Dlg Protein Is Required for Junction Structure, Cell Polarity, and Proliferation Control in *Drosophila* Epithelia

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Abstract. The Discs large (Dlg) protein of *Drosophila* is the prototypic member of a growing family of proteins termed membrane-associated guanylate kinase homologs (MAGUKs). The MAGUKs are composed of a series of peptide domains that include one or three DHR/PDZs, an SH3, and a region homologous to guanylate kinase (GUK). We have previously shown that the product of this gene, the Dlg protein, is localized at the septate junctions between epithelial cells, and that mutations in the gene cause neoplastic overgrowth of the imaginal discs. The dlg locus is therefore defined as a tumor suppressor gene. In this paper, we show that the Dlg protein is localized on the cytoplasmic face of the septate junction and is required for the maintenance of this structure. It is also required for proper organization of the cytoskeleton, for the differential localization of membrane proteins, and for apicobasal polarity of epithelial cells. However, these other functions can be uncoupled from Dlg's role as a tumor suppressor since mutations in two domains of the protein, the SH3 and GUK, cause loss of normal cell proliferation control without affecting the other functions of the protein. These results suggest that, besides regulating cellular proliferation, the Dlg protein is a critical component of the septate junctions and is required for maintaining apicobasal polarity in *Drosophila* epithelium.

MANY animal tissues are composed of polarized epithelial sheets, which are held together by a series of specialized apicolateral junctional contacts including, in vertebrates, tight and adherens junctions (Anderson et al., 1993). Epithelial cells of *Drosophila* and other arthropods also contain adherens junctions, but the tight junction is replaced by a more basolateral structure, the septate junction (Noirot-Timothee and Noirot, 1980; Wood, 1990; Woods and Bryant, 1993*b*; Bryant, 1994). These junctions are important for maintaining a variety of epithelial properties, including apicobasal polarity, the basolateral restriction of membrane components, and the transepithelial diffusion barrier (Anderson et al., 1993; Eaton and Simons, 1995). In many organisms, including *Drosophila*, epithelial apicobasal polarity is established during embryogenesis, but the mechanisms controlling this process are not well understood. Two *Drosophila* genes, *crumbs* (*crb*) and *stardust* (*sdt*), have been shown to be involved in establishment of polarity in the ectodermal epithelia (Tepass and Knust, 1993; Knust et al., 1993; Wodarz et al., 1995). However, these two genes are apparently not involved in maintaining apicobasal polarity since postembryonic loss of either gene has no effect on the structure of imaginal epithelia (Tepass and Knust, 1993). Both of these genes act at about the time that adherens junctions form in the embryo, but before the formation of septate junctions during late embryogenesis (Tepass and Hartenstein, 1994).

The *dlg* gene was originally identified based on its ability to act as a tumor suppressor in *Drosophila*. Its product, the Discs large (Dlg)[1] protein, was found to be localized to both smooth and pleated septate junctions (Noirot-Timothee and Noirot, 1980) and provided the first specific molecular marker for this structure (Woods and Bryant, 1991). Other molecules found at the pleated septate junction include the transmembrane cell adhesion molecule fasciclin III (Brower et al., 1980; Patel et al., 1987; Woods and Bryant, 1993*b*), the *Drosophila* homolog of the membrane cytoskeletal protein 4.1, Coracle (Fehon et al., 1994), and the cytoskeletal proteins tubulin and actin (Lane, 1992; Colombo et al., 1993). The Dlg protein is the prototypic member of growing family of proteins, the membrane-associated guanylate kinase homologs (MAGUKs), many of which are localized at junctional contacts (Woods and Bryant, 1994). All of these proteins contain a series of

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1. *Abbreviations used in this paper*: Cor, Coracle; Dlg, Discs large; Ex, Expanded; GMP, guanosine monophosphate; GUK, guanylate kinase; MAGUK, membrane-associated guanylate kinase; PKC, protein kinase C; TEM, transmission EM.

domains: one or three copies of an 80–90-amino acid motif called DHR/PDZ (Dlg Homologous Region/PSD-95, Dlg, ZO-1) (Cho et al., 1992; Bryant et al., 1993; Kornau et al., 1995), an SH3 domain (Ren et al., 1993), and a region with high similarity to guanylate kinases (GUK). The SH3 domain acts as a site for protein–protein interactions in other proteins (Ren et al., 1993), and the DHR/PDZ may also act as a site of protein–protein interaction (Sato et al., 1995). One subfamily of MAGUKs, the Dlg-R proteins, includes Dlg as well as the tight-junction proteins ZO-1 (Willott et al., 1993) and ZO-2 (Duclos et al., 1993; Jesaitis and Goodenough, 1994), the synaptic junction protein PSD-95/SAP90 (Cho et al., 1992; Kistner et al., 1993), and hDlg/p97 (Lue et al., 1994; Müller et al., 1995), which is localized on cell membranes as well as in the nucleus (Peel, D., unpublished data). The finding of structurally similar molecules in tight and septate junctions suggests that these two junctional contacts perform analogous functions (Woods and Bryant, 1993*a*). In the Dlg-R subfamily, there are three DHR/PDZ domains, and the GUK domain has a three–amino acid deficiency in the ATP-binding site, making it unlikely that they have guanylate kinase catalytic activity. The second subfamily, the p55-R proteins, contains the erythrocyte membrane protein p55 (Ruff et al., 1991), a similar gene product dlg2 (Mazoyer et al., 1995), and the *Caenorhabditis elegans* signal transduction molecule Lin-2 (Hoskins et al., 1995; Kim, 1995). The members of this subfamily have only one DHR/PDZ, and the GUK domain does not have the deficiency.

The availability of mutations in the *dlg* gene and antibodies against the protein allows us to test directly the function of the Dlg protein. In this paper, we analyze the requirement for Dlg protein in the maintenance of septate junctions and epithelial structure. We also show that specific domains of the protein are critical for growth and patterning during *Drosophila* development.

Materials and Methods

Drosophila Stocks and Crosses

All *Drosophila* strains were maintained on standard cornmeal-molasses medium. The various *dlg* alleles used in this study have already been described elsewhere (Perrimon, 1988; Woods and Bryant, 1991, 1989). The wild-type strain used was Oregon-R.

RNA and Protein Analysis

Isolation and Characterization of dlg cDNAs. To characterize the molecular alterations in the *dlg* alleles *XI-2*, *1P20*, and *m30*, RNA from homozygous mutant animals was isolated, and first-strand cDNA was made using the Ready-to-Go first-strand cDNA kit (Pharmacia Fine Chemicals, Piscataway, NJ). The first-strand cDNA was PCR amplified (Innis et al., 1990) using primers to the 5′ and 3′ halves of the *dlgA* transcript. The amplifed products were cloned into the TA vector (Invitrogen, San Diego, CA) and transformed into DH5α, and plasmids with inserts were isolated. At least three independent clones were characterized from each allele to reduce the chances of PCR-induced artifacts. The genomic interval of *1P20* and *m30* was also sequenced to verify the nucleotide alteration.

Western Blot Analysis. Imaginal discs, brains, and body walls were dissected out from late third-instar larvae homozygous for the *dlg* alleles *v59*, *m30*, and *m52*, and from OreR. These tissues were harvested, washed in PBS, and homogenized in sample buffer (125 mM Tris-HCl, pH 6.5, 2% SDS, 10% glycerol, 0.05% bromophenol blue, 5% mercaptoethanol) containing protease inhibitors (0.4 mM phenylmethylsulfonyl sulphate, 1 mM benzamidine, 5 mM EDTA, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 0.2 trypsin inhibitor unit/ml aprotinin). Protein levels were measured in a Bradford colorimetric assay using BSA as a standard and adjusted to 20 μg/ml, doubled in the case of v59 to 40 μg/ml, and the samples were separated on a 7.5% SDS-PAGE gel. The proteins were electroblotted onto nitrocellulose, blocked overnight in TTBS (100 mM Tris, pH 7.5, 0.9% NaCl, 0.1% Tween-20) containing 10% goat serum, 5% nonfat dried milk, 3% BSA, and then probed for 3 h at room temperature with a 1:400 dilution of Dlg affinity-purified antibodies in blocking buffer.

Dlg affinity-purified antibodies were prepared by running a Western blot, using the fusion peptide against which the antibody was raised. Vertical strips from this blot were cut out and probed with anti-Dlg sera. The area highlighted was marked, the strips aligned with the main blot, and the corresponding region cut out. These strips were then blocked in TTBS containing 5% nonfat dried milk, incubated overnight at 4°C with a 1:5 dilution of anti-Dlg sera, and washed in TTBS, and the bound antibody was stripped by incubating in a stripping buffer (0.2 M glycine, pH 2.0, 1% Tween-20, 0.1% SDS) for 1 h at room temperature. After incubation of the membrane with the affinity-purified antibodies, the membrane was then washed three times for 10 min each in TTBS and incubated for 2 h at room temperature with anti–rabbit HRP antibodies (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) diluted 1:5,000 in TTBS blocking buffer. The blots were visualized with an ECL chemiluminescence kit (Amersham Corp., Arlington Heights, IL).

Marker lanes containing biotinylated molecular weight markers were cut out before antibody incubations and incubated separately in a 1:5,000 dilution of streptavidin HRP (Jackson Immunoresearch Laboratories, Inc.) in TTBS. These were then visualized along with the main blot.

DNA Constructs and Transgenic Flies

Generation of pUAST–DlgA Transgenic Flies. To generate the pUAST–DlgA transformation construct, a cDNA fragment containing the full-length Dlg coding region, −240 to 2915 bp (Woods and Bryant, 1991), was subcloned into the pUAST P-element transformation vector (Brand and Perrimon, 1993). Germline transformation of *yw* embryos was carried out using the published method (Rubin and Spradling, 1982), and five independent lines were established. In three cases, the insert mapped to the second chromosome, and in two cases, it mapped to the third.

Activation of pUAST–DlgA Constructs by a GAL4 Enhancer Trap Line and Rescue of dlg Mutants with Transgenic DlgA. Flies expressing a full-length DlgA transgene were created by crossing a transformant carrying the pUAST–DlgA insert on the second chromosome to GAL4 enhancer trap line 32B (Brand and Perrimon, 1993). To determine if DlgA alone could rescue *dlg* mutants, male progeny from this cross, which produce DlgA relatively uniformly throughout imaginal tissue, were crossed to virgin female dlg^{m30}, dlg^{XI-2}, or dlg^{m52} mutant flies balanced over FM7. Nonbar male progeny were scored to determine rescue.

Preparation of Tissue for Light and Electron Microscopy

Drosophila larvae were dissected in Ringer's solution, and the tissues were isolated. For immunolocalization at the light level, isolated tissues were fixed with 4% formaldehyde for 30 min, washed twice with PBS, and incubated in block (PBS containing 2% BSA, 0.2% NP-40, and 0.02% sodium azide). For detection of Coracle, the tissues were fixed as described (Fehon et al., 1994). After 1 h, primary antibodies were added at the appropriate concentration, and the tissues were incubated overnight at 4°C. The stained tissues were then washed three times with block, and secondary antibodies were added. If the tissue was stained for filamentous actin, then rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) was also added at this time. After a 2-h incubation at 22°C, the tissue was washed three times with block and mounted in Vectashield (Vector Laboratories, Burlingame, CA) for viewing with the MRC600 confocal microscope (Bio Rad Laboratories, Hercules, CA). For EM, the tissues were dissected as described above, and then fixed in PBS containing 2% gluteraldehyde for 1 h at 25°C, washed with several changes of PBS, and treated as described (Watson et al., 1992).

For immunolocalization at the electron microscopic level, salivary glands of wild-type third-instar larvae were fixed in 1% paraformaldehyde in PBS for 30 min. The glands were cut longitudinally with a razor blade and permeabilized for an additional 30 min in PBS containing 1% Triton X-100. Samples were washed twice for 20 min, incubated for 30 min in PBS containing 1% BSA (PBS-BSA), and, finally, incubated for an additional 4 h at room temperature in anti-dlg antibody at a dilution of 1:100 in

PBS-BSA. The stained glands were washed twice in PBS-BSA for 30 min and incubated at room temperature with goat anti-rabbit IgG coupled to 10 nm gold (EM Gar 10; BioCell, Cardiff, NJ), which was diluted 20-fold with PBS-BSA. The glands were then washed in PBS three times for 30 min and fixed for 1 h in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After washing in cacodylate buffer, the samples were postfixed in 1% Os_2O_4 in cacodylate buffer for 30 min. After washing in distilled water, the glands were stained en bloc with 1% uranyl acetate, dehydrated in a graded ethanol series, and embedded in Polarbed (BioRad Laboratories, Hercules, CA). Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and observed in an electron microscope (CM10; Philips Electronic Instruments, Inc., Mahwah, NJ). Controls were performed by omitting incubation with the first antibody.

Results

Complementation Behavior of dlg Alleles

Table I shows the complementation matrix derived from studies of heteroallelic *dlg* genotypes. Based on these data, the *dlg* gene contains three groups of alleles. Group I includes the mutations that appear to represent null alleles (dlg^{m52} and dlg^{G3}, Table I; see also Woods and Bryant, 1989). These are homozygous and hemizygous lethal at late larval stages and result in overgrown imaginal discs. Group I alleles behave like deletions and fail to complement all group II and III alleles.

Group II contains a single allele, dlg^{m30}. Larvae homozygous for this allele resemble group I alleles, showing imaginal disc overgrowth and failure to pupariate. Heteroallelic combinations between group II and III result in varying levels of complementation (Table I), including wild-type adult viability and fertility with no visible mutant phenotype in dlg^{m30}/dlg^{1P20} animals. Females of this genotype produce viable offspring when fertilized with dlg^{-} sperm, demonstrating that the maternal Dlg product, produced by interallelic complementation, is also fully functional.

Group III alleles (dlg^{sw}, dlg^{1p20}, dlg^{v59}, and dlg^{XI-2}) are less severe than alleles from the other complementation groups. The dlg^{sw} allele (not shown in Table I) is adult viable, but mutant flies have minor bristle defects, including missing and duplicated bristles (Fig. 1, *c* and *d*), similar to those observed in animals overexpressing the numb protein (Rhyu et al., 1994). Embryos produced by dlg^{sw} homozygous females, if not provided with a wild-type zygotic copy of the *dlg* gene, fail to hatch and die during late embryogenesis with a failure of dorsal closure and terminal defects. dlg^{1P20} homozygotes die as pharate adults with severe bristle and eye defects (Fig. 1, *e* and *f*); antennae and legs of these animals have small overgrown regions, and

Table I. Genetic Complementation Matrix of dlg Locus

Alleles		XI-2	v59	1P20	m30	m52	G3
XI-2	III	0					
v59	III	0	0				
1P20	III	0	0	0			
m30	II	8	14	128	0		
m52	I	0	0	0	0	0	
G3	I	0	0	0	0	0	0

Complementation of dlg^{sw} is not shown in this matrix since this is a viable allele; it was assigned to group III by analyzing the phenotype (Fig. 1) of various heteroallelic combinations. The numbers are the percentage of expected progeny based on the number of recovered heterozygous adult sibling ($n > 100$ for each genotype).

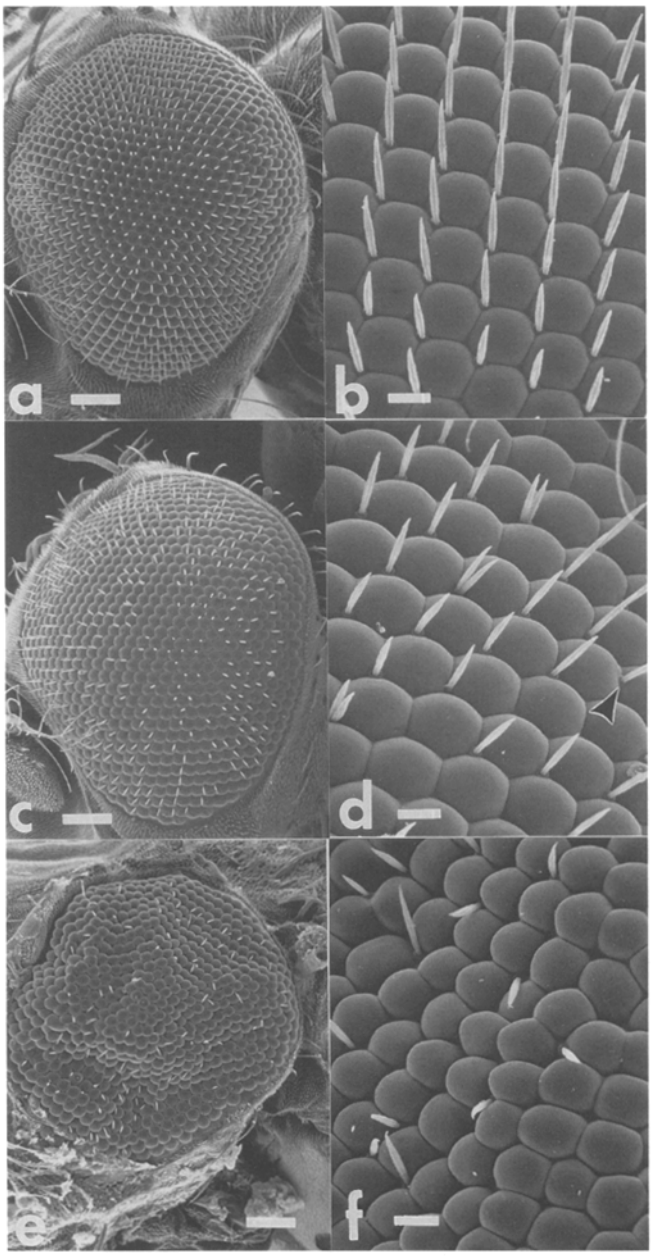


Figure 1. Scanning electron micrographs of male eyes from wild type and flies carrying mutations in the GUK domain of *dlg*. (*a*, *c*, and *e*) Low magnification views of adult eyes. (*b*, *d*, and *f*) Higher magnification views of the ommatidia and associated bristles. (*a* and *b*) Wild-type eyes display a very regular organization of ommatidia and interommatidial bristles. (*c* and *d*) Eye from a dlg^{sw} animal. Note the loss of bristles from the corners of many ommatidia as well as duplicated bristles. There is also an occasional bristle misplacement (*arrow* in *d*). (*e* and *f*) Eye from a dlg^{1P20} animal. The eye is severely deformed compared to wild type, with very few bristles (*e*) that have an apparently random organization (*f*). Bars: (*a*, *c*, and *e*) 50 μm; (*b*, *d*, and *f*) 10 μm.

the eyes show defects in the planar polarity of the ommatidial bristles since they are now found at random around the ommatidial cluster (Fig. 1 *f*). Larvae homozygous for dlg^{v59} show imaginal disc overgrowth and die during the early pupal period without forming adult cuticle. Homozygotes of the dlg^{XI-2} allele have overgrown imaginal discs

and fail to pupariate (Stewart et al., 1972; Woods and Bryant, 1989), a phenotype identical to that produced by group I and II alleles.

Molecular Analysis of the dlg Alleles

To further elucidate the functions of the Dlg protein, we have analyzed all three groups of *dlg* alleles at the molecular level. The group I allele dlg^{m52} is a null based on our genetic and Western blot assays (see Table I and Fig. 3). The molecular alteration in dlg^{m52} is a G to A transition at the splice donor for intron 5 (Fig. 2 *c*). This results in the introduction of a stop codon near the beginning of PDZ/DHR3 (immediately after R_{482}), and thus predicts a severely truncated protein (Fig. 2) that would not be recognized by antibodies to the Dlg fusion construct (Woods and Bryant, 1991).

The molecular alteration in the group II allele, dlg^{m30}, is a T to a C transition at 1895 bp, causing the change of a highly conserved leucine (L_{632}) to a proline in the SH3 domain. This alteration is not a naturally occurring polymorphism since the parental chromosome from which dlg^{m30} was derived has the same sequence as wild type.

The alterations found in the group III alleles are shown in Fig. 2. These alleles are all predicted to remove different amounts of the carboxyl end of the Dlg protein, and their phenotypic severity in homozygotes reflects the degree of truncation. Thus, the most severe alteration is seen in dlg^{XI-2}, which has a new splice acceptor site (a G to an A transition) at the beginning of exon 9. This change in exon 9 results in the formation of a novel transcript that is not detected in wild type and that has an additional 134 bp immediately upstream of the region encoding the GUK domain (Fig. 2, *a* and *b*). This results in the introduction of a stop codon 13 amino acids after the end of exon 8 and predicts a truncated protein lacking the GUK domain (Fig. 2 *b*). The dlg^{v59} allele shows a 14-bp deletion starting at bp 2528 that shifts the reading frame and is predicted to delete about two-thirds of the GUK domain (Woods and Bryant, 1991; Fig. 2 *a*). dlg^{1P20} is a C to a T transition at bp 2752, converting a glutamine (CAA) to a stop codon (TAA) that is predicted to eliminate the last third of the GUK domain without affecting the conserved putative guanosine monophosphate (GMP)–binding residues (Fig. 2 *a*). Finally dlg^{sw} is a 100-bp deletion starting at bp 2840 and is expected to cause a truncation COOH-terminal to the GUK domain (Woods and Bryant, 1991; Fig. 2 *a*).

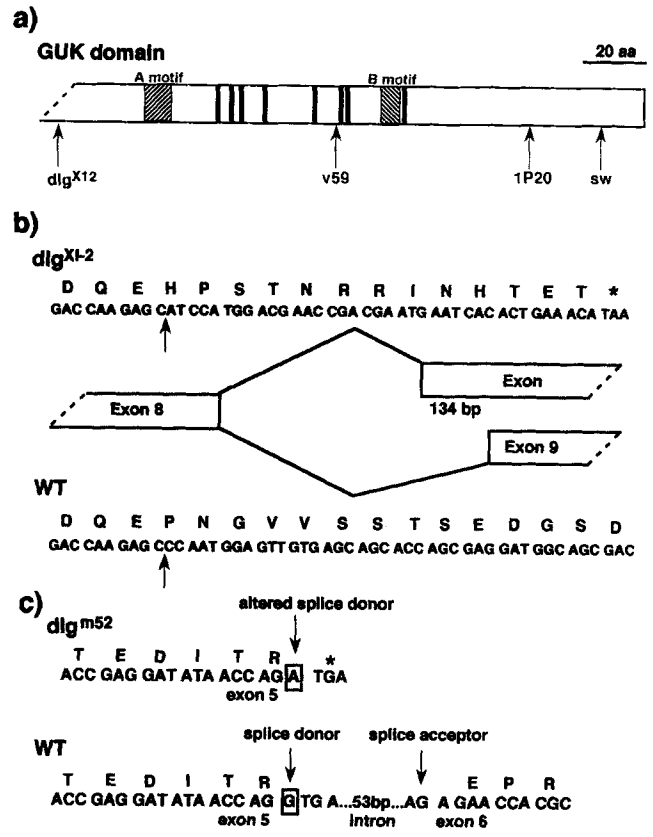


Figure 2. Molecular alterations of *dlg* alleles in complementation group I and III. (*a*) Truncations in the GUK domain caused by the various group III alleles. The A motif is the region that would bind ATP in a functional GUK, and the B motif is a Mg^{+2} binding site (Woods and Bryant, 1994). (*Black lines*) Conserved amino acids that would interact with GMP based on studies of yeast GUK (Stehle and Schulz, 1992). Each allele is predicted to truncate the protein, removing material to the right. (*b*) Molecular alteration in the Dlg protein caused by a splicing defect, which results in a 134-bp insert in exon 9 in transcripts from the dlg^{XI-2} allele. (*Arrow*) Junction of exon 8 and 9 in the cDNAs. Only the exon sequence is shown; the altered base in the intron has been spliced out. (*c*) Molecular alteration in the dlg^{m52} allele caused by a splicing defect at the beginning of PDZ/DHR3. *WT*, wild type.

Western Blot Analysis of the dlg Alleles

Affinity-purified antibodies to bacterially expressed Dlg fusion protein (Woods and Bryant, 1991) were used to perform a Western blot analysis of the Dlg protein from late third-instar tissues of wild-type and representative *dlg* alleles (Fig. 3). The antiserum recognizes two bands of ~110 and 116 kD in wild-type imaginal discs (Fig. 3), and an additional, higher molecular weight form (120 kD) in wild-type larval brains (Fig. 3). Neither tissue contained a 102-kD band, which would have been predicted based on the molecular characterization of the most abundant splice form, DlgA (Woods and Bryant, 1991), suggesting that the Dlg protein is posttranslationally modified to give rise to higher molecular weight peptides. Larval body wall, mainly composed of muscle, contains the 110-kD band as well as a lower molecular weight band of ~97 kD, which is in agreement with the results of Lahey et al. (1994). All of these bands are altered in mutant tissues in the way predicted from the molecular and genetic analyses of the *dlg* alleles (Table I and Fig. 2). Thus, dlg^{v59} tissues have a similar pattern of Dlg protein bands, compared with wild type, but with reduced molecular weights: 97, 101, and 105 kD in the discs and brains, and 82 and 101 kD in the body wall (Fig. 3). In addition, a smaller band at ~50 kD, which probably represents a breakdown product of the Dlg protein, is seen in imaginal discs. Since the dlg^{v59} animals also produce less Dlg protein, twice as much total protein was loaded into these lanes compared with the other samples. This reduction in Dlg protein was also noted by Lahey et al. (1994). Tissues isolated from dlg^{m30} animals have essentially normal amounts and sizes of Dlg protein, as would be predicted for a missense mutation (Fig. 3). Tissues from homozygous dlg^{m52} larvae, a null allele based on genetic

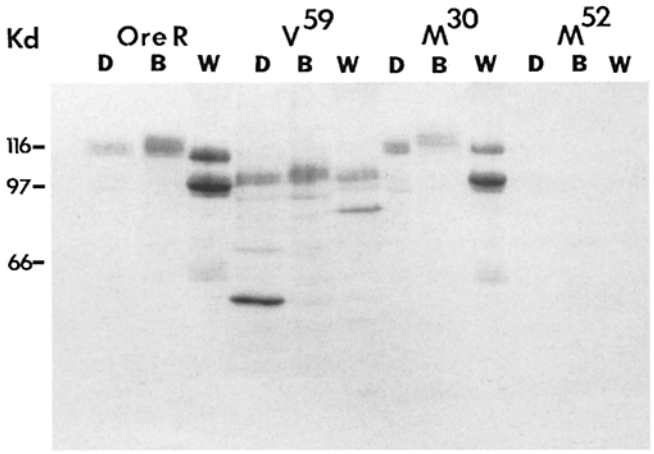


Figure 3. Western blot analysis of Dlg protein from tissues isolated from wild-type and several *dlg* mutants. *D*, wing imaginal discs from late third-instar larvae. *B*, brains from the same larvae. *W*, body walls from the same larvae.

complementation (Table I; see also Woods and Bryant, 1991) but based on our molecular data (Fig. 2), predicted to produce a truncated protein that would not be recognized by the Dlg antibodies (Woods and Bryant, 1991), have no detectable Dlg protein (Fig. 3). However, very long exposures of the blot (data not shown) reveal residual Dlg protein in the body wall, probably from maternally contributed Dlg, which is apparently stable in nonproliferating tissue (Perrimon, 1988; Woods and Bryant, 1991).

The DlgA Transcript Rescues Alleles of All Three Complementation Groups

Northern blot analysis of the *dlg* locus revealed the presence of at least five differently sized transcripts throughout development (Woods and Bryant, 1989). The most abundant form, *dlgA*, which encodes the DHR/PDZ, SH3, and GUK domains, has been the focus of our analysis. However, at least one minor alternatively spliced *dlg* transcript form, *dlgB*, does not contain the OPA or GUK-encoding exons (Woods and Bryant, 1991). To test whether loss of *dlgB* contributes to the *dlg* mutant phenotype, we transformed flies with a GAL4–UAS construct (Brand and Perrimon, 1993) containing a *dlgA* cDNA, with a complete open reading frame, that starts at 143 bp upstream of the initiating methionine and includes 40 bp of 3′ untranslated sequence. Flies carrying the *dlgA* construct were crossed to the GAL4 activator line 32B, which has moderate levels of expression throughout the larvae, including the imaginal discs, brain, gut, and salivary glands (data not shown; see also Brand and Perrimon, 1993). The heterozygous progeny were crossed to three sets of flies carrying various *dlg* alleles: dlg^{m52}, dlg^{m30}, or $dlg^{XI\text{-}2}$ that represent the three complementation groups of *dlg*. Progeny from this cross were scored for survival. The activated *dlgA* construct rescued to adulthood flies that contained each of the three *dlg* alleles, indicating that the DlgA protein alone can provide functional Dlg product for all three complementation groups.

Western blot analysis of this *dlgA* construct showed that both the 112- and 116-kD bands are present in imaginal discs but at several-fold higher levels than native Dlg (data not shown). No smaller-sized Dlg protein was observed on the blot. The presence of both of the higher molecular weight bands suggests that these two peptides are made by posttranslational modification of the DlgA splice form.

The Dlg Protein Localizes to the Cytoplasmic Face of Septate Junction in Salivary Glands

Using antibodies to the Dlg protein, we previously showed that, at the light microscopic level, the protein was localized at the position of the septate junction (Woods and Bryant, 1991). We have now studied its localization in salivary gland cells at the ultrastructural level using immunogold labeling and shown that the Dlg protein is localized at the cytoplasmic face of the septate junction (Fig. 4). The median distance between the gold particles and the midline of the septate junction in these experiments was 35–45 nm (n = 475 gold particles). No gold particles were found in the extracellular space or over the septa. A similar pattern of localization was found in the midgut (data not shown), which contains smooth rather than pleated septates (Tepass and Hartenstein, 1994).

Requirement of Dlg for Cell Polarity and Junctions

The apicobasal polarity of imaginal disc cells and the formation of septate junctions is dependent on the presence of the Dlg protein. The cells in a wild-type imaginal disc are highly columnar and have well-defined apical junctions (Figs. 5 *a* and 6 *a*). Imaginal disc cells homozygous for the complete loss-of-function *dlg* allele, dlg^{m52}, lack apicobasal polarity and septate junctions (Figs. 5 *c* and 6 *b*). However, the adherens junctions are still present; in fact, adherens junctions can be found at various ectopic positions on the cell membrane (see Fig. 6 *c*). In animals homozygous for the complementation group III allele $dlg^{XI\text{-}2}$, imaginal disc cells are much less columnar (Fig. 5 *b*), and they also show extensive overproliferation (Woods and Bryant, 1989). However, the apical junctions are still present, and the cells exhibit normal apicobasal polarity (Fig. 5 *b*; see also Abbott and Natzle, 1992).

The dependence of septate junction structure on functional Dlg is seen more clearly in the salivary gland, a differentiated epithelial tissue that grows during larval life by cell enlargement rather than proliferation. In wild-type larvae, salivary gland cells have enormous septate junctions that can extend over 100 μm of the lateral cell membrane (Fig. 7 *a*). Salivary gland cells of homozygous dlg^{m52} larvae still have obvious apicobasal polarity, but the septate junctions are reduced to a small fraction (~10%) of their normal length (Fig. 7 *b*). Spot adherens junctions (Tepass and Hartenstein, 1994) normally found at a more basal position on the lateral membrane are moved to a more apical location (Fig. 7 *b*), and the cells are rounder than normal (data not shown). The basolateral membrane is also highly disrupted in dlg^{m52} salivary glands since they are no longer tightly juxtaposed (compare Fig. 7 *c* and 7 *d*). In contrast, dlg^{v59} and dlg^{m30} show no obvious morphological or ultrastructural defects in the salivary glands.

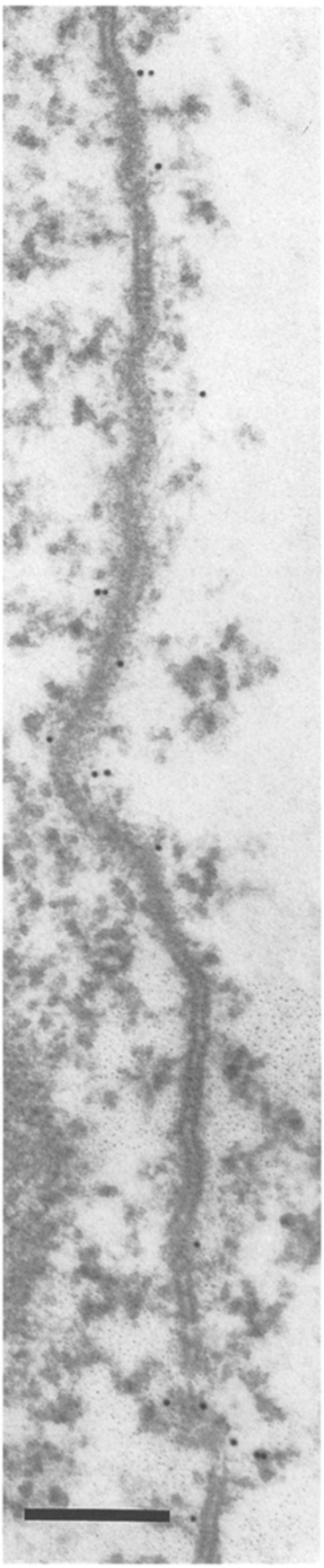


Figure 4. Ultrastructural localization of the Dlg protein shown by immunogold labeling in larval salivary glands. A transmission EM (TEM) section showing the septate junction. Note the presence of gold particles along the cytoplasmic face of the septate junction. Bar, 0.25 μm.

Role of the Dlg Protein in Controlling Localization of Other Proteins

Cytoskeletal Proteins. To determine how loss of apicobasal polarity reflects changes in the cytoskeleton, we examined the distribution of several different cytoskeletal proteins in epithelial cells from wild type and from larvae homozygous for the loss-of-function allele dlg^{m52}. Filamentous actin, which in wild-type epithelia is enriched at the apical end of the cell, is found throughout the cell in dlg^{m52} imaginal discs (Fig. 8, *a* and *b*). The organization of tubulin is also severely disrupted in the dlg^{m52} mutant tissue (Fig. 8, *c* and *d*). Therefore, both the microfilament and microtubule networks are disrupted by loss of the Dlg protein.

Two proteins in the band 4.1 family of membrane cytoskeletal proteins, Coracle (Cor), and Expanded (Ex), show a drastic alteration in distribution after loss of the Dlg protein. In wild type, Cor is highly enriched in the septate junctions of imaginal and some larval epithelia (Fig. 8 *e*; see also Fehon et al., 1994), whereas Ex is restricted to the apical end of the cell in a pattern similar to that observed with filamentous actin (Fig. 8, *a* and *e*; see also Boedigheimer and Laughon, 1993). Loss of the Dlg protein results in the distribution of Cor and Ex throughout the cell (Fig. 8 *f*). The effects of loss of Dlg are somewhat different in larval polytene cells. Cor antibodies fail to stain the salivary gland cells in wild type, but both Cor and Dlg are highly localized at the apicolateral membrane of cells in the proventriculus, presumably at the position of the septate junctions (Fig. 8 *e*). In this tissue, severe reduction of the Dlg protein in the dlg^{m52} allele results in the nearly complete loss of the Cor protein rather than mislocalization (Fig. 8 *g*).

Transmembrane Proteins. Loss of the Dlg protein also affects the distribution of fasciclin III (Brower et al., 1980; Patel et al., 1987) and neuroglian (Bieber et al., 1989), two transmembrane proteins thought to be involved in cell adhesion that are restricted to particular membrane domains in imaginal disc cells. Fasciclin III is highly enriched at the septate junction and is present in lower amounts in the lateral cell membrane, but it is excluded from the adherens junction (Fig. 9 *a*). Neuroglian is enriched at the apical end of the cell, reduced in the septate junction, and found again on the rest of the lateral cell membrane (Fig. 9 *c*). This restricted distribution is much more obvious for both proteins in the salivary gland. In this tissue, fasciclin III is found only in the septate junctions (Fig. 9 *e*), with neuroglian strictly excluded from these junctions but still enriched at the apical end of the cell (Fig. 9 *g*).

Localization of fasciclin III and neuroglian in both salivary glands and imaginal discs is dependent on Dlg. This is shown by staining mutant tissues from dlg^{m52} larvae with antibodies to the two proteins. When septate junctions are completely eliminated, as in the imaginal discs of dlg^{m52} homozygous mutant larvae (Fig. 6 *b*), both proteins are found apparently unrestricted along the cell membrane (Fig. 9, *b* and *d*). In fact neuroglian appears to have an elevated level of expression compared with wild type (Fig. 9, *c* and *d*). The large reduction but not complete loss of the septate junctions in salivary gland cells is associated with a concomitant reduction in the amount of fasciclin III (Fig. 9 *f*) and an expansion of neuroglian staining to most of the

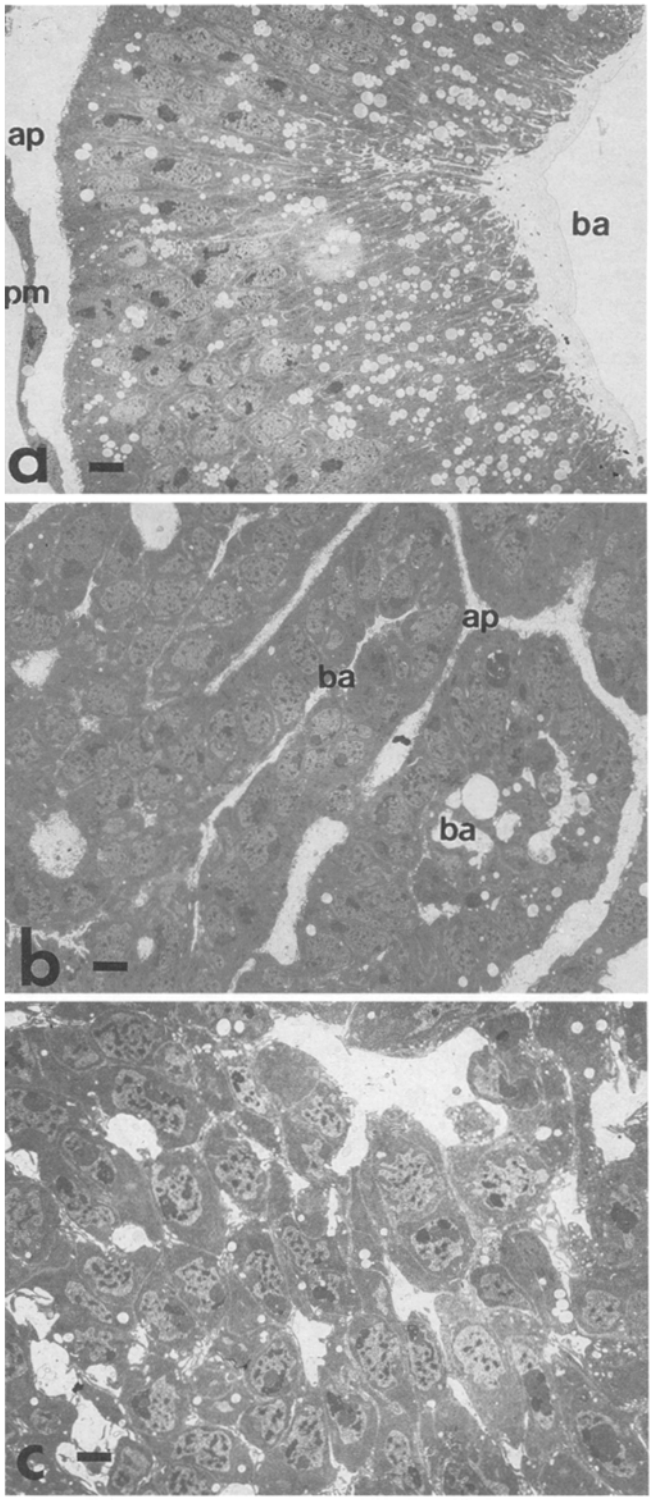


Figure 5. TEM of imaginal disc epithelia from wild-type and *dlg* mutants. (*a*) Wild-type wing imaginal disc. *ap*, apical; *ba*, basal; *pm*, peripodial membrane. (*b*) Wing disc from an animal homozygous for the $dlg^{X1\text{-}2}$ allele. Note the highly folded and overgrown but still recognizable epithelial structure. The cells still have an obvious apical and basal end. (*c*) Wing disc from an animal homozygous for the dlg^{m52} allele. Note the loss of epithelial organization. Bar, 10 μm.

lateral membrane (Fig. 9 *h*). This pattern of staining parallels the effect of dlg^{m52} on the septate junctions, which in salivary glands are reduced to <10% of the basolateral extent of wild type (Fig. 7 *b*).

Discussion

The dlg Locus Encodes a Multifunctional Protein

Our genetic and molecular data indicate that the Dlg protein has multiple functions, including the regulation of cell proliferation as well as the maintenance of septate junctions and apicobasal polarity of epithelial cells. Complete loss of the Dlg protein, caused by homozygosity for the group I allele dlg^{m52}, leads to overgrowth of imaginal discs and loss or severe reduction of the septate junctions in both imaginal and larval epithelia. Neuromuscular junctions are also reduced in larvae homozygous for dlg^{m52} (Lahey et al., 1994), indicating that Dlg plays a structural role in synaptic as well as septate contacts.

None of the alterations in *dlg* mutations were found to map to the DHR/PDZs, so our data do not address the functions of this domain. However, this domain has been found in at least 15 different proteins, including protein kinases, the Dishevelled protein, nitric oxide synthase, and several protein tyrosine phosphatases. Ponting and Phillips (1995) have suggested that, since the DHR/PDZ is found in several proteins associated with the cell cortex, it may act as a signal to localize proteins to their proper subcellular territory by binding to specific protein components. Data to support this model come from studies of the Fas-associated phosphatase 1 protein tyrosine phosphatase, in which one of the six DHR/PDZs has been shown to bind to the cytoplasmic domain of the Fas transmembrane receptor (Sato et al., 1995), from the demonstration of the binding of the *N*-methyl-D-aspartate receptor and Shaker-like K^+ channels to the DHR/PDZ2 domain in the rat homolog, PSD-95/SAP90 (Kornau et al., 1995; Kim et al., 1995), and from binding of the DHR/PDZ of the neuronal form of nitric oxide synthase to PSD-95/SAP90 and syntrophin (Brenman et al., 1996).

Analysis of group II and III alleles indicates that the two other conserved domains of Dlg, the SH3 and GUK, are necessary for growth regulation and maintenance of the septate junctions. A mutation in the SH3 domain (group II) causes loss of septate junctions and overproliferation in the imaginal discs but no obvious loss of septate junctions in the larval salivary glands. The SH3 motif has been found in many proteins and most of these appear to be involved in cell signaling cascades (Pawson, 1994). This domain acts as a region of protein–protein interaction by binding specific proline-rich motifs (Ren et al., 1993). A change of the conserved leucine to a proline in the SH3 domain of dlg^{m30} produces a mutant phenotype, demonstrating that this domain in Dlg is crucial for normal cell proliferation control, and raising the possibility that the mutant phenotype is due to disrupted binding of an unidentified protein. Another possibility suggested from the allelic complementation results is that Dlg normally acts as a multimer and that SH3 mediates a homotypic interaction between subunits. Accordingly, if the individual Dlg proteins are unable to form a multimer because of a disrup-

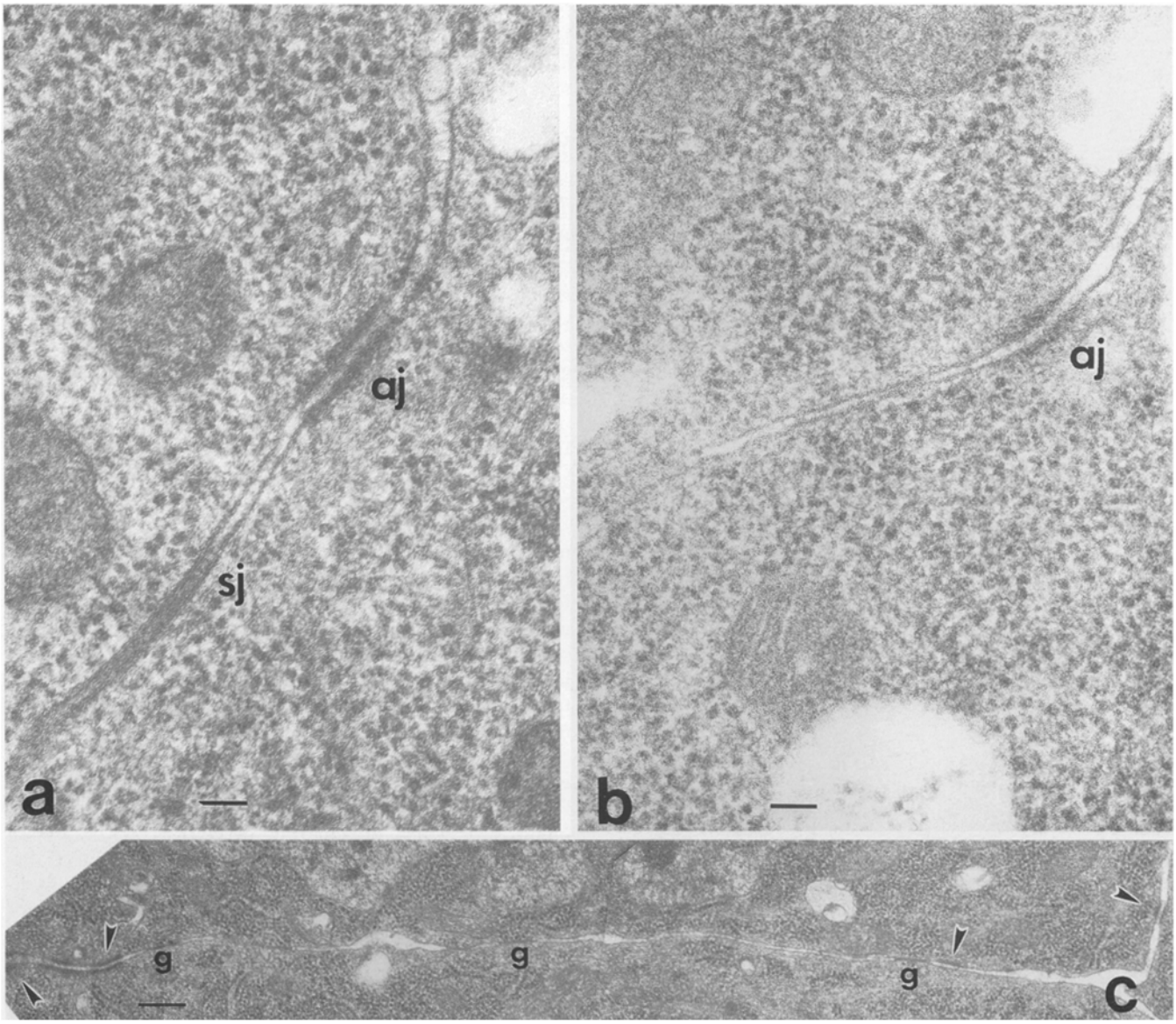


Figure 6. TEM of apicolateral cell junctions in wing discs. (*a*) Wild type. *aj*, adherens junction; *sj*, septate junction. (*b*) dlg^{m52}. Note the adherens junction (*aj*) but the lack of any organized septate junction. (*c*) A lower magnification view of dlg^{m52} wing disc cells showing multiple adherens-type junctions (*arrowheads*) on various parts of the cell. *g*, gap junctions. Bars: (*a* and *b*) 0.1 μm; (*c*) 1.0 μm.

tion of the SH3, then Dlg would be nonfunctional. In either case, the SH3 would act as a site of direct protein–protein binding.

Alleles causing deletions in the GUK domain (group III) cause epithelial overgrowth without affecting septate junction structure or cell polarity, and the severity of phenotype is correlated with the size of the deletion. Thus, animals carrying the $dlg^{X1\text{-}2}$ allele, which completely deletes the GUK domain, have overgrown imaginal discs that fail to differentiate into adult cuticle. A smaller truncation of the GUK domain, as in dlg^{sw} and dlg^{1P20}, results in essentially normal imaginal discs that can differentiate into adult structures, but the resulting adults have severe bristle and eye defects. The dlg^{1P20} mutation also affects planar polarity in the eye, as can be observed in the position of the bristle of each ommatidial cluster. Since the group II and III *dlg* alleles show complementation, the SH3 and GUK domains may have distinct functions, e.g., controlling subcellular localization vs regulating growth.

The GUK domain has a much more limited distribution than DHR/PDZ and SH3; it has thus far been found only in members of the MAGUK family of proteins. This domain is identified by its homology to the enzyme guanylate kinase, but it is not known whether it has similar catalytic activity. Our data on group III *dlg* alleles have shown that, unlike complete loss of Dlg, deletions in the GUK domain cause loss of normal growth and pattern without affecting epithelial structure. We previously suggested (Woods and Bryant, 1991) that this domain could function as a guanylate kinase and regulate the levels of guanine nucleotides at specific junctional contacts, thus leading to changes in the activity of ras-like proteins. In support of this model, it

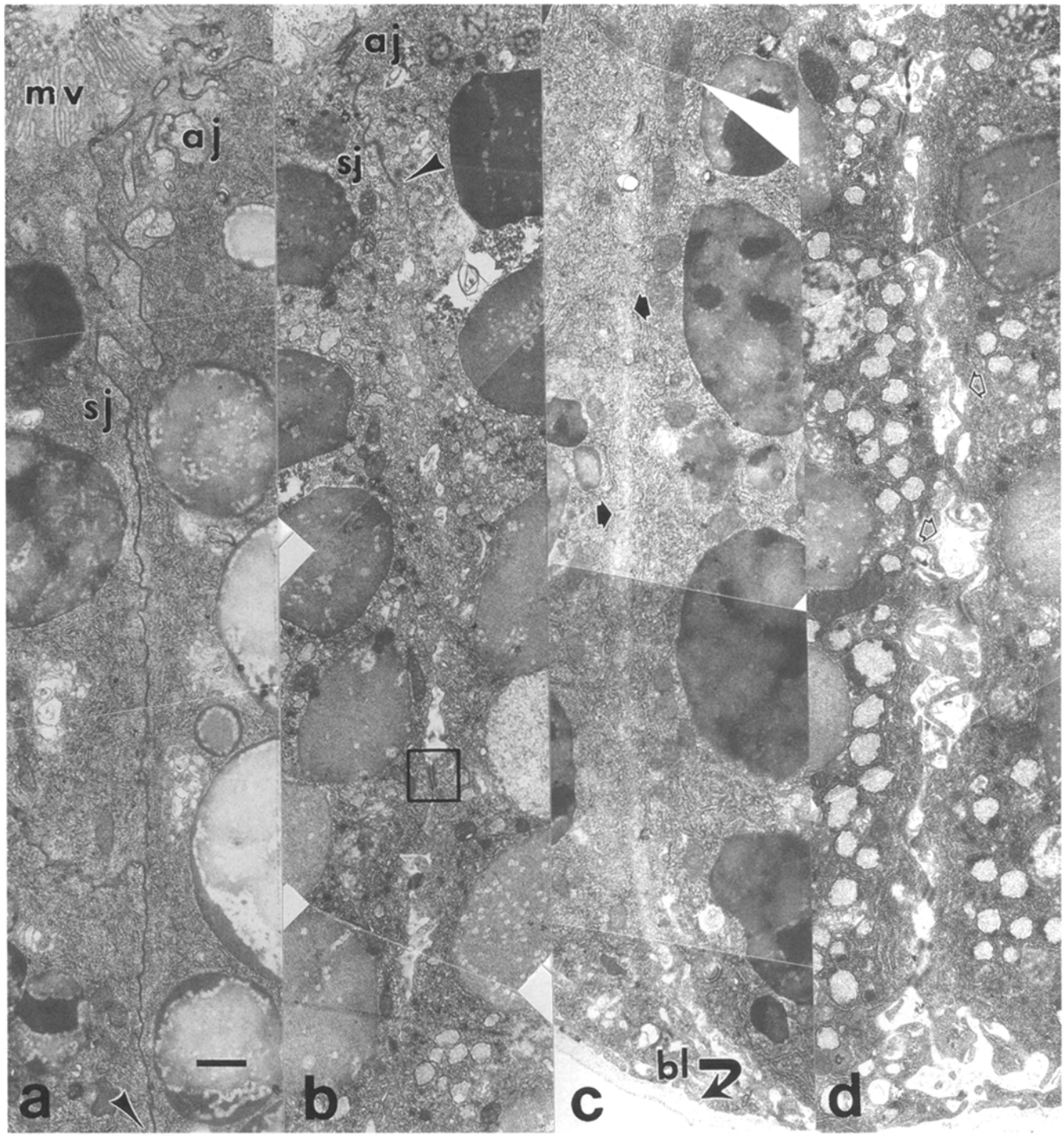
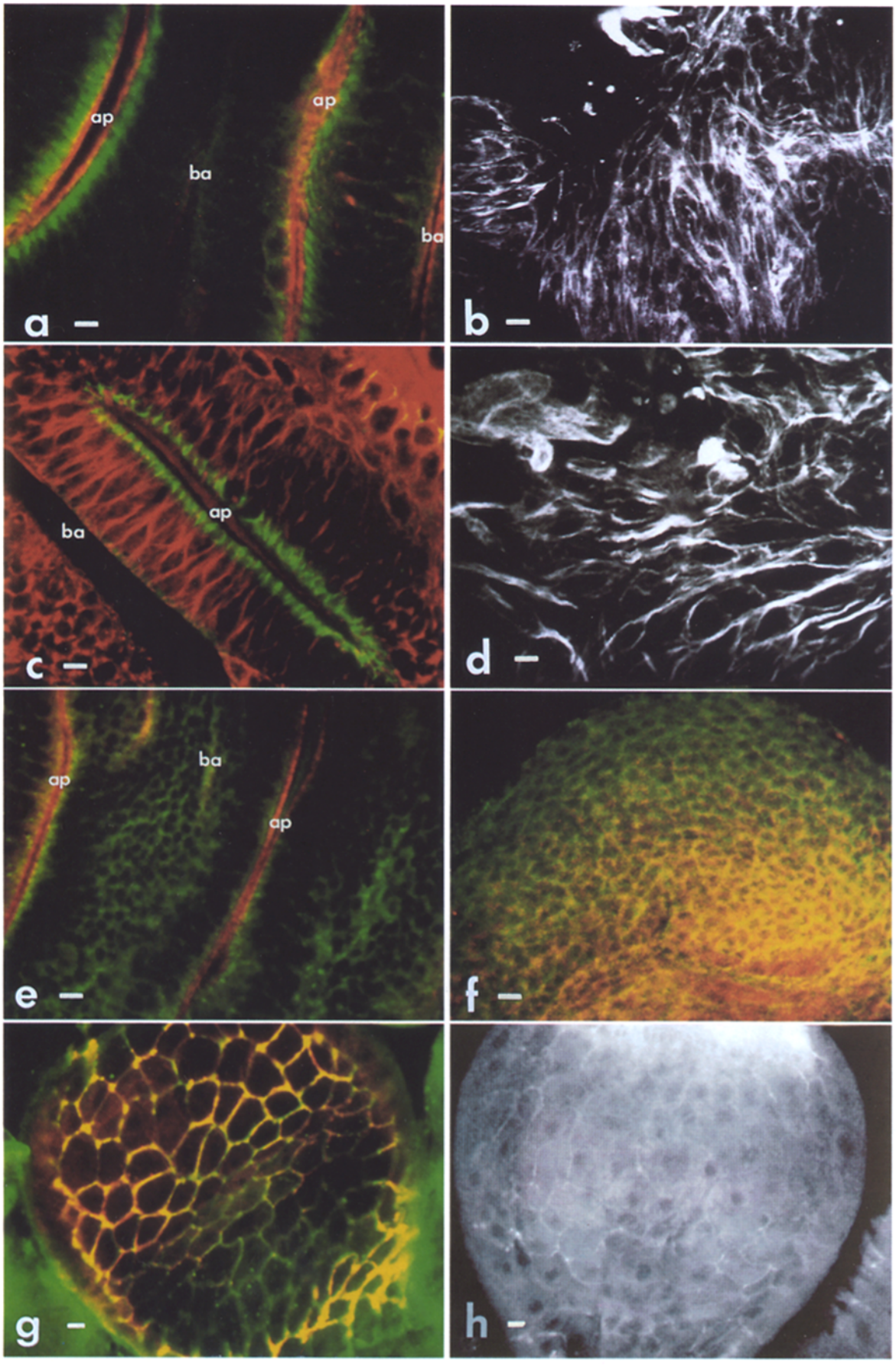


Figure 7. TEM of salivary glands from wild-type and dlg^{m52} larvae. All four images are at the same magnification, and apical is at the top. (*a*) Apicolateral region of two cells showing the apical microvilli (*mv*), the adherens junctions (*aj*), and the very extensive septate junctions (*sj*) extending to the end of the photograph (*arrowhead*). (*b*) A similar region from a dlg^{m52} gland. The septate junctions are reduced to ~10% of their normal length (*arrowhead*). A spot adherens junction is present at an abnormal apical position (*box*). (*c*) Basolateral end of the same cells as in *a*. *bl*, basal lamina. Note the tight association of the lateral membrane (*arrows*). (*d*) Basolateral end of dlg^{m52} cells. Note the highly folded lateral membrane and the nearly complete loss of membrane contact (*open arrows*). Bar, 4 μm.

has recently been shown that a small G-protein, rab13, is localized with the MAGUK ZO-1 to the tight junction (Zahraoui et al., 1994), and that the MAGUK p55 possesses measurable GUK activity (Chishti, A., personal communication). However, this kinase model may not be applicable to Dlg because of the three–amino acid deletion within the A motif, which in yeast GUK binds phosphate donor, ATP, used in the kinase reaction. These results have led to a second suggestion for the function of the GUK domain in Dlg; it could use GMP and/or gua-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nosine diphosphate (GDP) to modulate activation of a signal transduction pathway at the junction, analogous to the way that GTP and GDP regulate Ras-mediated signaling (McCormick et al., 1991). In support of this hypothesis, it recently has been demonstrated that the GUK domain of one of the vertebrate MAGUKs, hDlg/p97, binds GMP at very high affinity but lacks detectable GUK activity (Kistner et al., 1995). Both ZO-1 and ZO-2 have small deletions within the GUK region that should result in loss of GMP binding, so these MAGUKs are unlikely to be either guanylate kinases or GMP-binding proteins.

The Dlg Protein Is Required for Apicobasal Polarity in the Imaginal Epithelia

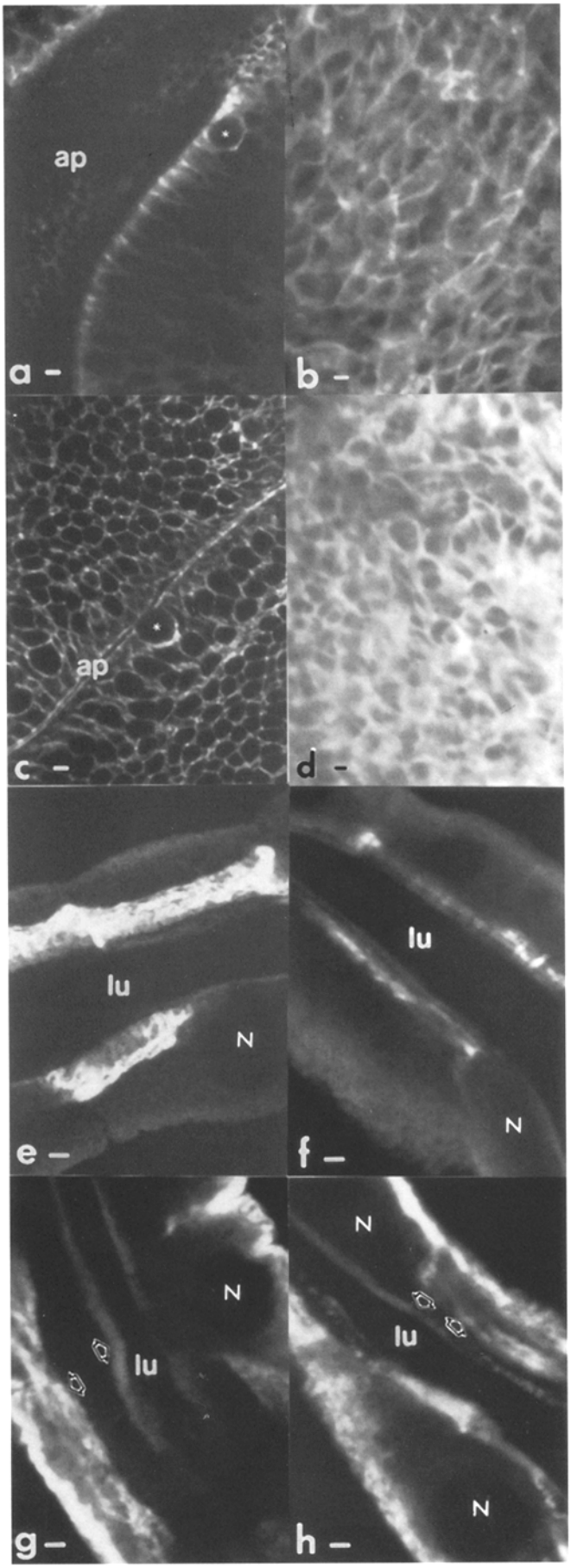
Our data clearly indicate that Dlg is necessary to maintain the septate junctions, apicobasal polarity, and normal internal structure of imaginal disc epithelial cells. This is the first example of the genetic loss of a septate junctional component causing structural and developmental changes. These changes include rearrangement of the cytoskeletal components (actin and tubulin) and proteins with a polarized distribution (Ex and Cor), abnormal localization of transmembrane proteins (fasciclin III and neuroglian) as well as loss of septate junctions, normal growth control, and the ability to differentiate (Stewart et al., 1972; Woods and Bryant, 1989). However, adherens and gap junctions are still present in *dlg* mutant imaginal discs. This indicates an important role for Dlg, and possibly the septate junctions, in maintaining the apicobasal axis and basolateral organization of both membrane and cytoskeletal structures. A similar role for tight junctions, the putative vertebrate equivalent of septate junctions, is indicated by experiments in which tight junctions were eliminated by the removal of Ca^{2+} (Cereijido et al., 1989; González-Mariscal et al., 1990; Contreras et al., 1992). Removal of Ca^{2+} also causes the loss of adherens junctions (Gumbiner, 1990), as well as altering the activity of many proteins such as protein kinase C (PKC), and recent work on the mammalian MAGUK ZO-1 has shown that PKC phosphorylation plays an important role in regulating tight junctions and epithelial structure (Balda et al., 1993). Furthermore, treatments that activate PKC function can allow the formation of tight junctions, accumulation of actin at the membrane, and normal apicobasal polarity under conditions that block adherens junction formation, such as low extracellular Ca^{2+} (Balda et al., 1993). Dlg appears to be highly phosphorylated, possibly on both serine/threonine and tyrosine (Wu, J.W., D. Woods, and P. Bryant, unpublished results), and has at least two potential PKC phosphorylation sites (Woods, D., unpublished results). We are now characterizing and investigating the potential function of these phosphorylations. The results presented in this paper on Dlg, and the previously published data about ZO-1, suggest that junctions containing MAGUKs, the septate junctions in *Drosophila* and the tight junctions in vertebrates, are necessary for proper apicobasal polarity in epithelial cells, and that Dlg and ZO-1 are required for the organization of these junctional complexes.

The MAGUKs p55 and hDlg/p97 have been shown to directly bind vertebrate protein 4.1 (Marfatia et al., 1994; Lue et al., 1994), and the regions of interaction are conserved in both Dlg and *Drosophila* protein 4.1, Cor, which colocalizes with Dlg to the septate junction of imaginal and some larval epithelia (Fehon et al., 1994; Fig. 8 *g*). Loss of Dlg leads to loss of Cor localization, and homozygous mutations in *cor* cause early lethality, giving rise to embryos with a dorsal cuticle hole or "topless" phenotype similar to that observed in embryos derived from females homozygous for dlg^{sw}. Based on the paradigm provided by the p55/4.1/glycophorin C interaction (Marfatia et al., 1994), we expect that this Dlg complex should also include a transmembrane component, as yet unidentified. The complete loss of Dlg would cause disruption of this network, resulting in loss of epithelial structure and cell polarity. However, residual maternal Dlg product and therefore septate junctions, as found in the larval epithelial tissues from dlg^{m52} mutant animals, is enough to control the localization of other proteins in the cell, including neuroglian and fasciclin III, and to maintain cell polarity.

Another gene in *Drosophila* in which mutations cause overproliferation and some loss of epithelial structure is *lethal(2)giant larvae (lgl)* (Gateff, 1978). The Lgl protein has been shown to be part of the cytoskeleton (Strand et al., 1994*b*) and directly associated with nonmuscle myosin II heavy chain (Strand et al., 1994*a*). However complete loss of the Lgl protein does not disrupt the adherens or septate junctions (Gateff et al., 1984), and the imaginal disc cells still maintain a polarized epithelial structure based on protein markers (Woods, D., unpublished results).

The establishment of apicobasal polarity in the ectoderm during embryonic development of *Drosophila* seems to involve mechanisms distinct from maintenance of polar-

Figure 8. Cytoskeletal proteins of wild-type and dlg^{m52} epithelia seen in confocal optical sections. (*a*, *c*, *e*, and *g*) Wild type. (*b*, *d*, *f*, and *h*) dlg^{m52} homozygotes. Apical (*ap*); basal (*ba*). (*a*) Section of a wing imaginal disc stained with antibodies to Dlg (*green*) and rhodamine-phalloidin (*red*) to visualize filamentous actin. Since the tissue contains several folds, more than one section of epithelium is visible. Note the location of filamentous actin apical to Dlg. (*b*) Mutant wing imaginal disc stained with rhodamine-phalloidin, showing delocalization of actin. (*c*) Wing imaginal disc stained with antibodies to Dlg (*green*) and tubulin (*red*). Tubulin is found in filaments that run apicobasal in the cell and start near the septate junction. (*d*) Mutant wing disc stained with antibodies to tubulin. Note the loss of organization within the cells. (*e*) Wing imaginal disc stained with antibodies to Cor (*green*) and Ex (*red*). Cor is highly enriched at the level of the septate junction, whereas Ex is more apical. (*f*) Wing disc mutant for Dlg stained for Cor and Ex. Note the lack of any obvious localization and the overlap in staining (*yellow*). (*g*) Section near the apical end of the proventricular epithelium, a larval tissue, stained with antibodies to Dlg (*green*) and Cor (*red*). The honeycomb-like appearance is expected from a protein localized to the septate junctions. Note the extensive overlap in staining (*yellow*). (*h*) Similar section of a proventriculus from Dlg mutant larva. The image was intentionally overexposed to reveal Cor staining, resulting in background cytoplasmic signal. The tissue fails to stain with Dlg antibodies, and only a residual amount of Cor staining is detectable. However, the staining is still localized to the apical end of the cells. Bars: (*a–c*, *e*, and *f*) 20 μm; (*d*, *g*, and *h*) 10 μm.



ity in the imaginal epithelium. For example, Crumbs (*crb*) has been shown to be involved in setting up polarity of embryonic epithelium (Wodarz et al., 1995), but mutations in *crb* show no effect on differentiation in homozygous mitotic-recombination clones in the imaginal discs, suggesting that Crb is not necessary to maintain apicobasal polarity in imaginal epithelium (Tepass and Knust, 1993; Knust et al., 1993). Similarly, Dlg is maternally loaded into the egg and has been shown, by analysis of germ-line clones, to be important for embryonic development well before formation of the septate junctions (Perrimon, 1988; Woods and Bryant, 1991; Tepass and Hartenstein, 1994). Loss of Dlg in the embryo results in a variety of phenotypes, including terminal and segmentation defects, but the ectoderm cells are still able to form polarized sheets since they can secrete cuticle (Woods and Bryant, 1991). Milder phenotypes include loss of polarity in the basal migration of cells during neuroblast segregation and failure of dorsal closure, which results in a hole in the dorsal cuticle (Perrimon, 1988). However, most of the affected developmental events occur before the appearance of septate junctions, indicating that the Dlg protein has a function during embryogenesis that is not related to the junctional structure or apicobasal polarity.

Maintenance of apicobasal polarity and apical junctional contacts is crucial for the normal development of epithelial tissues (Woods and Bryant, 1993*b*). One reason for this may be that localization of signal transduction proteins to junctional contacts increases the cellular sensitivity to a transmitted signal by concentrating the receiving proteins to one part of the cell. Our hypothesis is that the Dlg protein acts in such a localized signaling pathway to control growth and patterning in the imaginal disc. We are now isolating proteins that interact with Dlg to further characterize this signaling pathway and to identify other components of the septate junction.

Figure 9. Membrane proteins of wild-type and dlg^{m52} epithelia seen in confocal optical sections. (*a*, *c*, *e*, and *g*) Wild type. (*b*, *d*, *f*, and *h*) dlg^{m52} homozygotes. Apical (*ap*); luminal space (*lu*). (*a*) Wing imaginal disc stained with antibodies to FasIII. Note the enrichment of staining near the apical end of the epithelium at the level of the septate junctions. *, dividing cell. (*b*) Mutant wing disc stained with antibodies to FasIII. Note the loss of FasIII localization. (*c*) Wing disc stained with antibodies to neuroglian. The antibodies stain most of the membrane except for a reduction of staining in the apicolateral area that, based on double staining with antibodies to Dlg (data not shown), is at the level of the septate junction. *, dividing cell. (*d*) Mutant disc stained with antibodies to neuroglian. Note the staining throughout the cell membranes. (*e*) Late third-instar wild-type salivary gland stained for FasIII. A band of staining is found along the apicolateral membrane at the level of the septate junction. *N*, nucleus. (*f*) Mutant salivary gland stained with FasIII antibodies showing the severe reduction in protein. (*g*) Wild-type salivary gland stained with antibodies to neuroglian. All of the cell membrane stains except the region that contains the septate junctions (*arrows*). (*h*) Mutant gland showing the expansion of neuroglian to include most of the lateral membrane. Note the loss of unstained lateral membrane (compare the distance between the *arrows* in *g* and *h*). Bars: (*a–d*) 10 μm; (*e–h*) 25 μm.

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