hold up Is Required for Establishment of Oocyte Positioning, Follicle Cell Fate and Egg Polarity and Cooperates with Egfr during Drosophila Oogenesis

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ABSTRACT

In Drosophila the posterior positioning of the oocyte within the germline cluster defines the initial asymmetry during oogenesis. From this early event, specification of both body axes is controlled through reciprocal signaling between germline and soma. Here it is shown that the mutation hold up (hup) affects oocyte positioning in the egg chamber, follicle cell fate and localization of different markers in the growing oocytes. This occurs not only in dicephalic egg chambers, but also in oocytes normally located at the posterior. Generation of mosaic egg chambers indicates that hup has to be at least somatically required. Possible interactions of hup with Egfr, the Drosophila epidermal growth factor receptor homolog, have been investigated in homozygous double mutants constructed by recombination. Stronger new ovarian phenotypes have been obtained, the most striking being accumulation of follicle cells in multiple layers posteriorly to the oocyte. It is proposed that the hup gene product is a component of the molecular machinery that leads to the establishment of polarity both in follicle cell layer and oocyte, acting in the same or in a parallel pathway of Egfr.

A NTERIOR-POSTERIOR (A/P) and dorsal-ventral (D/V) axis formation of the Drosophila embryo is established during oogenesis and requires reciprocal cell signaling between germline, oocyte and nurse cells and somatic follicle cells of the egg chamber (reviewed in Spradling 1993). The first visible asymmetry during the egg chamber development is the positioning of the oocyte posteriorly in the cyst, and it has been recently proposed that this early event plays a key role in the establishment of both major body axes (Gonzalez-Reyes et al. 1995; Roth et al. 1995; Ray and Schüpbach 1996). Mutations affecting oocyte determination and positioning within the cyst have been identified (Lohs-Schardin 1982; Peifer et al. 1993; Gonzalez-Reyes and St Johnston 1994). Most of the corresponding genes, whose function has been demonstrated to be required in the germline, have not yet been isolated, with the exception of homeless (Gillespie and Berg 1995).

The posterior localization of the oocyte relative to the nurse cells in the egg chamber allows a reciprocal cross-talk between oocyte and the polar follicle cells. The key signaling pathway is composed of a ligand, the transforming growth factor (TGFα) homologue Gurken (Grk), present in the oocyte, and a receptor, the Drosophila epidermal growth factor (EGF) receptor homolog (Egfr) (Price et al. 1989; Schejter and Shilo 1989; Neuman-Silberberg and Schüpbach 1993), expressed by somatic follicle cells. The interaction between these two central components, with a number of other factors that regulate or amplify this ligand/receptor activity (Lane and Kalderon 1994; Roth et al. 1995; Goode et al. 1996a,b) leads the adjacent follicle cells to adopt a posterior fate.

Signaling from the follicle cells back to the oocyte induces a polarization of the cortical cytoskeleton leading to the formation of a microtubule network with the plus end directed toward the posterior pole. This orientation of the microtubule network is involved in the intracellular transport of RNA and protein molecules (Theurkauf et al. 1993; Cooley and Theurkauf 1994; Knowles and Cooley 1994; Lehmann 1995). A possible model explaining the asymmetric localization of proteins and mRNAs is that a plus-end-directed microtubule motor could be responsible for posterior localization of oskar RNA, while association with a minus-end-directed microtubule motor protein could be responsible for localization of bicoid RNA to the anterior cortical ring, even if this model has not been proven and the specific motor proteins have not yet been identified. A fusion protein composed of the β-galactosidase joined to part of the plus-end-directed microtubule motor kinesin accumulates at the posterior pole of stage 8 and 9 oocytes (Clark et al. 1994) and is used as a marker for the microtubule arrangement in the oocyte. As a consequence of the microtubule reorganization induced by the somatic signaling to the oocyte, the asymmetric movement of grk mRNA and the oocyte nucleus defines...
the future dorsal-anterior corner of the oocyte (Neuman-Silberberg and Schüpbach 1993; Roth et al. 1995)

The observation that Grk-Egfr signaling mediates both A/P and D/V patterning raises the question of how different two responses are produced by the same molecules. This seems to involve the Notch-Delta pathway that restricts the competence of follicle cells to respond to this signaling (Ruohola et al. 1991; Ruohola-Baker et al. 1994; Ray and Schüpbach 1996). It is known that the polar follicle cells appear at early stages as a restricted group within the follicle epithelium that is competent to respond to the Grk/Egfr signal. The polar or terminal follicle cells are therefore considered different from the main body follicle cells and behave as two equivalent groups that, only after the oocyte positioning posteriorly in the egg chambers, acquire different fates.

Despite the intensive investigation and the exciting recent findings described above, the somatic factors involved in the early events during oogenesis are as yet unknown, and the molecular mechanisms underlying the regulation of posterior positioning of the oocyte in the germline cluster remain to be elucidated. In addition to our previous studies on Drosophila oogenesis (Gargiulo et al. 1991; Gigliotti et al. 1993; Malva et al. 1994), we report in this paper the effects produced by the female semisterile mutation hold up (hup), isolated by L. Sandler (1977), on oocyte positioning, determination of follicle cell fate and establishment of both major body axes. The described hup and hup Egfr homozygous double mutants phenotypes indicate that hup is an essential component of the molecular machinery that leads to the establishment of polarity both in follicle cell layer and oocyte and cooperates with Egfr in assuring the monolayer integrity of follicle cells.

MATERIALS AND METHODS

Fly strains: Drosophila melanogaster stocks were maintained on standard medium at 18°C. Wild-type flies were Oregon R and Canton S. hup1/ CyO were obtained from L. Sandler (Department of Genetics, University of Washington, Seattle). hup2 was isolated in our laboratory during an EMS mutagenesis on the multiple marked chromosome dp cl b cn sp and was maintained as dp cl hup b cn sp/ Cy stock. For the analysis of the ovarian phenotypes, the stocks were raised at 25°C. The ovarian phenotype of hup2 is very similar to that described in this paper for hup1. The stocks T(2;4)DTD38 Cy and Df(2L)J2/ CyO, T(2;4)DTD38 (DTD38) does not complement hup and behaves as a stronger allele, while T(2;4)DTD38 Cy flies behave as wild type. The same translocation, when placed in trans to other mutations that map in the region interested by the rearrangement, such as grk, gives a wild-type phenotype. Df(2L)J2 does not complement the hup sterility (Sinclair et al. 1992) and increases the severity of the ovarian phenotype. Recombinant hup1 Egfr topCl (topCl) chromosome was obtained by crossing hup1/ cn Egfr topCl bw females to hup1/ cn Egfr topCl bw males. Homozygous bw males were selected and singly crossed with Inv(2LR)SM 6a2 Cy dp cn27 sp2/ Sco females. Phenotypically

on Cy females and males from the same vial were crossed and the bx progeny were scored for the presence of the hup and topCl phenotype. Three independent recombinant lines were established and further analyzed.

For complete description of the stocks, see Lindsay and Zimm (1992).

Generation of hup homozygous germline clones: Germline clones were generated as described by Chou et al. (1993) by crossing P(hsFLP)/P(hsFLP);hup1/ P(ryHsp70:neFRT40A)/ CyO females (derived from three independent recombinants) to P(w+12)FLRT/CyO males. The progeny of these crosses was heat shocked every day for 1 hr at 37°C after the onset of the second instar larval stage. Females of genotype P(hsFLP)/+; hup1FLRT/ovoD2 2FLRT were crossed to hup1/CyO males and, after scoring the vials for the presence of progeny, were dissected for ovarian phenotype classification.

Cuticle preparation: The procedure was essentially as described by Schüpbach and Wieschaus (1986). The eggs were mounted in Hoyer’s lactic acid (1/1) and heated to 65°C to clear.

Immunohistochemistry: For 4’,6-diamidino-2-phenylindole (DAPI) staining, fixed ovaries were incubated for 5 min with DAPI (1 μg/ml), washed in phosphate-buffered saline (PBS) and mounted in 50% glycerol/PBS. For the detection of β-galactosidase activity, ovaries were fixed in 0.5% glutaraldehyde and stained as described (Bellen et al. 1989).

Double-staining ovaries, fixed as described in Gillespie and Berg (1995), were incubated with anti-α-tubulin antibody and fluorescein-conjugated secondary antibody. Nuclei were visualized with propidium iodide or DAPI. All tissues were analyzed with conventional epifluorescence or with Zeiss (Thornwood, NY) laser confocal microscope attached to a Zeiss Axiofot microscope. The images were processed in Photoshop 3.05 (Adobe Systems).

Whole-mount in situ hybridization: This procedure was performed according to Tautz and Pfeifle (1989), with minor modifications. Ovaries were dissected into ovarioles before staining and mounted in 50% glycerol/PBS or dehydrated through ethanol series and xylene and mounted in Permount. DNA probes were labeled with digoxigenin (Boehringer Mannheim; Indianapolis).

RESULTS

Mutation in hup affects oocyte localization and egg-shell appendage formation: We investigated the ovarian phenotype induced by the hup mutation. We also used hup1-bearing chromosomes placed in trans to the translocation T(2;4)DTD38 (DTD38), which does not complement the hup phenotype and behaves as a stronger allele. In addition, the deficiency Df(2L)J2 does not complement the hup sterility (Sinclair et al. 1992) and Df(2L)J2/ hup1 or hup2 females also show an increase in severity of the ovarian defects, with respect to those observed in hup1/ hup1 and hup2/ hup2.

The phenotype of eggs laid by hup mothers, either hup1/ hup1 or hup1/ DTD38, is variable (Figure 1), and dorsallized or ventralized eggs are observed; 100% show abnormal dorsal appendages, 83% shorter, 14% fused, 3% absent appendages. In dissected hup1/ DTD38 mutant ovaries, the oocyte is displaced from its posterior localization in 15.5% of the egg chambers (n = 2177), showing a lateral (5%), central (9.5%; Figure 2, A and B) or anterior (1%) localization in the egg chamber. In
10% S14 egg chambers (n = 202) from dissected ovaries, a micropyle is present at each pole (Figure 2C). Dicephalic egg chambers or eggs with two micropiles were never observed in hup1/hup1 ovaries, while oocytes with an anterior localization were occasionally detected. From now on all the phenotypes reported for mutant flies will be derived from flies of the genotype hup/DTD38, unless differently specified.

**hup is involved in induction of posterior follicle cell fate:** In wild-type egg chambers, 6–10 follicle cells (border cells) migrate from the anterior tip between the nurse cells to reach the anterior of the oocyte. These cells, that collaborate with the anterior follicle cells to produce the micropyle, express the slow border cell gene (Montell et al. 1992) and are also marked by β-galactosidase expression in a line obtained in our laboratory and called alfa15 (Figure 3A). This line was used, with the appropriate crosses, to investigate the follicle cell fate in a hup background. In mutant egg chambers, alfa15-positive cells are also present at the posterior end of the egg chambers, and this occurs not only in all dicephalic egg chambers (Figure 3C), but also in 47% of egg chambers (n = 102) with the oocyte correctly positioned (Figure 3B). In this last case, only a subset of the polar follicle cells at the posterior seems to adopt an anterior fate, and this indicates that within the polar follicle cells there are fine differentiative distinctions that have not yet been elucidated.

**hup is at least somatically required for proper oogenesis:** Taken together, these observations suggest that the hup function is required not only for the correct positioning of the oocyte at the posterior end of the germ-line cluster, but it is also necessary for the proper determination of the somatic follicle cells in response to oocyte signal(s). Germ cell hup1 homozygous clones were produced by the FLP-FRT system (Chou et al. 1993) through induced mitotic recombination in developing females trans-heterozygous for the hup1 mutation and the dominant female sterile mutation ovoD1. Adult females bearing hup mutant germline clones, crossed to heterozygous hup1/Cy males, produced hup1 and Cy progeny. This result appears to indicate that homozygosity in the germline does not cause a mutant phenotype, but allows the production of normal eggs and offspring. However, since the hup1 allele used in these experiments does not behave as a null allele, the observed fertility could be the result of escapers. We therefore dissected and carefully scored ovoD1/hup1 ovaries for the ovarian phenotype after induction of mitotic recombination. Out of 219 ovaries, 190 contained fully wild-type egg chambers, 26 were fully ovoD1 and only three contained some egg chambers with hup ovarian characteristics, such as oocyte misplacement and alterations of dorsal appendages. The presence of this last class could be explained by the contemporary occurrence of recombination events both in germline and soma, according to previous observations (Margolis and Spradling 1995). These results indicate that hup must be at least somatically required, because ho-

**Figure 1.—D/V defects in hup eggs.** (A) Wild-type eggshell showing the characteristic dorsal appendages. (B–C) hup1 homozygous S14 egg chambers with shorter appendages. (D–F) Stronger phenotypes of eggshell produced by heterozygous hup1/DTD38 females. In (F), a moderate ventralized egg is shown and the forked appendages arise from a single base on the midline.

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**Figure 2.—hup induces oocyte mislocalization and A/P defects.** Surface view of a dicephalic egg chamber produced by hup/DTD38 females in bright field (A) or stained with the DNA dye DAPI (B). Large nuclei correspond to polyploid nurse cells and the smallest ones to the follicle cells surrounding the oocyte localized in the center between the nurse cells. In (C), a mature mutant egg with a second micropyle at the posterior instead of the normal aeropyle is shown.
Mozygosity for hup only in the germline does not confer any hup phenotypic characteristic to the egg chambers.

**hup is required for oocyte polarity and microtubule organization:** To investigate whether the defect in the A/P follicle cell asymmetry, observed in hup egg chambers, produces any consequence on the establishment of oocyte A/P polarity and microtubule integrity, the distribution of oskar, bicoid and grk mRNAs and the kinesin-β-galactosidase activity (Clark et al. 1994) were examined in hup ovaries. This analysis was performed in

**Figure 3.**—The induction of posterior follicle cell fate requires hup. X-Gal staining of the line alfa15 in wild-type or hup background, showing alfa15-positive follicle cells. (A) In the wild type the positive border cells migrate from the anterior tip of the egg chamber to reach the anterior margin of the oocyte. In hup mutant egg chambers, a second set of alfa15-positive follicle cells develops at the posterior either in apparently normal (B) or in bipolar (C) egg chambers.

**Figure 4.**—hup function is required for polarization of the A/P axis of the oocyte. The bicoid mRNA is localized at the anterior margin of wild-type oocyte (a); in the mutated bipolar egg chamber, bicoid mRNA accumulates at the two poles of the oocyte (b). When the oocyte is normally localized, bicoid mRNA is at the proper anterior position and only in 1% of these egg chambers (c) heaps all around at the oocyte periphery, grk mRNA in wild type (d) is linked to the oocyte nucleus at the dorsal-anterior corner. In bipolar hup egg chambers (e), grk mRNA localization depends on the position of the oocyte nucleus. In the mutant egg chambers with the oocyte normally located posteriorly, grk mRNA accumulates with broad cortical localization (f). The normal posterior localization of oskar mRNA in a wild-type egg chamber is shown (g). Both dicephalic and hup egg chambers with the oocyte normally located (h and i) show oskar mRNA in the center. Kinesin-β-galactosidase fusion protein in wild-type S9 egg chambers is localized posteriorly (l), while in hup egg chambers (m and n) it is localized in the center of the oocytes.
bipolar egg chambers as well as in those where the oocyte is normally located at the posterior (Figure 4).

In dicephalic egg chambers, bicoid mRNA accumulates at both poles of the oocyte (Figure 4b) rather than at anterior, as in wild type (Figure 4a), whereas oskar mRNA localizes to the center of the oocyte (Figure 4h) instead of at the posterior pole (Figure 4g). grk mRNA localization follows the position of the oocyte nucleus (Figure 4, d and e). In wild-type S9 egg chambers, the kinesin-β-galactosidase activity is detected at the posterior pole (Figure 4i), while it is localized at the center of hup bipolar egg chambers (Figure 4m). The simplest explanation for all these data is that in dicephalic egg chambers the oocyte is not in contact with the polar follicle cells but surrounded by nurse cells, and therefore the cross-talk between follicle cells and oocyte is affected. As a consequence, the polar follicle cells are unable to acquire a posterior fate and to signal back to the oocyte where, in turn, a mirror symmetric arrangement of microtubule network is formed.

The analysis of mutant egg chambers with the oocyte normally positioned posteriorly (Figure 4, c, f, i and n), indicates that defects appear also when the oocyte normally contacts the posterior follicle cells. bicoid mRNA is at the proper anterior position, although showing a punctuated aspect, and very rarely (less than 1%) is located also posteriorly (Figure 4c). grk transcript was found appropriately localized in all hup early egg chambers and in the majority of egg chambers at later stages, presumably reflecting the correct positioning of the oocyte nucleus, while in 18% of these egg chambers, distribution and/or level at the anterior corner was affected to a different extent (Figure 4f). These slightly lower levels of grk message or its mislocalization could be responsible for the most evident D/V defects observed in mature hup eggs and could be an effect of disrupted arrangement of microtubule network. In 40% of egg chambers, oskar mRNA mislocalization is observed (Figure 4i), while in the remaining 60% oskar mRNA is not tightly linked at the posterior pole. At a high percentage (85%) kinesin-β-galactosidase activity is detected in the center of the oocyte (Figure 4n).

**hup cooperates with Egfr in specification of monolayer follicle integrity:** To investigate the possible interaction of hup with Egfr in the follicle cells to specify their fate and/or maintain their epithelial characteristics, we performed recombination experiments using the topCj allele of Egfr and isolated recombinant hup1 topCj lines. The analysis of the mutant egg chambers derived from double homozygous females (Figure 5) from three independent lines revealed phenotypes different from those observed in the single homozygous hup1 or hup2 (this paper) or topCj (Roth et al. 1995); 16% of clear dicephalic egg chambers (n = 145) were observed, and an additional 12.5% of egg chambers, either dicephalic or with the oocyte normally localized, showed degenerated nurse cells. These phenotypes were not observed in homozygous hup or topCj homozygous female ovaries and dicephalic egg chambers were present only in hup/DTD38 at a lower percentage (9.5%). The most striking phenotype was the presence of multiple layers of follicle cells at the posterior in 10% of egg chambers, from stage 9 to stage 12 (Figure 5, b, d, e and g). In some cases, these follicle cells try to migrate centripetally at
the posterior splitting the cytoplasm of the oocyte (Figure 5g), in a more dramatic way than that described for some grk alleles (Gonzalez-Reyes and St Johnston 1994); 25% of S14 retained eggs with two micropiles at the two ends were also observed (n = 51), present at 11% in topC/Df(2R)top18A (Roth et al. 1995) and at 10% in hup/ DTD38.

DISCUSSION

In this paper we analyzed the involvement of hup in egg chamber development. A requirement for the hup product seems essential in germlarium for the correct positioning of the oocyte at the posterior of the cyst and for the specification of posterior follicle cells. The early defects resemble those observed in didýchallic (Lohs-Schardin 1982), spindleC (Gonzalez-Reyes and St Johnston 1994) and homeless (Gillespie and Berg 1995) mutants. In spindleC mutants, like in hup, didýchallic egg chambers are observed in which the change in follicle cell fate generates a mirror image with an anterior structure, the micropyle, produced at both egg poles. The difference in the phenotypes induced by these two mutations is the higher penetrance of hup in cyts where the oocyte lies normally at the posterior. In hup ovaries, egg chambers with normal oocyte localization are nevertheless abnormal because an anterior follicle cell marker appears also at the posterior. In homeless egg chambers, displacements of oocyte in the cyst and of molecular markers in the oocyte have been reported, but posterior follicle cell fate determination seems unaffected. The mosaic experiments suggest that hup is required at least in the soma, while spindleC and homeless work in the germline.

Because the hup alleles are hypomorphic, some mutant cysts are able to surmount the first defect in the oocyte-follicle cell communication, but later, even in presence of the correct signal(s) from the oocyte, the follicle cells are unable to respond to the Grk/ Egfr signaling for the acquisition of their posterior fate. Thus, specification of the A/P polarity in the follicle cell layer appears to require the hup function. Microtubule disruption and the subsequent defects in the transport and localization of selected mRNA observed in hup background can be a consequence of the failure of polar follicle cells to acquire their posterior fate, in agreement with the hypothesis that this initial step determines all the subsequent events in the establishment of A/P and D/V asymmetries (Gonzalez-Reyes et al. 1995; Ray and Schüpbach 1996).

The observation of stronger phenotypes in the double hup Egfr mutant seems to indicate that hup and Egfr somehow cooperate for A/P and D/V patterning, mediating follicular morphogenesis, and that they can be involved in the same pathways. Another possibility is that, besides Grk/Egfr, other parallel, but partially overlapping pathways exist, as suggested by the recent results with brainiac and egghead (Goode et al. 1996b), and hup could participate in this novel signaling regulating germline-follicle cell adhesion and migration. Our current analysis does not allow us to unravel the hierarchical relationships among hup, Egfr and/ or brainiac signaling, but opens new questions that will be elucidated by interaction studies between these genes and those working downstream in the signaling pathways.

Only very recently the studies on the establishment of A/P and D/V axes have got back to the point in which the oocyte is originally defined and positioned. The role of follicle cells in directing the posterior positioning of the oocyte is unknown, but at some level, cellular communications within follicle cells and between follicle cells and oocyte should be required. For example, a signal from posterior polar follicle cells to the oocyte could be involved to direct this movement. However, this would imply that the posterior follicle cells are already different from the anterior polar follicle cells, before the Grk/ Egfr signaling. On the other hand, it seems difficult to explain this signaling only by a ligand/receptor interaction because, in the formation of the germline cluster, it is reported that the oocyte lies in the center and is not immediately in contact with the follicle cells. Different types of molecular cues and/or different components in the nurse cells intercalating between the oocyte and the posterior polar follicle cells could therefore be involved in this signaling. Neither spindleC and didýchallic, which affect positioning of the oocyte, nor the hup component reported here has yet been isolated. Their molecular characterization may provide new leads for the elucidation of the very early events that ensure the asymmetry of egg and embryo.

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