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The tumor suppressor gene, *lethal(2)giant larvae (l(2)gl)*, is required for cell shape change of epithelial cells during *Drosophila* development

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SUMMARY

Inactivation of the *lethal(2)giant larvae (l(2)gl)* gene results in malignant transformation of imaginal disc cells and neuroblasts of the larval brain in *Drosophila*. Subcellular localization of the *l(2)gl* gene product, P127, and its biochemical characterization have indicated that it participates in the formation of the cytoskeletal network. In this paper, genetic and phenotypic analyses of a temperature-sensitive mutation *(l(2)gl^{ts3})* that behaves as a hypomorphic allele at restrictive temperature are presented. In experimentally overaged larvae obtained by using mutants in the production of ecdysone, the *l(2)gl^{ts3}* mutation displays a tumorous potential.

This temperature-sensitive allele of the *l(2)gl* gene has been used to describe the primary function of the gene before tumor progression. A reduced contribution of both maternal and zygotic activities in *l(2)gl^{ts3}* homozygous mutant embryos blocks embryogenesis at the end of germband retraction. The mutant embryos are consequently affected in dorsal closure and head involution and show a hypertrophy of the midgut. These phenotypes are accompanied by an arrest of the cell shape changes normally occurring in lateral epidermis and in epithelial midgut cells. *l(2)gl* activity is also necessary for larval life and the critical period falls within the third instar larval stage. Finally, *l(2)gl* activity is required during oogenesis and mutations in the gene disorganize egg chambers and cause abnormalities in the shape of follicle cells, which are eventually internalized within the egg chamber. These results together with the tumoral phenotype of epithelial imaginal disc cells strongly suggest that the *l(2)gl* product is required in vivo in different types of epithelial cells to control their shape during development.

Key words: *Drosophila*, tumor suppressor gene, cell shape, cytoskeleton

INTRODUCTION

A mutant animal deficient for the gene *l(2)gl* develops tumors that appear at the end of the third instar larval stage (Gateff, 1978). These tumors affect the optic lobes in the larval brain and the imaginal discs. The brain tumors comprise undifferentiated neuroblasts and ganglion mother cells while the tumors of the imaginal discs consist of amorphic masses of cells, which have lost their characteristic cell polarity and are unable to differentiate (Gateff and Schneiderman, 1969, 1974). *l(2)gl* and *lethal(1)disc large (dlg)* are, among the characterized genes, the only two that lead to a neoplastic overgrowth of the discs while all the other tumor suppressor genes are classified as hyperplastic in their action (Bryant and Schmidt, 1990). Transplantation experiments of *l(2)gl* mutant brains or imaginal tissues into abdomens of adult hosts have demonstrated that *l(2)gl* tumoral cells have a metastatic behaviour resulting in a massive overgrowth of the implant and subsequent invasion of the tissues, which finally kill the hosts (Gateff and Schneiderman, 1969). In addition, although *l(2)gl* is expressed very early in embryogenesis, tumors arise in homozygous mutants much later in development, during the end of larval life, and the observed phenotype might be the consequence of a series of complex processes following an early primary event, as is generally assumed for vertebrates (Fearon and Vogelstein, 1990; Marshall, 1991). These observations suggest that a detailed study of the *l(2)gl* mutation could provide a simplified model system to approach the understanding of tumorous growth and metastasis.

The *l(2)gl* gene encodes a polypeptide of 127×10^3 M_r and 1,161 amino-acids (P127) whose sequence shows no similarity to any other known protein (Jacob et al., 1987) and, therefore, gives no clues as to the eventual function of this protein. However, a search for structural repeats has revealed the presence of different types of potential motifs for protein-protein interaction that are conserved in *Drosophila* and diptera species; one of these shares partial similarities with a sequence found in β subunits of G proteins (Török et al., 1993). This observation has prompted Török et al. (1993) to postulate that P127 could interact with other proteins and/or with itself to

form a multimeric protein complex. Subcellular localization of P127 on the inner plasma membrane at sites of cell-cell contact, biochemical and cell fractionation experiments are all consistent with the hypothesis that the protein participates in a cytoskeletal network (Strand et al., 1994a). Among the proteins that coprecipitate in a complex with immuno-purified P127 or that can be isolated by virtue of their affinity for P127, Strand et al. (1994b) have identified nonmuscle myosin heavy chain. These data allow speculation that, in the absence of P127, the overgrowth phenotype could result from a partial disruption of the cytoskeletal network leading to a loosening of cell-cell adhesion or to a gap in the signal transduction pathway.

A murine homolog of *l(2)gl*, *mlg-1*, has been identified as a target for the homeotic gene product Hox-C8 (Tomostune et al., 1993) and, more recently, a human homolog has also been isolated (Strand et al., 1995). These proteins probably define a new family of cytoskeleton-associated proteins.

Recent observations have established a correlation between tumorigenesis and malfunctioning of cytoskeleton-associated proteins, such as cadherins (Vleminckx et al., 1991; Oda et al., 1994; Mahoney et al., 1991), integrins (Giancotti and Ruoslahti, 1990; Fan Qian et al., 1994), DCC (Fearon and Vogelstein, 1990), vinculin (Rodriguez Fernandez et al., 1992), α-actinin (Gluck et al., 1992) and tropomyosin (Prasad et al., 1993), some of them being transmembrane adhesion molecules, others being localized in the cytoplasm. In addition, the existence of a transduction pathway coupling cytoskeletal architecture at sites of cell junctions and control of cell proliferation has been suggested (Lo et al., 1994). Further correlation is possible between the human tumor suppressor gene of neurofibromatosis type II, which is present at junctional sites and can bind to actin and thus stabilize the actin network (NF2; Trofatter et al., 1993; Rouleau et al., 1993), and its *Drosophila* homolog, the gene *expanded* (Boedigheimer and Laughon, 1993; Boedigheimer et al., 1993). Disruption of the membrane cytoskeleton due to the inactivation of the gene NF2 could therefore promote tumor growth (Algrain et al., 1993). Additionally, the adenomatous polyposis coli (APC) protein, which has been implicated in genetic susceptibility to adenomatous polyposis (Powell et al., 1992), has a subcellular localization similar to that of the NF2 product and interacts directly with β catenin and perhaps with α catenin (Rubinfeld et al., 1993; Su et al., 1993).

A description of *l(2)gl* function during early development could bring some important information on the cellular process in which the gene is involved. The first point to elucidate is the absence of a clear *l(2)gl* function during embryogenesis, although it is abundantly expressed throughout this period (Mechler et al., 1985). Embryogenesis of a *l(2)gl* amorphic homozygous mutant proceeds apparently normally and the animal dies as a late larva. On the contrary, it has been suggested that establishment of the tumoral potential takes place during the early hours of development (Merz et al., 1990). To reconcile these two observations, one can propose that the activity of the *l(2)gl* product deposited by the heterozygous mother in the egg is sufficient to allow a normal development of the embryo. This hypothesis, however, could not be confirmed by studying germ-line clones for *l(2)gl*, since *l(2)gl* function is also required during oogenesis and no eggs were collected from mosaic females (Szabad et al., 1991).

To address these different questions, the defects induced by temperature-sensitive mutations of *l(2)gl* (Hanratty, 1984a,b) have been examined during development. In this paper, it is shown that *l(2)gl* activity is required during embryogenesis and that its inactivation leads to an embryonic phenotype. Phenotypic analyses reveal that the absence of *l(2)gl* activity produces abnormalities in cell shape changes of epithelial cells from different origins.

MATERIALS AND METHODS

Drosophila strains

Fly stocks were raised at all temperatures on standard media. Wild-type embryos and larvae were from the Oregon R strain.Three *l(2)gl* temperature-sensitive alleles have been identified and two of them described by Hanratty (1984a,b). For null *l(2)gl* alleles, two deficiency strains were used: *l(2)gl*^{4}*/Cyo* (Mechler et al., 1985) and *Dfnet 62/Cyo* (Korochkina and Golubovsky, 1978). To carry out rescue experiments, a fragment of the genomic region that includes the entire *D. pseudoobscura l(2)gl* gene (Török et al., 1993) was used. For phenotypic studies, mutant stocks were balanced over *Cyo* marked with a P(y^{+}) (obtained from Dr A. Shearn) in a y^{-} background, resulting in the absence of *y* expression in the *nonCyo* progenies. Since in the case of the temperarure-sensitive allele, *ecd*^{1}, temperature shifts performed during embryogenesis led to embryonic lethality, *ecd*^{1} and the double mutant were reared at restrictive temperature only during larval stages.

Cleaning of the initial chromosome bearing the temperature-sensitive allele *l(2)gl*^{ts3} *dp*^{ovn} *or* has been carried out by recombination with a *net cn sp* homozygous viable chromosome. Three *l(2)gl*^{ts3} *cn sp* independent chromosomes were recovered and tested for their ability to be rescued by the *l(2)gl* gene from *D. pseudoobscura*. One of these cleaned chromosomes has been used in all the studies described herein.

In the case of the *l(2)gl*^{ts1} and *l(2)gl*^{ts2} temperature-sensitive alleles, the chromosomes have been cleaned by recombination with a *al dp b cn sp* chromosome. The recovered *l(2)gl*^{ts} *dp b cn sp* chromosomes showed an authentic temperature-sensitive behaviour and were totally rescued by introduction of the *l(2)gl* gene from *D. pseudoobscura.*

To establish lines of transgenic flies expressing the *l(2)gl* cDNA under control of the heat-shock promoter, the *l(2)gl* cDNA (from Professor B. Mechler) was introduced into the pHS-Casper plasmid vector and coinjected with helper plasmid pUChsπΔ2-3 into preblastoderm wild-type embryos (Rubin and Spradling, 1982). Ectopic overexpression of the *l(2)gl* transcript and protein was checked by northern blot, western blot, in situ hybridization and immunohistocytochemistry analyses. A similar procedure was used to establish lines of transgenic flies expressing the *l(2)gl* cDNA under control of the UAS (Upstream Activating Sequence) element promoter (Brand and Perrimon, 1993).

In situ hybridization

l(2)gl cDNA probes were labelled according to the protocol 'DIG cDNA Labeling Mixture' (Boehringer, Mannheim). Embryos were treated for hybridization as described by Tautz and Pfeifle (1989). The fragment used for *in situ* hybridization was the *l(2)gl* cDNA (from Professor B. Mechler).

Immunohistochemistry and other staining procedures

Polyclonal anti-P127 antibodies were provided by B. Mechler and D. Strand and described in Strand et al. (1994a). The various antibodies utilized in this work were diluted as follows: anti-P127 (1/200), FASIII (1/500) (from Dr C. Goodman), anti-α-Spectrin (1/1000) (from Dr D. Kiehart).

Embryos were dechorionated in 50% chlorox and fixed in 4% formaldehyde in a 0.1 M Hepes Buffer (pH 7.0) containing 1 mM EGTA, 2 mM $MgSO_4$ and 50% (v/v) heptane. Subsequent immunohistochemistry was performed essentially as previously described (Ashburner, 1989). In the case of anti-FASIII primary antibody, the activity of the secondary antibody was revealed by using a Vectastain

ABC kit (Vector Laboratories, CA). In the case of anti-P127 and α-spectrin primary antibodies, the secondary antibodies were coupled to a fluorescent FITC marker. The DNA nuclear structure in ovaries was revealed by staining with DAPI or Hoechst.

Temperature-shift experiments

Temperature shifts to define the critical period for *l(2)gl* activity during both embryogenesis and larval life have been carried out between 22°C and 29°C, which are the permissive and restrictive temperatures, respectively. The effects of temperature shifts on embryogenesis were measured by an estimation of embryonic lethality relative to viability of embryos of the same cross maintained at 22°C (see Table 1 for the viability of the different stocks). In larval life, the effects of temperature shifts were correlated to the number of hatching larvae. In both experiments, embryos or larvae were reared at a given temperature, shifted at different moments to an other temperature and maintained at that latter temperature throughout development.

Cuticle preparation and semi-thin sections

Eggs were collected for 6 hours periods from $l(2)gl^{ts3}$ homozygous or hemizygous females and $l(2)gl^{ts3}$ homozygous males and allowed to develop for 24 hours at 29°C. The embryos thus obtained were dechorionated, devitellinized in a heptane/methanol solution (v/v) and mounted in Hoyer's medium to examine the cuticle phenotype. Controls were eggs from wild-type flies prepared under the same conditions.

Semithin sections of ovaries were processed as described in Kerridge and Thomas-Cavallin (1988).

RESULTS

Temperature-sensitive alleles of *l(2)gl*

Temperature-sensitive alleles used in this work have been isolated in a screen previously described by Hanratty (1984a). Two of them, $l(2)gl^{ts1}$ and $l(2)gl^{ts2}$, have been extensively characterized (Hanratty, 1984a,b) while the properties of the third one, $l(2)gl^{ts3}$, have not yet been described.

As shown in Table 1, the three alleles, in *trans* of a deficiency for the *l(2)gl* gene ($l(2)gl^4$ and $Dfnet^{62}$), produced no progeny at 29°C. At 22°C, a substantial number of *trans*-heterozygous adults were recovered and, in the case of $l(2)gl^{ts3}$, this number corresponded to the number expected for a fully viable conditional allele. A transposon carrying the entire *l(2)gl* gene from *Drosophila pseudoobscura* (Török et al., 1993) was able to rescue the lethality at 29°C of the three temperature-sensitive alleles.

Table 1. Temperature sensitivity of the *l(2)gl* alleles

Crosses Female×Male	Temperature °C	*nonCyO* Progeny (% of expected survival)	
$l(2)gl^{ts1}/CyO \times l(2)gl^4/CyO$	22	19.1	sterile
	27	0	–
	29	0	–
$l(2)gl^{ts2}/CyO \times l(2)gl^4/CyO$	22	34.2	sterile
	27	0	–
	29	0	–
$l(2)gl^{ts3}/CyO \times l(2)gl^4/CyO$	22	108	fertile
	27	91	male sterile
	29	<9	sterile
$l(2)gl^{ts1}/CyO \times l(2)gl^{ts1}/CyO$	22	34.2	sterile
	27	0	–
	29	0	–
$l(2)gl^{ts2}/CyO \times l(2)gl^{ts2}/CyO$	22	47.3	sterile
	27	0	–
	29	0	–
$l(2)gl^{ts3}/CyO \times l(2)gl^{ts3}/CyO$	22	100	fertile
	27	81.8	male sterile
	29	15.1	sterile
$l(2)gl^{ts1}/CyO \times l(2)gl^{ts2}/CyO$	22	72.7	sterile
	27	0	–
	29	0	–
$l(2)gl^{ts3}/CyO \times l(2)gl^{ts1}/CyO$	22	98.5	fertile
	27	100	male sterile
	29	81.8	sterile
$l(2)gl^{ts3}/CyO \times l(2)gl^{ts2}/CyO$	22	105.2	fertile
	27	100.1	male sterile
	29	60.6	male sterile

Approximately 200 progeny issued from each cross were examined. Experiments have also been performed with the $Df\ net^{62}$ deficiency and give similar results.

The $l(2)gl^{ts1}$ and $l(2)gl^{ts2}$ alleles did not complement but were both capable of a partial complementation of the $l(2)gl^{ts3}$ mutation. Strand et al. (1994b) have recently demonstrated that P127 comprises independent functional domains that can participate in the formation of dimers or oligomers. A chimeric protein dimer resulting from the combination of different alleles located in different domains of the protein would be functional and could explain the observed intragenic complementation.

Table 2. Critical periods for the activity of *l(2)gl*

Crosses at 29°C Female×Male	Laid eggs	Phenotype of progeny	Hatched larvae		Adults	
			number	% of the expected percentage	number	% of the expected percentage
(a)						
$y\ ;\ l(2)gl^{ts3}/CyO\ y^+ \times y\ ;\ l(2)gl^{ts3}/l(2)gl^{ts3}$	423	y	168	79.4	0	0
		y^+	255	120.5	220	96.7
(b)						
$y\ ;\ l(2)gl^{ts3}/l(2)gl^4 \times y\ ;\ l(2)gl^{ts3}/l(2)gl^4$	334	y	0	0	0	0
(c)						
$y\ ;\ l(2)gl^{ts3}/l(2)gl^4 \times y\ ;\ l(2)gl^{ts3}/CyO\ y^+$	439	y	0	0	0	0
		y^+	42	19.1	38	17.3
(d)						
$y\ ;\ l(2)gl^{ts3}/l(2)gl^4 \times +/+$	259	y^+	60	23.1	42	16.2
(e)						
$+/+ \times y\ ;\ l(2)gl^{ts3}/l(2)gl^4$	402	y^+	300	74.6	245	60.9

Larvae with wild-type *l(2)gl* activity are identified with the aid of the y^+ dominant phenotype carried by the balancer *CyO*. The phenotype of homozygous larvae for *l(2)gl* mutations is *y*. Mating was allowed at permissive temperature, then the flies were kept at 29°C for 4 hours before the eggs were collected. Growth was carried out at 29°C. The low recovery of progeny in (e) is probably due to a partial sterility of the $l(2)gl^{ts3}/l(2)gl^4$ males.

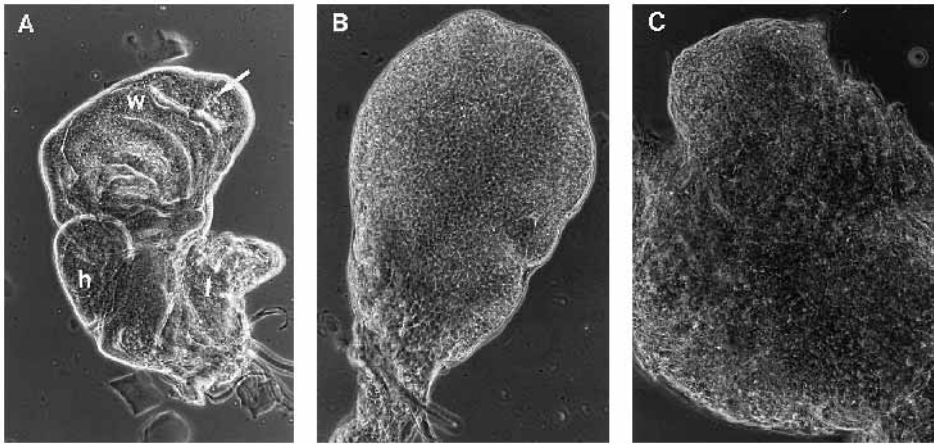

Fig. 1. Imaginal discs from double mutants $ecd^1/l(2)gl^{ts3}$. ecd^1/ecd^1 larvae subjected to a temperature shift failed to pupariate and progressively became necrotic and died after one or two weeks. Their imaginal discs were essentially normal (A) but slightly hyperplastic (arrow). The folded structure was easily recognized. Double mutant $ecd^1/l(2)gl^{ts3}$ larvae (B,C), when shifted at 29°C, did not pupariate and became giant and translucent, very similar to null mutants for *l(2)gl*. Their dissected imaginal discs (B) showed typical tumoral growth. (C) Discs from $ecd^1/l(2)gl^{ts3}$ mutants, which had fused and appeared coalescent. w, wing disc; h, haltere disc; l, leg disc; arrow, hyperplasia.

Another interesting phenotype was the male sterility of hemizygous $l(2)gl^{ts3}$ adults reared at 27°C. This phenotype was also observed with *trans*heterozygous $l(2)gl^{ts1}/l(2)gl^{ts3}$ and $l(2)gl^{ts2}/l(2)gl^{ts3}$ males reared at 29°C. Male sterility could directly be attributed to a visible abnormality of the genitalia and the anal plate, which had not rotated by 180°C when compared to that of a wild-type male and displayed a disorganized protruding appearance. Hadorn and Gloor (1942) and Gloor (1943) had also observed malformations of the gonads in larvae deficient for *l(2)gl* function.

$l(2)gl^{ts3}$ has essentially been used in this work because it behaves as a pure conditional allele of *l(2)gl*. Homozygous mutant larvae produced at 29°C from a $l(2)gl^{ts3}/CyO$ strain pupariated according to a correct timetable and then died during the early pupal stages. Giant larvae or tumorous brain or imaginal discs were never observed either in $l(2)gl^{ts3}$ or in $l(2)gl^{ts1}$ or $l(2)gl^{ts2}$ mutants and this was in contrast with null alleles of *l(2)gl* that correspond to deficiencies of the entire gene (Mechler et al., 1985). One hypothesis is that a tumoral phenotype could not be observed in $l(2)gl^{ts3}$ tissues essentially because normal pupariation of mutant larvae did not allow enough time for tumors to reach a detectable size. The inability to pupariate of the *l(2)gl* null alleles probably originates from a lack of ecdysone secretion caused by a malformation of the ring gland, which does not differentiate normally in mutant larvae (Aggarwal and King, 1969). The ring gland appeared normal in $l(2)gl^{ts3}$ larvae. Attempts to delay the puparation of $l(2)gl^{ts3}$ animals have therefore been performed by decreasing the ecdysone production. A strain carrying the $l(2)gl^{ts3}$ mutation and a temperature-sensitive ecd^1 mutation in the *ecdysoneless* gene was constructed. Homozygous ecd^1 larvae, when shifted from 22°C to 29°C in the middle of third instar larval stage failed to pupariate and survived as long as 3 weeks as larvae (Garen et al., 1977; Henrich et al., 1993). A similar shift experiment performed on $l(2)gl^{ts3}$; ecd^1 homozygous double mutants also prevented puparation but the delay in larval life resulted here in the formation of bloated and giant larvae. Such a phenotype was reminiscent of the phenotype encountered in a *l(2)gl* null mutant and was different from the ecd^1 homozygous mutant phenotype (Garen et al., 1977). Moreover, wing imaginal discs dissected from surviving $l(2)gl^{ts3}$; ecd^1 larvae showed neoplastic transformations (Fig. 1). Weak $l(2)gl^{ts3}$ mutants, like null mutants were thus able to display a tumoral phenotype when a longer larval life was permitted. Whether tumor formation was due to a prolonged life that, by itself, allowed tumors to develop or, rather and more indirectly, to the lack of ecdysone, remains to be clarified. These results suggest however that the temperature-sensitive mutation $l(2)gl^{ts3}$ has a tumoral potentiality.

Function of *l(2)gl* in embryogenesis and mutant phenotype

The $l(2)gl^{ts3}$ allele has been used to reinvestigate the function of *l(2)gl* in development (Table 2). Homozygous $l(2)gl^{ts3}$ embryos from crosses between $l(2)gl^{ts3}/CyO$ females and homozygous $l(2)gl^{ts3}$ males reared at 29°C developed into viable homozygous mutant larvae, most of them dying as early pupae (Table 2a). Strong reduction of the maternal contribution to *l(2)gl* expression by using homozygous $l(2)gl^{ts3}$ females led

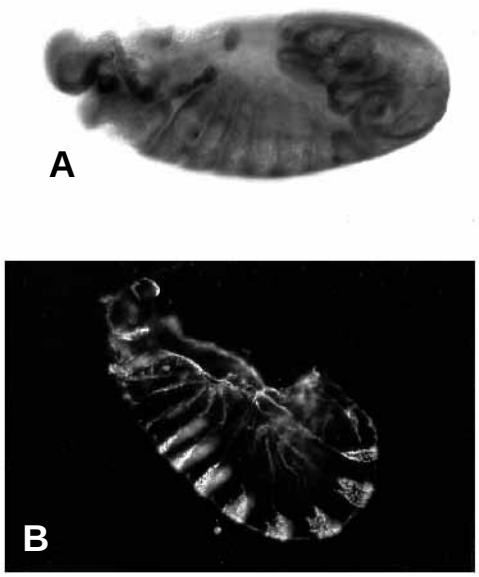

Fig. 2. *l(2)gl* mutant phenotype at restrictive temperature. Embryos of a homozygous $l(2)gl^{ts3}$ strain after (A) 12 hours at 29°C, labelled with an anti-Fasciclin III antibody. Development was blocked at germ-band shortening and the involution of the head was also prevented. (B) Cuticle preparation showing the extreme phenotype observed in $l(2)gl^{ts3}$ mutants at restrictive temperature. In a mutant blocked at germ-band retraction, the cuticle had differentiated but a dorsal opening in the cuticle and defects in the organization of the head and of the telson were systematically observed.

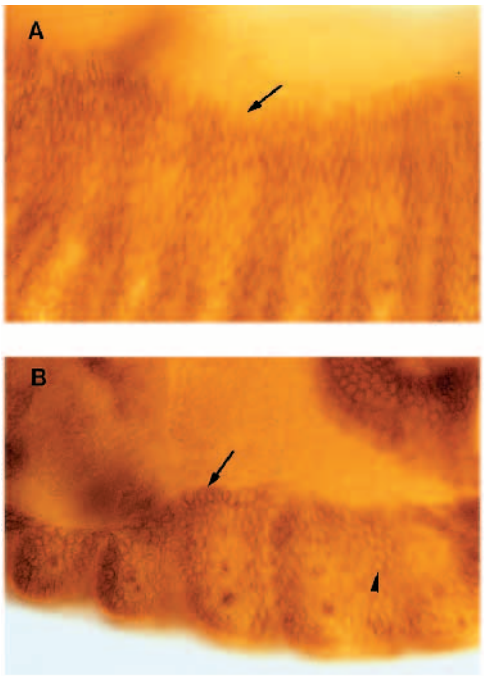

Fig. 3. Shape of ectodermal cells in *l(2)gl* mutants. Abnormal cell shapes in *l(2)gl* mutants obtained from a homozygous $l(2)gl^{ts3}$ strain were visualized in whole-mount embryos stained with an anti-Fasciclin III antibody. (A) A stage 14 wild-type embryo displaying the elongated shape of the dorsal most epidermal cells (arrow). Propagation of the change from a cuboïdal to an elongated cell shape became apparent along the dorsoventral axis. Fasciclin III was expressed in regions of cell-to-cell contacts and not in the part of the dorsalmost ectodermal cells facing the amnioserosa. (B) In a $l(2)gl^{ts3}$ homozygous mutant, slightly older than the embryo in A, cell shape change has not occurred and the ectodermal cells remained cuboïdal in form (arrow). This was particularly evident in the dorsalmost ectodermal cells (arrowhead). These cells, in which Fasciclin III was now expressed on the membrane facing the amnioserosa, had not acquired their normal polarity. Note that the embryo shown in B, although older than that presented in A (late stage 15), has not initiated its cell shape change.

to an embryonic lethality of eggs deposited at 29°C (Table 2b). Reduction in maternal component could partially be complemented by introduction in the zygote of a wild-type copy of the gene (Table 2c,d). It can therefore be concluded that *l(2)gl* activity is required in embryogenesis. The maternal contribution to *l(2)gl* expression is sufficient for the embryogenesis to proceed normally and, in its absence, a partial zygotic rescue can be obtained, suggesting that the maternal and zygotic expressions are functionally equivalent.

Eggs laid at 29°C by $l(2)gl^{ts3}$ homozygous females or $l(2)gl^{ts3}$/*Df net62* females crossed with $l(2)gl^{ts3}$ homozygous males were allowed to develop at 29°C and examined for embryonic defects resulting from a loss of function of *l(2)gl*. These embryos had a greatly reduced maternal component and were not rescued by the mutant zygote. In this situation, development of mutant embryos was blocked at germ band shortening (Fig. 2A). Fig. 2B shows a cuticle preparation of a typical mutant 'shrimp-shaped' embryo. The involution of the head and the distal region of the embryos were also affected, but segmentation appeared normal. The observed phenotype was almost 100% penetrant and indistinguishable for embryos of $l(2)gl^{ts3}/l(2)gl^{ts3}$ or $l(2)gl^{ts3}/Df\ net^{62}$ genotypes.

During dorsal closure, cells of the lateral epidermis undergo a change in shape as the epidermal sheet spreads over the amnioserosa. The epidermal cells become thinner along the anteroposterior axis and longer along the dorsoventral axis (Martinez-Arias, 1993; Young et al., 1993). This change is completed at dorsal closure when all epidermal cells have acquired their final elongated shape. The movement is in fact initiated in the dorsal edge of epidermal cells (Fig. 3A) and is later transmitted from cell to cell along the dorsoventral axis. As shown in Fig. 3, the dramatic change in shape of ectodermal cells did not occur in a *l(2)gl* mutant. This phenotype is highly reminiscent of that of *zipper* mutants that are affected in cytoplasmic myosin II heavy chain (Young et al., 1993) or of *scab* mutants (Nüsslein-Volhard et al., 1984; our own unpublished observations).

In addition to cell shape change in a wild-type embryo, there is a relocalization of cell junction proteins such as Fasciclin III (Martinez-Arias, 1993). During dorsal closure, the protein present in the ectodermal cells of the dorsal edge is not initially expressed on the membrane facing the amnioserosa. When the two edges of the ectodermal cells join at dorsal midline, Fasciclin III becomes expressed uniformly at sites of cell-to-cell contacts in ectodermal cells of the dorsal edge as it is usually the case in all other ectodermal cells. In *l(2)gl* mutants, ectodermal cells of the dorsal edge remained round and showed, in many instances, an absence of polarity (Fig. 3B). As a consequence, Fasciclin III was expressed on the entire surface of these cells. Transmission of cell shape change to lateral and ventral epidermal cells was also affected in mutant embryos (Fig. 3B).

Another major defect appeared in embryos that had spent 12 hours or more at 29°C: they displayed hypertrophy of the midgut, which did not develop normally (Fig. 4). Progression of intestinal hypertrophy during development was visualized with the aid of the *l(2)gl* cDNA which is still efficiently expressed in $l(2)gl^{ts3}$ mutants. In late wild-type embryos, *l(2)gl*

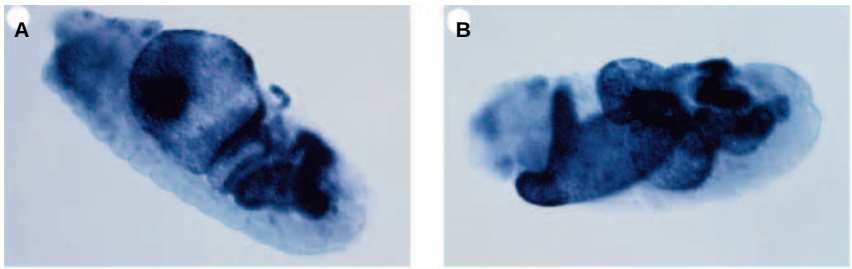

Fig. 4. Midgut phenotype of *l(2)gl* mutants. Whole-mounts embryos from $l(2)gl^{ts3}$ homozygous flies at 29°C were hybridized with DIG-labelled *l(2)gl* cDNA probes. In $l(2)gl^{ts3}$ alleles, *l(2)gl* mRNA was expressed normally at 29°C and, therefore, its expression could be followed during development of the mutant. Initially, development was normal, but, later on, the anterior lobe seemed to continue to grow and swelled out of the embryo by the dorsal opening (A). The final state was often a multiglobular and hypertrophied midgut (B). The mutant embryos shown in the figure are aged to a corresponding wild-type stage 17 embryo.

transcripts (not shown) and P127 (Strand et al., 1994a) were mainly found in the midgut endodermal cells. In the mutant, midgut formation had initiated correctly with the three constrictions appearing at the correct time even though germ band shortening and dorsal closure were blocked. However, instead of the lengthening and convolution of the midgut that normally occur in later stages (Skaer, 1993), the mutant midgut enlarged to a size 2 to 3 times greater than that of the wild type, leading to a swelling of the embryos, especially in the dorsal part where the epidermis failed to form. In this process, the anterior lobe was systematically larger than the others (Fig. 4A,B). Semithin sections showed that the yolk, which is digested at the end of embryogenesis in wild-type animals, was still present inside the intestine (not shown). These results suggest that the intestinal cells that are responsible for the hydrolysis of the yolk had not differentiated properly. A labelling with antibodies directed against Fasciclin III did not detect abnormalities in the formation of the visceral mesoderm of mutant embryos (data not shown) and this eliminated a possible cause for midgut epithelium malformation (Tepass and Hartenstein, 1994). It is noteworthy that, in other mutants, such as *scab* for example, which are also affected in dorsal closure, the midgut protrudes from the embryo, although shape and differentiation of intestinal cells appear normal with no sign of hypertrophy (see Fig. 5A,B).

The most conspicuous aspect of the mutant phenotype was a profound shape alteration of intestinal cells (Fig. 5). Tepass and Hartenstein (1994) have described three successive changes in the shape of precursors of midgut epithelial cells. They assumed that these modifications were related to a modulation of cell-to-cell adhesiveness during midgut development. Analysis of *l(2)gl* mutant phenotypes suggests that the two first steps, formation of an epithelium from mesenchymal precursor midgut cells and their flattening with reduced cellular adhesiveness, occurred correctly at stage 14. Later in embryogenesis, wild-type midgut epithelial cells became thinner and longer to constitute a tube-like and highly convoluted larval intestine. In *l(2)gl* mutants, this transformation did not occur and the epithelial cells seemed to have arrested their evolution at the previous stage. In several regions of the midgut, epithelial cells were rounder and flatter than the cells from a wild-type embryo of the same age (Fig. 5C,D). This defect could in part explain the apparent hypertrophy of the midgut, each cell occupying a larger area than in the wild-type embryo at the same stage.

No attempt was made to check whether midgut epithelial cells continued to divide but, in *l(2)gl* mutant embryos, the midgut adult precursor cells appeared to be in greater number (Tepass and Hartenstein, 1994) (Fig. 5C,D) and this overproliferation could also participate to some extent in the hypertrophied midgut phenotype.

Critical period for *l(2)gl* activity during embryogenesis and larval life

Temperature-sensitive alleles of the *l(2)gl* gene have been used to determine the sensitive period for embryonic lethality. In these experiments, it has been assumed that a temperature upshift produces an inactive *l(2)gl* protein, probably by a conformational change, and that this process is immediate and

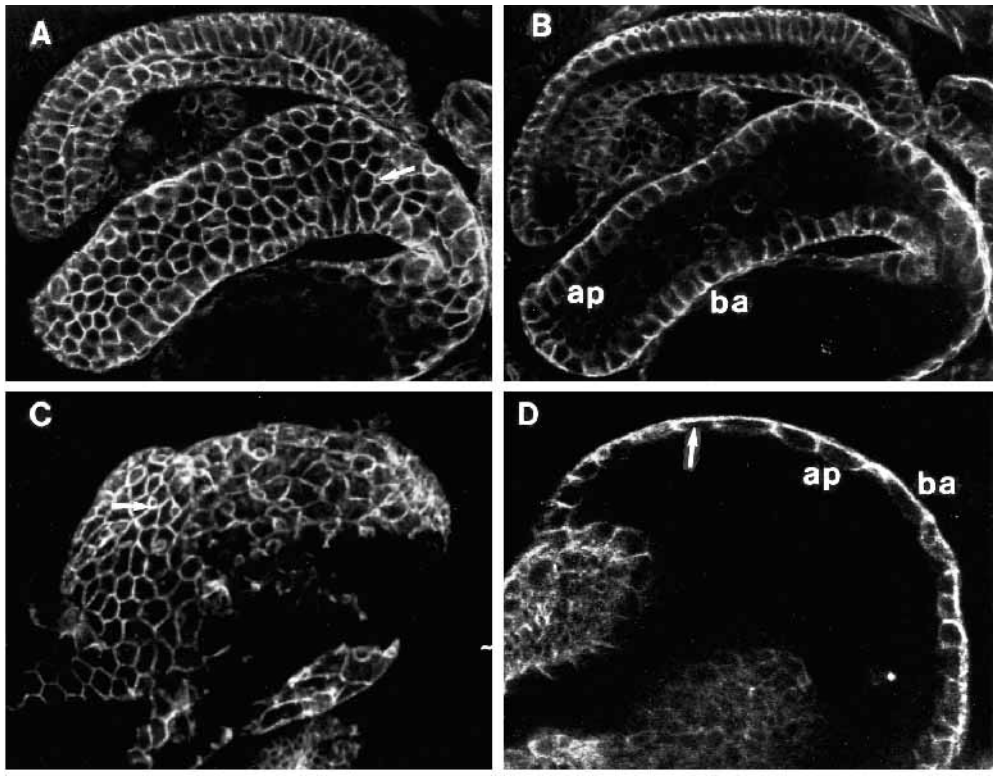

Fig. 5. Shape of midgut epithelial cells in *l(2)gl* mutants. Embryos from a homozygous $l(2)gl^{ts3}$ strain were stained with an anti-α spectrin antibody (Lee et al., 1993) to decorate the basolateral membranes of epithelial cells. The epithelial midgut is essentially composed of two types of cells (Tepass and Hartenstein, 1994): the principal epithelial midgut cells, which stop their division at stage 10 and become polyploïd, and the adult precursor cells, which are smaller and diploïd but that do not divide after stage 14-15 (Campos-Ortega and Hartenstein, 1985; Smith and Orr-Weaver, 1991). Confocal images of a late stage 16 wild-type (A,B) or of a $l(2)gl^{ts3}$ mutant embryo (C,D) of the same age as in A and B. Two optical sections of the epithelial midgut are shown for each sample. (A,C) Tangential sections; (B,D) transverse sections. Compare the sac-like structure in the mutant (C) with the elongated and convoluted midgut observed in the wild type (A). The adult precursor cells appeared to be in greater number in the mutant (arrow in C) than in the wild type (arrow in A). The transverse sections showed that the epithelial mutant cells (arrow in D) did not acquire their prismatic shape (compared B and D). Staining with the anti-α spectrin antibody was systematically slightly weaker in the mutant (not shown), but its polarized localization was not affected. ap: apical membrane; ba: basal membrane.

possibly reversible (Suzuki, 1970). Indeed, a protein whose size was indistinguishable from those of the wild-type component was produced by the *l(2)gl^{ts3}* mutant at 29°C, as illustrated in Fig. 7.

Shifts between permissive (22°C) and restrictive (29°C) temperature were applied to 1 hour egg-layings obtained from *l(2)gl^{ts3}/l(2)gl^{ts3}* flies. Fig. 6 shows that the gene activity is required very early in embryogenesis and the critical period extends from stage 8 to stage 12. Embryos that had spent 5 to 6 hours at 29°C (late stage 11, germ-band shortening) were no longer able to pursue their development, even if they were placed back at 22°C. Therefore some irreversible events had occurred during this period, indicating that *l(2)gl* is involved in some early stages of embryogenesis. By contrast, temperature upshifts performed after 10 hours of development did not have deleterious effects on development of homozygous *l(2)gl^{ts3}* embryos, which evolved into larvae with apparently normal midguts, as reinforced by confocal microscopy and(or) sectioning experiments; however, they died at the end of the third instar larval stage if maintained at 29°C. This result was somehow unexpected because, at this stage, *l(2)gl* is abundantly expressed in precursor cells of the epithelial midgut in which morphological defects have been shown to appear when the mutant embryos were maintained at 29°C from the time of fertilization.

The hypomorphic character of the *l(2)gl^{ts3}* allele seems to be in part responsible for this behaviour. This is reflected by Fig. 6 where the upshifts experiments were performed on descendants of a cross between *l(2)gl^{ts3}/Dfnet 62* females and homozygous *l(2)gl^{ts3}* males. In this case, the curve was systematically situated below that obtained in the case of a homozygous *l(2)gl^{ts3}* strain. A wide variety of phenotypes was observed depending both on the time at which the upshift was performed and the genotype of the embryos. The effect on germ-band shortening ranged from the extreme phenotype shown in Fig. 1 in the case of an early temperature upshift to an almost complete dorsal closure when the temperature upshift was applied later.

In the same line, the effect of the *l(2)gl^{ts3}* mutation could be strengthened by performing temperature upshifts at 31°C. For example, more than 90% of the *l(2)gl^{ts3}* embryos showed an arrest in dorsal closure when the temperature upshift was made at stage 11, shortly before the beginning of dorsal closure. By comparison, less than 50% of mutant embryos displayed a similar phenotype when temperature upshifts were carried out at 29°C. By contrast, the intestinal phenotype described in Fig. 5 could only be observed in a small proportion of mutant embryos when the upshift at 31°C was made at early stage 15. This latter observation might suggest that the intestinal phenotype would not be a direct consequence of the loss of function of *l(2)gl*.

However, due to the fact that *l(2)gl^{ts3}* is a hypomorphic allele at 29°C, a small proportion of the protein produced by the mutant could still be functional. In addition, the active conformation of the protein could be stabilized by its integration into a multicomponent complex (Strand et al., 1994b) which would then be difficult to displace once formed. As illustrated in Fig. 7, the amount of *l(2)gl^{ts3}* protein recovered in an insoluble fraction, which is indicative of its complexed form (Strand et al., 1994b), was compared when different conditions of temperature upshifts were imposed to *l(2)gl^{ts3}*. Mutant embryos persistently grown at 29°C contained no insoluble form of the P127 protein (Fig. 7B) whereas embryos that had been submitted to the temperature upshift after 11-13 hours at 25°C contained a substantial proportion of this same insoluble protein (Fig. 7A), even though to a lesser extent than in wild-type embryos (not shown; see also Strand et al., 1994a). This observation could explain why the late temperature upshifts presented in Fig. 6 did not produce the phenotypes that were routinely observed when the embryos were grown at non-permissive temperature. The complex formed at permissive temperature at early stages would be stable and functional and not able to be displaced when the temperature is raised.

l(2)gl function is also required during larval life and Fig. 8 shows that the sensitivity period fell within the third instar larval stage. These data were in disagreement with previous

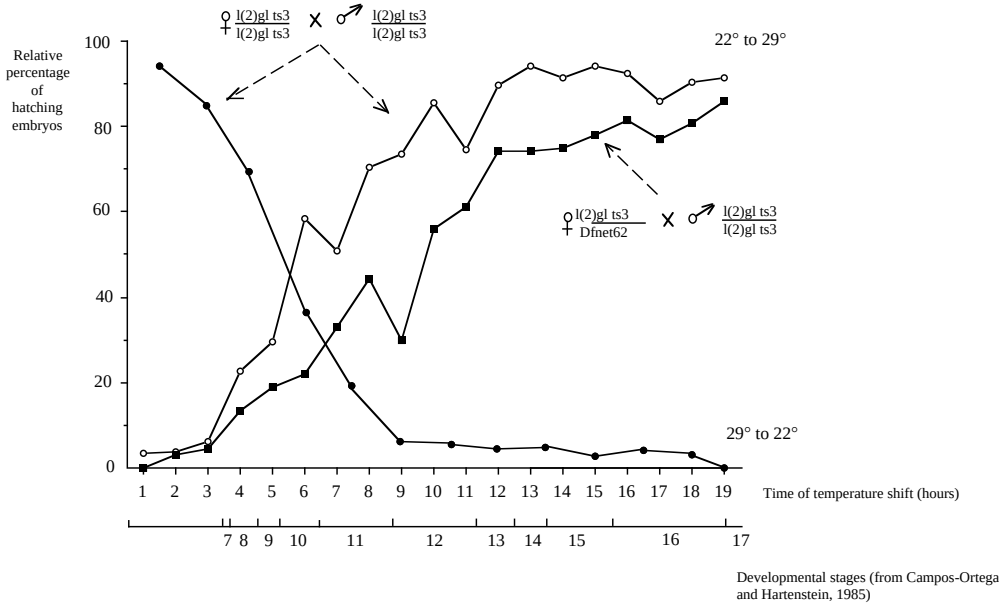

Fig. 6. Critical period for *l(2)gl* activity during embryogenesis. Effects of temperature upshifts on embryonic lethality in the progeny of *l(2)gl^{ts3}/ l(2)gl^{ts3}* (open circles) or *l(2)gl^{ts3}/ Dfnet^{62}* females (squares) crossed with *l(2)gl^{ts3}/ l(2)gl^{ts3}* males and temperature downshifts (filled circles) in the progeny of a *l(2)gl^{ts3}/ l(2)gl^{ts3}* strain. 100% refers to the number of embryos that hatch in the case of the same cross maintained at 22°C. Under these conditions, around 95% of laid eggs hatch. Flies are placed at the desired temperature 4 hours before the first shift. Approximately 200 embryos were counted for each point. The curves have been normalized to development at 25°C.The abscissa show the time of the shift.

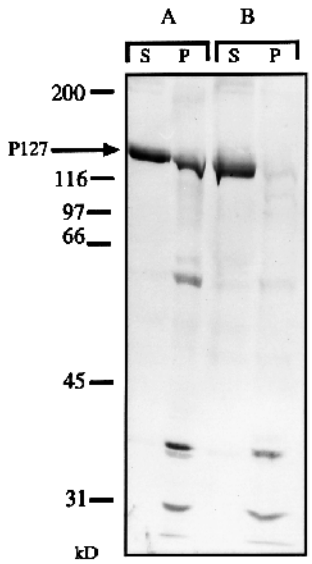

Fig. 7. Subcellular distribution of P127 in homozygous *l(2)gl*^{ts3} embryos submitted to different conditions of temperature upshifts. Extracts of 50 embryos in PBS containing protease inhibitors were fractionated into a soluble (S) and a pellet (P) fraction under the conditions described in Strand et al. (1994a) and the proteins of each fraction were submitted to SDS-PAGE electrophoresis and western blotting. The blot was probed with an anti-P127 antiserum and the reaction was revealed with a secondary antibody coupled to alkaline phosphatase. (A) Homozygous *l(2)gl*^{ts3} embryos were collected during 2 hours at 25°C, then left 11 hours at 25°C before being placed at 29°C for 6 hours before homogenization. A substantial fraction of P127 was recovered in the insoluble fraction. (B) Homozygous *l(2)gl*^{ts3} embryos were collected during 2 hours at 29°C and left 13 hours at 29°C before the homogenization step. No P127 protein was recovered in the insoluble fraction. Note that the P127 antibody recognized the mutant protein on western blot. The bands of lower molecular weights are components not related to P127 which cross-react with the antibody.

experiments carried out using genetic mosaics that had shown that larval expression of *l(2)gl* was not required for the viability and hatching of the pupae (Merz et al., 1990). The different rationale underlying these two types of experiments could explain these opposite results. Two other observations lend support to our conclusion. By crossing a strain carrying a UAS-*l(2)gl* cDNA construct with a 69B-GAL4 strain in which the GAL4 transcription factor is specifically expressed in the precursors and the derivatives of the ectoderm layer (Brand and Perrimon, 1993), it has been possible to regulate, by temperature shifts, temporal *l(2)gl* expression in the progeny of this cross. The experiments were performed in a *l(2)gl*^{4} mutant background, which displayed a null phenotype. The cross reared at 22°C, a temperature at which GAL4 was weakly active, never led to the recovery of *l(2)gl*^{4} homozygous viable adults. By contrast, at 29°C, GAL4 was fully active and consequently viable although sterile homozygous adults were recovered in the expected proportion. In this case, also, temperature-shift experiments allowed the determination of the same critical period for the *l(2)gl* gene activity. The weak activity of the GAL4 system at 22°C during embryogenesis and first larval stages could be sufficient to prevent the formation of tumors in late larvae. The same type of results was obtained with transgenic larvae carrying the *l(2)gl* cDNA under the control of a heat-shock promoter. Heat-shock delivered at the third instar larval stage was able to rescue a small percentage of adults. The absence of a tumoral phenotype in such animals reared at 22°C was also interpreted by a leaky expression of the heat-shock promoter sufficient to prevent the formation of tumors.

Function of *l(2)gl* during oogenesis

The results presented above suggest that a *l(2)gl* product is maternally provided to the embryo and that this expression is responsible for normal development of the homozygous mutant embryos at least until late larval stages. This implies expression of the *l(2)gl* gene during oogenesis and Szabad et al. (1991) have shown that this expression is indeed required both in germ-line and follicle cells.

The temperature-sensitive allele *l(2)gl*^{ts3} has been used to study the function of *l(2)gl* in oogenesis. The fertility of *l(2)gl*^{ts3} homozygous females maintained at 29°C progressively decreased, with a reduction in egg-laying of about 50% after 3 days and of 90-95% after 7 days at this temperature. This effect was not observed in homozygous *l(2)gl*^{ts3} females carrying a transposon containing the *Drosophila pseudoobscura* gene which is, as previously described (Török et al., 1993), capable of rescuing the lethal phenotype associated to the conditional *l(2)gl*^{ts3} mutation.

Fig. 9 shows an ovary from a homozygous mutant female grown at 29°C for 6 days. Oogenesis appeared to be blocked at stage 7-8. Older egg chambers had a necrotic aspect and did not develop normally. Egg chambers blocked at stages 7-8 all showed the same phenotype, a multilayered accumulation of cells, probably of follicular origin, at their anterior and posterior tips. As depicted in Fig. 9E, these cells had lost the correct polarity of follicular cells. The mutant cells were rounder than in the wild-type and, more interestingly, had internalized into the egg chamber within the space usually occupied by the ooplasm.

Another phenotype, although not totally penetrant, showed

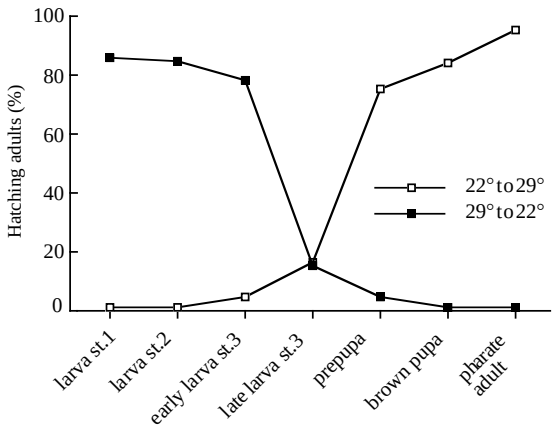

Fig. 8. Critical period for *l(2)gl* activity during larval life. Effect of temperature upshifts (open squares) or temperature downshifts (filled squares) on hatching of larvae issued from *l(2)gl*^{ts3} flies. First instar larvae, freshly hatched from embryos that had developed at 22°C, were immediately placed at 29°C (downshifts) or maintained at 22°C (upshifts) until the time of the temperature shift represented in abscissa. Each point corresponds to 200 animals.

a fusion of the germarium with the youngest egg chambers (Fig. 9C,D). This phenotype was better visualized by using the amorphic $l(2)gl^4$ mutation. As reported earlier, overexpression of the *l(2)gl* cDNA in a homozygous $l(2)gl^4$ background at third instar larval stage led to recovery of a small proportion of viable adults. The females were however sterile. Their ovaries appeared very small and the early egg-chamber fusion phenotype was observed in almost all the ovarioles (Fig. 9F). Under these conditions therefore, the same phenotype was obtained for the hypomorphic $l(2)gl^{ts3}$ allele and the null $l(2)gl^4$ allele carrying the transgene with, however, a better penetrance in the latter case.

DISCUSSION

In this paper, the use of temperature-sensitive mutants has demonstrated that the activity of the *l(2)gl* gene is required during *Drosophila* embryogenesis. The maternal contribution is sufficient by itself to permit a normal embryonic and early larval stages development. *l(2)gl* activity appears critical during oogenesis and is also probably very important for gametogenesis and/or the formation of male genital organs as revealed by the phenotype observed at 27°C in $l(2)gl^{ts3}$ homozygotes.

$l(2)gl^{ts3}$ behaves as a pure conditional allele and several observations suggest that it is a hypomorphic allele. It is, therefore, likely that the observed phenotypes are only partial and that more extreme defects could have been obtained by using a null allele. For example, *l(2)gl*, which is expressed with a high level of activity before the blastoderm stage, probably plays a role in the gastrulation process. During this morphogenetic event, blastoderm cells undergo a change in shape (Costa et al., 1993), reminiscent of that occurring during dorsal closure. Indeed, the fraction of protein remaining active at 29°C could be sufficient for a normal gastrulation to take place but yet too low to allow germ-band retraction or dorsal closure.

In spite of pleiotropic aspects of the mutations in *l(2)gl*, which are compatible with a rather ubiquitous expression of this gene product, the study presented herein has uncovered some features shared by all phenotypes. First, mutations in *l(2)gl* essentially affect cells committed to an epithelial fate. Second, they apparently alter neither determination nor identity of embryonic cells but rather the differentiation state of epithelial cells. The process of shape remodeling that occurs in ectodermal cells during dorsal closure or in the midgut epithelial cells is abolished in *l(2)gl* mutants. An alteration of cell adhesiveness and loss of cell polarity is observed during oogenesis in follicle cells of the egg chambers

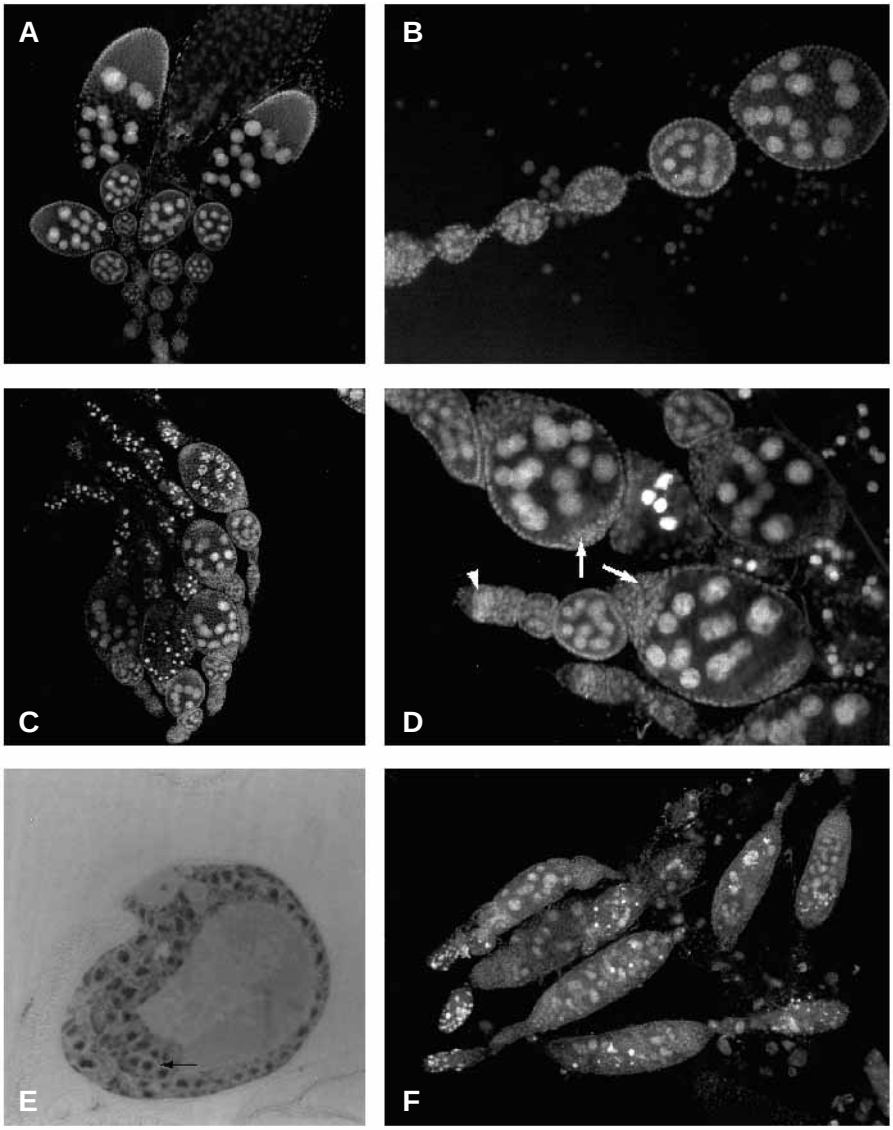

Fig. 9. Effects of *l(2)gl* mutations on oogenesis. Ovaries from wild-type females (A,B) or homozygous $l(2)gl^{ts3}$ females (C-F) were stained with DAPI. (A) Whole-mount ovary showing four ovarioles composed of a succession of egg chambers of different stages, the oldest ones being on the top of the picture. (B) Magnification of an ovariole with egg chambers from stage 2 to stage 7 (from left to right). (C) In mutant ovaries from females left 6 days at 29°C, oogenesis was blocked at stage 7-8. (D) No egg chambers from an older stage could be recovered. Mutant ovarioles showed two characteristic phenotypes with a penetrance depending on the time the flies had spent at 29°C. The egg chambers were fused (arrowhead in D) instead of being clearly separated by the stalk cells as it is shown in B. Also, there was an accumulation of cells, probably of follicular origin, at both extremities of egg chambers (arrows). A thin-section of the posterior region of a stage 8 mutant egg chamber is presented in E. Cells had invaded the ooplasm and they did not possess the characteristic epithelial structure of follicular cells. (F) A very strong fusion phenotype obtained with an almost total penetrance in viable females of $l(2)gl^4/l(2)gl^4$ genotype rescued by a *l(2)gl* transgene under the control of heat-shock promoter (see text). The heat-shock was made during the third larval stage and the ovaries of 2-day-old females were dissected and stained with DAPI.

(this work), in tumorous imaginal discs (Gateff, 1978) and in salivary glands (not shown).

The cytoskeleton has been extensively implicated in control of cell shape during *Drosophila* development (Karr and Alberts, 1986; Theurkauf et al., 1992; Young et al., 1993; Postner and Wieschaus, 1994; Thomas and Kiehart, 1994; Deng et al., 1995). Our results and the fact that *l(2)gl* has been shown to be a cytoskeletal protein (Strand et al., 1994a), strongly suggest that *l(2)gl* functions during development as a regulator of cytoskeleton organization in epithelial cells.

Genes whose mutations lead to analogous phenotypes are expected to act either separately or in cooperation in the same cellular process. Mutations in three other genes, *zipper* (Young et al., 1993), *coracle* (Fehon et al., 1994) and *scab* (Nüsslein-Volhard et al., 1984) hamper dorsal closure in a manner that is analogous although not identical to that prevailing in *l(2)gl* mutants, the phenotype in epidermis of *scab* mutants being the closest (unpublished observations). However, the *scab* gene has not yet been cloned and the nature of the gene product is unknown. The *zipper* gene encodes the cytoplasmic myosin heavy chain, which is considered as a driving force for change in shape of the dorsal leading edge ectodermal cells (Young et al., 1993). Furthermore, a direct molecular interaction between the non-muscle heavy chain and P127 has been observed (Strand et al., 1994b). The *coracle* gene codes for a protein associated with septate junctions homologous to the membrane-cytoskeleton protein 4.1 and it has been suggested by Fehon et al. (1994) that it associates with non-muscular myosin.

Two *Drosophila* genes coding for the Ras-related small GTP-binding proteins, *DracA* and *DracB*, homologous to mammalian *Rac1* and *Rac2*, have been identified (Luo et al., 1994; Harden et al., 1995). Expression of transgenes bearing a dominant inhibitory version of the *DracA* cDNA under control of the *hsp70* promoter causes a high frequency of defects in dorsal closure which are due to disruption of cell shape changes in lateral epidermis (Harden et al., 1995). These effects are associated with an altered localization of actin and myosin probably caused by cytoskeleton perturbations. Actually, no components downstream of these Rho proteins and directly acting on the actin cytoskeleton have been identified (Reinhard et al, 1995). P127 could be one of these components and regulate the actin-myosin network necessary for cell-shape changes during epidermal development. Finally, P127 is found associated in a multicomponent complex containing one protein with protein kinase A activity (Strand et al., 1994b). It has been shown that protein kinase A may regulate microfilament integrity through phosphorylation and inhibition of the myosin light chain kinase activity in non- muscle cells (Lamb et al., 1988) and it could form a link between the cytoskeleton and the signal transduction regulating the actin-myosin pathway.

These observations suggest that P127 might interact with these different gene products to generate a network connecting cytoskeleton and plasma membrane. Absence of the protein would result in a loosening of the network and eventually in a loss of cell adhesiveness and cell polarity. To support this hypothesis, experiments clearly demonstrating a direct interaction of the implicated proteins as well as a genetic interaction between the different genes should be performed.

By delaying the puparation with the aid of an ecdysone temperature-sensitive mutation, it has been demonstrated that the hypomorphic $l(2)gl^{ts3}$ allele has a tumoral potentiality. Double mutant $ecd^{1}/l(2)gl^{ts3}$ larvae never pupariate and can stay alive for 2-3 weeks. This delay could be the cause of the formation of tumors which then have had enough time to grow (Bryant and Levinson, 1985). Alternatively, low ecdysone titer conditions could also be involved in tumor growth resulting from a lack of *l(2)gl* activity in *l(2)gl* larvae, as already suggested (Gateff and Schneiderman, 1974). Another important conclusion is that *l(2)gl* activity is required during larval development to prevent the overgrowth phenotype, in disagreement with previous reports (Gateff, 1978; Merz et al., 1990). In $ecd^{1}/l(2)gl^{ts3}$ mutants, the temperature shift at 29°C was performed during the first instar larval stage, such that embryogenesis had proceeded under conditions of normal zygotic expression of *l(2)gl*.

The phenotype in the gut leading to a hypertrophy of the organ and that in the egg chambers resulting in a higher number of cells of follicular origin at each pole, suggest also a loss of regulation of cell proliferation in these two tissues. Experiments are in progress to strengthen this hypothesis. A confirmation of such misregulation in cell division would mean that a phenotype similar to that previously described in imaginal discs and in brains of third instar larvae could also be observed in embryos. These observations should facilitate the description of the different steps resulting in uncontrolled cell proliferation in tumors mediated by a tumor suppressor gene.

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