaries were washed 4× 15 min in PBT, and incubated for 2 hr at room temperature with fluorescein or Texas Red-conjugated secondary antibodies (Cappel), which were preabsorbed against fixed ovaries and diluted 1:500, for 2 hr at room temperature.

Ovaries for microtubule staining were fixed for 10 min in −20°C methanol, and washed and stained as above. A monoclonal antibody against α-tubulin (Sigma) was used at 1:500. The degree to which oocyte microtubules could be detected varied, depending on the degree of microtubule preservation and antibody penetration. Data were collected from preparations in which the degree of microtubule preservation allowed for the visualization of mitotic spindles in the gerarium and dividing follicle cells, and antibody penetration permitted readily visible microtubule staining at the oocyte anterior by conventional fluorescence microscopy. Confocal images were obtained from egg chambers between stages 7 and 8, when the oocyte nucleus was free of cytoplasmic microtubules.

Mosaic analysis

To induce germ-line clones, first-instar larvae (36-48 hr old) from the cross yw, DC0/Cyo; KZ503× P[ovoP1]/Cyo were γ-irradiated at a constant dose of 1000 rads. x indicates DC0 alleles B3 and H2, which are null alleles, and the parent non-mutagenized chromosome, S6 (Lane and Kalderon 1993), and P[ovoP1] indicates the insertion 13×13 described by Chou et al. [1993]. Chromosomes carrying DC0P1 and DC0P2 are free of other lethals, as both can be rescued by a DC0 genomic transposon (Lane and Kalderon 1993; M.E. Lane and D. Kalderon, unpubl.). Adult females of the genotype DC0P1/P[ovoP1] were collected, crossed with males, and kept in vials with wet yeast for 3–5 days. Ovaries from 100–200 females of each genotype were dissected. Clones of germ-line cells homzygous for S6 or DC0 mutations could be identified easily by the presence of ovarioles with vitellogenic egg chambers, which are not produced by nonrecombinant females (Chou et al. 1993). Ovaries were stained with rhodamine isothiocyanate (RITC) phallolidin and anti-β-galactosidase antibodies as described above.

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