Assembly of *C. elegans* apical junctions involves positioning and compaction by LET-413 and protein aggregation by the MAGUK protein DLG-1

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SUMMARY

Specialised subapical junctions play a critical role in maintaining epithelial cell polarity and tissue integrity, and provide a platform for intracellular signalling. Here we analyse the roles of *C. elegans* genes *let-413* and *dlg-1*, a homologue of *Drosophila* lethal discs large, in the assembly of the *C. elegans* apical junction (CeAJ), and provide the first characterisation of this structure. We have identified *dlg-1* as an essential gene in an RNA interference screen against *C. elegans* homologues of genes encoding proteins involved in tight or septate junction formation. We show that DLG-1 colocalises with the junctional protein JAM-1 at CeAJs in a unit distinct from HMP-1/α-catenin, and apical to the laterally localised LET-413. Loss of *dlg-1* activity leads to JAM-1 mislocalisation and the disappearance of the electron-dense component of the CeAJs, but only mild adhesion and polarity defects. In contrast, loss of *let-413* activity leads to the formation of basally extended discontinuous CeAJs and strong adhesion and polarity defects. Interestingly, in LET-413-deficient embryos, CeAJ markers are localised along the lateral membrane in a manner resembling that observed in wild-type embryos at the onset of epithelial differentiation. We conclude that the primary function of LET-413 is to correctly position CeAJ components at a discrete subapical position. Furthermore, we propose that DLG-1 is required to aggregate JAM-1 and other proteins forming the electron-dense CeAJ structure. Our data suggest that epithelial adhesion is maintained by several redundant systems in *C. elegans*.

Key words: *C. elegans*, epithelial cell polarity, adherens junction, MAGUK protein, α-catenin, cell adhesion, LET-413

INTRODUCTION

During the development of an organism, epithelial tissues provide the basis for major morphogenetic changes. Epithelial cells possess particular characteristics that are essential for their function, such as specialised cell junctions and a distinct apico-basal polarity. Two major types of subapical junctions can be distinguished by electron microscopy in epithelial cells (for recent reviews, see Muller, 2000; Tsukita et al., 1999; Yeaman et al., 1999). Tight junctions (TJs) in vertebrates and septate junctions (SJ) in *Drosophila* are involved in controlling paracellular solute transport, otherwise known as the gate function. In addition, TJs maintain apico-basal polarity. Adherens junctions (AJs) in vertebrates and invertebrates are essential for cell adhesion. These junctions have been studied extensively in cell culture and *Drosophila*, but it is still not fully understood how they are organised or how the junctional components contribute to their function.

Cell junctions are specialised domains of the plasma membrane where transmembrane (TM) proteins are clustered and linked to the cytoskeleton via cytoplasmic proteins, thereby ensuring tight cell contacts and efficient cell adhesion. Aggregation of proteins at cell junctions is also thought to facilitate intracellular signalling. Occludin and claudins are TM proteins of TJs (Tsukita et al., 1999), whereas neurexin IV (NrxIV) is the only known TM protein in *Drosophila* SJs (Bauemgartner et al., 1996). Proteins belonging to the membrane-associated guanylate kinase-like (MAGUK) family appear to act as central players at the interface between junctional TM proteins and signalling pathway components or the cytoskeleton (Dimitratos et al., 1999). These proteins typically have 1 or 3 PDZ (PSD-95/Dlg/ZO-1) domains, an SH3 (src homology 3) domain, and a GUK (guanylate kinase-like) domain, all of which are involved in protein-protein interactions (Deguchi et al., 1998; Pawson and Scott, 1997; Ponting et al., 1997). Previously characterised MAGUKs include *Drosophila* Lethal, Discs Large (Dlg) and its human homologue hDlg/SAP97, as well as vertebrate Zonula Occludens-1 (ZO-1) and the *Drosophila* homologue Tamou (Lue et al., 1994; Takahisa et al., 1996; Willett et al., 1993; Woods and Bryant, 1991). Their function has been investigated extensively in the nervous system where they have been implicated in the clustering of TM receptors and ion channels at synapses, and in facilitating signal transduction (Garner et al., 2000). Genetic studies in *Drosophila* reveal that, in epithelia, the SJ-associated protein Dlg is required for SJ and AJ formation and the maintenance of epithelial polarity, while mutations inactivating other SJ components, such as NrxIV and Coracle (Cor), only affect the gate function (Bauemgartner et al., 1996; Lamb et al., 1998; Woods et al., 1996). There are
other functionally significant TJ/SJ-associated proteins, some of which are conserved between vertebrates and *Drosophila*. These include the PDZ-containing vertebrate AF-6/afadin or its fly homologue Canoe, the afadin-binding protein ponsin, cortactin/Dcoractin, band 4.1 protein/Coracle and symplekin (Asakura et al., 1997; Ikeda et al., 1999; Katsube et al., 1998; Keon et al., 1996; Lamb et al., 1998; Mandai et al., 1999; Matsuo et al., 1999; Zhadanov et al., 1999).

Within AJs, the TM protein cadherin emerges as the main adhesion molecule. Cadherins form homophilic bonds through their extracellular domains and interact with the actin cytoskeleton via cytoplasmic α- and β-catenins. Genetic studies in mouse and *Drosophila* have shown that AJs are essential for the maintenance of cell adhesion and epithelial polarity (for reviews, see Aberle et al., 1996; Knust and Leptin, 1996; Tepass, 1999; Yap et al., 1997). In *C. elegans*, the cadherin/β-catenin complex encoded by the genes *hmr-1*, *hmp-1* and *hmp-2* respectively, is essential for anchoring the actin cytoskeleton to the plasma membrane (Costa et al., 1998). However, it does not appear to support the adhesion and polarity functions that are attributed to the cadherin/catenin complex in other organisms.

Time-lapse imaging and genetic analyses suggest that the assembly of epithelial junctions is a complex and sequential process. In cultured MDCK cells, it has been shown that cell-cell contacts initiate the segregation of proteins into different domains of the membrane via Ca2+-dependent cadherin cell adhesion (Adams et al., 1998). In *Drosophila*, the assembly of AJs depends on the activity of two sets of proteins that act on either side of the future AJ. Basal to the AJ, Scribble (Scrib) has recently been implicated in maintaining epithelial cell integrity and apico-basal polarity (Bilder et al., 2000; Bilder and Perrimon, 2000). These functions have also been attributed to the basolateral Scrib homologue, LET-413, in *C. elegans* (Legouis et al., 2000). Scrib probably functions in a common pathway with Dlg and Lethal giant larvae (Lgl), as there are strong genetic interactions between the genes encoding these proteins, and as Scrib and Dlg colocalise to SJs and overlap with the membrane domain of Lgl (Bilder et al., 2000). Apical to the *Drosophila* AJ, it has been proposed that the TM protein Crumbs and its interacting partner, the multi-PDZ-domain protein Discs Lost, form a scaffold that allows the assembly of proteins leading to mature AJs (Bhat et al., 1999; Klebes and Knust, 2000; Wodarz et al., 1995).

We have been using *C. elegans* as a model system to investigate the epithelial cell characteristics of apico-basal polarity and specialised cell junctions. In contrast to other organisms, *C. elegans* epithelia possess only one type of apical junction, which has been referred to as a belt desmosome (Priess and Hirsh, 1986) or an AJ (Costa et al., 1998; Mohler et al., 1998; Podbilewicz and White, 1994; Raich et al., 1999) due to its ultrastructure, but which we will call the *C. elegans* apical junction (CeAJ). Two sets of proteins, the above-mentioned cadherin/catenin complex (Costa et al., 1998) and a protein called JAM-1 (Priess and Hirsh, 1986; Francis and Waterston, 1991; Podbilewicz and White, 1994; Mohler et al., 1998), have been reported to localise to CeAJs, based on their subapical localisation and analogy with other systems (for the cadherin/catenin complex; Costa et al., 1998), or on immunogold experiments (for JAM-1; D. Hall, personal communication). LET-413 is the only protein implicated to date in CeAJ assembly and apical-basal polarity maintenance in *C. elegans* epithelial cells (Legouis et al., 2000). *C. elegans* possesses homologues of proteins associated with TJ/SJs or involved in their assembly, although a structure resembling these junctions has not been described. We thus decided to investigate whether these homologues were involved in generating functional epithelial cells using an RNA interference (RNAi) approach (Fire et al., 1998). We found that the only gene encoding an essential epithelial protein is the *C. elegans* homologue of *Drosophila* dlg, which we have named *dlg-1*. As the phenotype of DLG-1-deficient embryos resembled that of let-413 mutants, we investigated whether these genes act in the same pathway.

Employing newly developed tools, we analysed the composition of CeAJs in LET-413- and DLG-1-deficient embryos compared with the wild-type situation. We show that DLG-1 is required for CeAJ assembly, but not to maintain cell polarity in the same way as LET-413, and does not colocalise with LET-413. We propose a model for CeAJ assembly and function.

**MATERIALS AND METHODS**

**Strains and GFP markers**

Animals were maintained as described (Brenner, 1974). The wild-type reference strain is Bristol N2 (Brenner, 1974). The following markers were used: *jcs1 [unc-29 (+) – rol-6 (su1006) – jam-1::gfp]*; the pML624 plasmid, which is a che-14::gfp translational fusion; and the pML801 plasmid, which is a functional *let-413::gfp* translational fusion (Legouis et al., 2000; Michaux et al., 2000; Mohler et al., 1998). To obtain LET-413-deficient embryos we performed RNAi against *let-413*, which has been shown to result in the same phenotype as strong loss of function or null *let-413* alleles (Legouis et al., 2000).

**Molecular biology**

We carried out Blast searches to identify possible homologues of proteins involved in TJ/SJ formation in the *C. elegans* genome (The *C. elegans* sequencing Consortium, 1998). Specific regions of genes encoding these homologues (Table 1) were PCR-amplified using primers carrying a T3 promoter sequence (A TTAACCCTCACTAAAGATCATC::promoter sequence up to the preceding gene) we cloned almost all the *C. elegans* coding sequence and 7 kb of 5′UTR RNAi). To obtain *let-413* mutants, we employed newly developed tools, we analysed the composition of CeAJs in LET-413- and DLG-1-deficient embryos compared with the wild-type situation. We show that DLG-1 is required for CeAJ assembly, but not to maintain cell polarity in the same way as LET-413, and does not colocalise with LET-413. We propose a model for CeAJ assembly and function.
Assembly of C. elegans apical junctions

Table 1. RNAi screen for C. elegans homologues of proteins involved in TJ/SJ formation

<table>
<thead>
<tr>
<th>Vertebrate/Drosophila homologue</th>
<th>C. elegans homologue</th>
<th>E-value*</th>
<th>Position of RNAi primers†</th>
<th>dsRNA length</th>
<th>RNAi phenotype</th>
</tr>
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<tbody>
<tr>
<td>hDlg/SAP97; Dlg</td>
<td>C25F6.2</td>
<td>5e-142</td>
<td>X</td>
<td>35540-36916</td>
<td>1159 &gt;99% EmbI</td>
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<tr>
<td>ZO-1; Tamou</td>
<td>Y105E8B.Z</td>
<td>e-100</td>
<td>I</td>
<td>21133-21901</td>
<td>783 nop**</td>
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<tr>
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<td>Cortactin/D-cortactin</td>
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*Expectation value, indicating the number of Blast hits one would expect to find by chance in a Blast search carried out with each C. elegans predicted ORF; ‡‡C. elegans linkage group; §¶Refers to the position of primers on the corresponding cosmid sequence used to generate dsRNA; ¶¶embryonic lethality; **no obvious phenotype; ‡‡pooled dsRNAs for ZK270.2, C48D5.2A, T04C9.6 and H05G16.1 injected together gave no phenotype either.

RESULTS

C25F6.2, a putative homologue of Drosophila Dlg, is necessary for epithelial cell junction integrity and morphogenesis

There are three main classes of epithelia in the C. elegans embryo: epidermal, intestinal and pharyngeal marginal cells. The epidermis is essential for morphogenetic changes during embryogenesis. Soon after terminal differentiation, epidermal cells extend around the developing embryo forming new cell contacts at the ventral surface, a process known as enclosure (Sulston et al., 1983; Williams-Masson et al., 1997). This is followed by a fourfold elongation of the embryo, which is driven by contraction of circumferentially aligned actin bundles in the epidermis (Priess and Hirsh, 1986; Sulston et al., 1983). The developmental stage of a wild-type embryo is determined by its shape, indicating the stage of elongation it has achieved, thus, comma stage (end of enclosure), 1.5-fold stage (beginning of elongation; Fig. 1A), two-fold and three-fold stages (mid-elongation) and pretzel stage (end of elongation; Fig. 1B).

We used RNAi (Fire et al., 1998) to screen predicted genes encoding homologues of proteins involved in TJ/SJ formation in order to investigate their functions in C. elegans (Table 1). Distribution of the subapical protein JAM-1 and Dlg/hDlg respectively. F25G6.2(RNAi) embryos (i.e. embryos that lack F25G6.2 function as a result of RNAi) arrested prior to the 150-cell stage with rounded cells, indicating a possible cell adhesion problem, and no visible tissue differentiation (data not shown).

Due to the early embryonic arrest phenotype of F25G6.2(RNAi) embryos, we decided to concentrate instead on C25F6.2, which we have named dlg-1. RNAi against dlg-1 resulted in lethality during embryonic morphogenesis. dlg-1(RNAi) embryos arrested at the two-fold stage of elongation.

Staining and microscopy

To assess the difference in DLG-1::GFP levels between uninjected and dlg-1(RNAi) dlg-1::gfp transgenic animals, fluorescent images of expressed GFP were captured using a Zeiss Axiophot microscope and the Coolsnap acquisition system (version 1.2). Immunocytochemistry was performed either as described (Labouesse et al., 1996) or using the freeze-crack, methanol/acetic acid fixation method (Miller and Shakes, 1995). Images were captured on a Leica TCS3 C confocal microscope (immunostaining) or on a Zeiss Axiophot microscope (Nomarski, 1986). To obtain transverse sections in the Z-axis starting from confocal images, we captured 25 or 36 lateral sections spaced 0.25 μm apart, covering on average one third to one half of the embryonic thickness. Subsequently, these images were processed using the TCSTK program, an interactive tool for processing and viewing confocal microscopy data (J.L.V., unpublished). One of its functions is the 3D-projection mode, which allowed us to view orthogonal slices at different positions within the embryo. Each transverse slice along the apico-basal axis had a depth of 25 or 36 sections and a width of 512 pixels. In order to score the spread (extent) and the position of JAM-1 or HMP-1 in order to investigate their functions in C. elegans (Table 1). Distribution of the subapical protein JAM-1 was used to denote the basal extent of the lateral membrane for each cell. The spread of JAM-1 or HMP-1 was calculated as a percentage of the total membrane length. To assess position, each lateral membrane was divided into three compartments along its height (top, middle and bottom) and the detected proteins were scored as present or absent in each section.

Electron microscopy

Preparation of embryos and electron microscopy analysis were carried out as described (Legou et al., 2000). All embryos were harvested between the 1.5- and two-fold stages. Blocks of four embryos were sectioned and analysed for wild-type and RNAi embryos, with sections containing between one and four embryos. Sections were taken either transversely or longitudinally. The presence or absence of electron-dense apical junctions was scored only where apposing cell membranes were clearly visible between neighbouring epithelial cells.
with vacuoles in epithelial tissues and leakage of a small amount of cytoplasm from the tip of the tail and ventral surface (Fig. 1C,D). Immunostaining with the monoclonal antibody MH27 (which recognises JAM-1; Francis and Waterston, 1991; Mohler et al., 1998) showed a completely discontinuous pattern in dlg-1(RNAi) embryos (Fig. 1G,H), in contrast to the belt-like staining observed in wild-type embryos (Fig. 1E,F). This punctate MH27 pattern is strikingly similar to that of let-413 mutant embryos, which display a more severe phenotype, either arresting around the 1.7-fold stage of elongation or rupturing from the ventral surface (Legouis et al., 2000). These results suggest that epithelial integrity is compromised due to abnormal CeAJs in dlg-1(RNAi) embryos.

**Fig. 1.** dlg-1(RNAi) embryos show abnormal morphogenesis and epithelial defects. (A-D) Nomarski interference microscopy of wild-type and dlg-1(RNAi) embryos. (E-H) Confocal images showing immunostaining with the monoclonal antibody MH27, which recognises CeAJs in the epidermis, pharynx (arrows) and intestine (arrowheads). (C,E,G) External focal plane showing the epidermis. (A,B,D,F,H) Internal focal plane showing the pharynx and intestine. (A,E,F) 1.5-fold stage wild-type embryos (6-7 hours development). (B) Wild-type pretzel stage embryo (9-10 hours development). (C,G,H) dlg-1(RNAi) embryos at 6-7 hours development. (D) dlg-1(RNAi) embryos at 9-10 hours development. In dlg-1(RNAi) embryos, CeAJ staining is punctate and worsens with age; epidermal CeAJs are more abnormal than in the pharynx or intestine. In C and D, white arrows indicate epithelial vacuoles. Also in C a black arrowhead indicates abnormal bulges at the embryo surface and in D a white arrowhead indicates loose cells that have leaked out of the embryo. In this and all following figures unless otherwise stated, the embryos are orientated with the anterior to the left and dorsal to the top of the image. Scale bar,10 μm.

**dlg-1 encodes a member of the MAGUK family of proteins**

ACeDB (A C. elegans DataBase) predictions, cDNA sequencing and RT-PCR reveal that dlg-1 (Fig. 2Ai) encodes a protein of 967 amino acids with closest homology to the *Drosophila* and human MAGUK proteinsDlg and hDlg/SAP97 respectively (Fig. 2Aii). Pfam database analysis indicated that DLG-1 possesses three PDZ domains (of 86, 85 and 80 amino acids respectively), an SH3 domain of 62 amino acids, and a GUK domain of 102 amino acids. In several MAGUK proteins, the region between the SH3 and GUK domains, known as the HOOK domain, is required for the localisation of Dlg to the SJs in *Drosophila* and interaction with the cytoskeleton band 4.1 protein in vertebrates (Hough et al., 1997; Lue et al., 1994; Marfatia et al., 1996). This region in DLG-1 shows only 32% similarity/43% identity with Dlg and hDlg/SAP97 isoform 2 and the configuration of lysine residues thought to bind protein 4.1 is not conserved, but it includes a particularly conserved FSRKFPF motif of unknown function (Lue et al., 1994; Marfatia et al., 1996).

**DLG-1 is localised to the subapical membrane of epithelial cells**

To determine the expression pattern and subcellular localisation of DLG-1, we constructed a translational fusion between dlg-1 and a cDNA coding for green fluorescent protein (GFP), which was inserted in the penultimate exon of the dlg-1 coding sequence (Fig. 2Ai, large arrows). As the dlg-1::gfp construct is 106 bases shorter than the full coding sequence, and there were no available dlg-1 mutants, we determined the functionality of the DLG-1::GFP using an RNAi approach. We designed dsRNA against the 3’UTR sequence of dlg-1 (Fig. 2Ai, small arrows) and verified that the dlg-1(3’UTR RNAi) phenotype was as described above for dlg-1(RNAi). The dlg-1::gfp plasmid does not possess the dlg-1 3’UTR sequence and therefore should not be affected by the 3’UTR dsRNA. In this way we specifically inactivated endogenous DLG-1 and examined whether DLG-1::GFP was able to ‘rescue’ the dlg-1(3’UTR RNAi) phenotype. The results are shown in Table 2 and clearly establish that the DLG-1::GFP is functional.

To assess the efficiency of dlg-1(RNAi), we injected dsRNA against the dlg-1 coding region (Fig. 2Ai, arrowheads) into dlg-1::gfp transgenic animals and examined GFP expression in the progeny. We found that DLG-1::GFP expression was greatly reduced in the progeny of injected animals compared to the progeny of uninjected controls and that a dlg-1(RNAi) phenotype was produced, which indicates that dsRNA against

<table>
<thead>
<tr>
<th>Table 2. ‘Rescue’ of dlg-1 3’UTR RNAi phenotype by transgenic DLG-1::GFP</th>
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<tr>
<td><strong>Percentage embryonic lethality</strong>*</td>
</tr>
<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>dlg-1::gfp transgenic worms</td>
</tr>
</tbody>
</table>

*The embryonic lethal phenotype in all cases is that described as the dlg-1(RNAi) phenotype. ‡95.2% of these dlg-1::gfp transgenic worms grew normally to adulthood and were fertile.
dlg-1 specifically and significantly reduces DLG-1 levels in the embryo (Fig. 2B,C).

DLG-1::GFP was first detected at the 350-cell stage in differentiating epithelial cells and neuroblasts (data not shown). The neuronal expression was transient and its functional significance was not investigated. After ventral enclosure, DLG-1::GFP was detected in the epidermis, pharynx and intestine (Fig. 2D,E), forming a continuous belt around epithelial cells at a subapical position, which persists throughout embryonic, larval and adult development (Fig. 2F,H). In adults, the DLG-1::GFP protein was also detected in epithelial cells contributing to the reproductive system: the vulva, uterus and spermatheca (Fig. 2G).

Our results indicate that the observed DLG-1::GFP expression pattern reflects that of the endogenous protein, although dlg-1 may also be expressed at very low levels in other cells.

Epithelial cell polarity is affected in LET-413- but not in DLG-1-deficient embryos
To determine whether the epithelial defects seen in dlg-1(RNAi) embryos resulted from apico-basal polarity abnormalities, we examined the localisation of both basal and apical proteins. The basal marker, MH46, which recognises the TM protein myotactin (Francis and Waterston, 1991; Hresko et al., 1999), showed a normal distribution (data not shown). To mark the apical surface of the embryonic pharynx and intestine, we used antibodies against the PDZ-containing proteins PAR-3 and PAR-6 (Fig. 3A,A',D,D'); Leung et al., 1999). Similarly, a GFP reporter construct of the multi-TM protein CHE-14 (Michaux et al., 2000) was used to mark the apical surface of a subset of epidermal cells (Fig. 3G). We observed that these proteins all remained at the apical surface in dlg-1(RNAi) embryos (Fig. 3B,B',E,E',H), although PAR-3 appeared to move to a position coincident with, or just apical to, the CeAJ (marked by HMP-1) in the intestine (Fig. 3B'), and small areas of subapical localisation were also detected for CHE-14::GFP in the epidermis (arrows in Fig. 3H). In contrast, PAR-3 and PAR-6 became progressively mislocalised basally along the lateral membrane of intestinal cells in let-413(RNAi) embryos (Fig. 3C,F,C',F'). Similarly, CHE-14::GFP was
progressively mislocalised to the lateral cell membrane in LET-413-deficient embryos (Fig. 3I).

In conclusion, these results show that DLG-1 does not play a major role in localising apical proteins in epithelia and confirm that LET-413 is required for the maintenance of apico-basal polarity (Fig. 3J).

DLG-1 is required for the maintenance of the CeAJ electron-dense structure

To further examine the let-413(RNAi) phenotype, we performed electron microscopic analysis of let-413(RNAi) embryos. In wild-type embryos, the visible component of CeAJs is denoted by a precisely defined, electron-dense subapical structure in the epidermis (Fig. 4A) and intestine (Fig. 4F). Among 30 sections containing single wild-type embryos, 86 electron-dense junctions were observed in the epidermis. In contrast, among 44 sections containing 2-4 let-413(RNAi) embryos, only 4 slightly extended electron-dense structures were observed (Fig. 4C). In these embryos the majority of cell-cell contacts were devoid of any electron-dense structure (Fig. 4B), although the epidermal cell membranes remained correctly apposed, as in wild-type embryos. Intestinal cells did not appear to be as badly affected as epidermal cells, although intestinal electron-dense structures were often missing (Fig. 4G,H). These structural defects differ from the CeAJs seen in let-413(RNAi) embryos, which show that the electron-dense structure is either discontinuous and greatly extended along the lateral membrane (38 junctions observed in 21 sections containing single embryos; Fig. 4D,I) or absent (Legouis et al., 2000). In double let-413(RNAi) dllg-1(RNAi) embryos, no laterally extended electron-dense structures were observed in 12 sections containing single embryos (Fig. 4E).

The ultrastructural analysis demonstrates that DLG-1 function is necessary for the formation or maintenance of the CeAJ electron-dense component while LET-413 appears to function in the compaction and positioning of these junctions.

CeAJ-associated proteins are distributed along the lateral membrane of differentiating epithelial cells

In order to understand the CeAJ defects resulting from the absence of DLG-1 and LET-413, we investigated the process of CeAJ formation in wild-type embryos, focusing on the epidermis, which is the most severely affected tissue. We examined the subcellular distribution of three CeAJ-associated proteins (henceforth referred to as CeAJ proteins) during epithelial cell differentiation by producing transverse sections of projected confocal images along the Z-axis as schematised in Fig. 5A (see Materials and Methods). HMP-1/α-catenin is expressed ubiquitously from the beginning of embryogenesis and is concentrated at regions of contact between blastomeres but, by the end of enclosure, it is restricted to a subapical belt in epidermal, intestinal and pharyngeal cells (Costa et al., 1998). We observed a punctate HMP-1 distribution along the lateral membrane of most cells, including all LIN-26-expressing cells, at stages preceding (Fig. 5B,B') and coincidental with (Fig. 5C,C') epidermal differentiation.

JAM-1 expression begins at the time of epithelial
differentiation, showing punctate localisation around epidermal cells (Podbielwicz and White, 1994). Using UNC-70, the *C. elegans* β-G spectrin, as a lateral membrane marker to indicate the extent of the epidermal cell layer (Moorthy et al., 2000), we found that JAM-1 was localised towards the base of the lateral membrane when it was initially expressed (Fig. 5D,D′). Using the *dlg-1::gfp* transgene as a marker, we established that the onset of DLG-1 expression was temporally and spatially coincident with JAM-1 expression within epithelial cells (Fig. 5E,E′). In contrast, we observed that HMP-1 and DLG-1, were distributed in a mutually exclusive manner (Fig. 5C,C′). Small patches of yellow staining were observed (Fig. 5C′), which may indicate areas of overlap, but this was impossible to resolve within the limits of confocal microscopy.

These results indicate that CeAJ proteins are initially distributed along the lateral membrane rather than in a discrete subapical region (Fig. 5F). In addition, these proteins form distinct membrane domains, containing either DLG-1 and JAM-1, or HMP-1.

**CeAJ proteins localise to distinct components of mature epidermal CeAJs**

To continue this CeAJ analysis, we investigated the localisation of CeAJ proteins and LET-413 in mature (fully differentiated) wild-type epidermal cells using Z-axis projections, as schematised in Fig. 6A,B. At this stage, DLG-1 and JAM-1 remained colocalised at the mature junction in a subapical belt (Fig. 6C) and HMP-1 formed a slightly punctate belt separate from, and generally apical to, the DLG-1/JAM-1 belt (Fig. 6D). Further evidence that HMP-1 is distinct from IAM-1/DLG-1 comes from observations of HMP-1 and JAM-1 localisation in
dlg-1(RNAi) and let-413(RNAi) embryos. The JAM-1 belt was completely punctate and disorganised in both dlg-1(RNAi) and let-413(RNAi) embryos (Fig. 6E-G). However, the HMP-1 belt was similar to wild-type in most dlg-1(RNAi) embryos, specifically 86% of wild-type (n=50) and 50% of dlg-1(RNAi) (n=50) embryos displayed a slightly punctate HMP-1 belt while this belt was more interrupted in remaining embryos. HMP-1 was more interrupted, but still much less abnormal than JAM-1, in let-413(RNAi) embryos (Fig. 6H-J).

In conclusion, mature CeAJs in epidermal cells are heterogeneous, with JAM-1 and DLG-1 (and associated proteins) forming a compartment distinct to that of HMP-1 (and associated proteins), which can be independently affected.

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In conclusion, mature CeAJs in epidermal cells are heterogeneous, with JAM-1 and DLG-1 (and associated proteins) forming a compartment distinct to that of HMP-1 (and associated proteins), which can be independently affected.
CeAJ proteins are mislocalised differently in *dlg-1(RNAi)* and *let-413(RNAi)* embryos

To investigate the possible functions of DLG-1 and LET-413 further, we analysed the distribution of either JAM-1 or HMP-1 in *dlg-1* or *let-413* deficient embryos and scored both the spread (extent) of each detected protein and its position along the cell membrane (Fig. 7A,B; and see Materials and Methods). We found that the spread of JAM-1 (where present) and HMP-1 in *dlg-1* or *let-413* embryos was as follows. Wild-type: 71 (JAM-1), 88 (HMP-1); *dlg-1(RNAi)*: 57 (JAM-1), 80 (HMP-1); *let-413(RNAi)*: 78 (JAM-1), 85 (HMP-1); early wild-type: 85 (JAM-1). (C) Schematic diagram of a transverse section through the mature epidermal layer of a wild-type embryo (see Materials and Methods). We found that the spread of JAM-1 (where present) and HMP-1 in *dlg-1* or *let-413* embryos was as follows. Wild-type: 71 (JAM-1), 88 (HMP-1); *dlg-1(RNAi)*: 57 (JAM-1), 80 (HMP-1); *let-413(RNAi)*: 78 (JAM-1), 85 (HMP-1); early wild-type: 85 (JAM-1). (C) Schematic diagram of a transverse section through the mature epidermal layer of a wild-type embryo (see Materials and Methods). We found that the spread of JAM-1 (where present) and HMP-1 in *dlg-1* or *let-413* embryos was as follows. Wild-type: 71 (JAM-1), 88 (HMP-1); *dlg-1(RNAi)*: 57 (JAM-1), 80 (HMP-1); *let-413(RNAi)*: 78 (JAM-1), 85 (HMP-1); early wild-type: 85 (JAM-1).

To assess the subcellular position of these proteins in a different manner, we also scored the fraction of lateral membranes where HMP-1 or JAM-1 could be detected in the top, middle or bottom part of the membrane (Fig. 7B,C). In the mature wild-type embryos, both HMP-1 and JAM-1 staining was observed at the top and middle of the lateral membrane, but never at the bottom (Fig. 7B, green bars, and D,G). In *dlg-1(RNAi)* embryos, although the HMP-1 position was essentially as wild type, there was also a small percentage of membranes where the protein was detected at the bottom of the lateral membrane (Fig. 7B, red bars, and H). Where present, the distribution of JAM-1 was more abnormal in these embryos, detected at the middle section in the majority of membranes and at the bottom section in a small percentage (Fig. 7B, red bars, and E). In *let-413(RNAi)* embryos, both HMP-1 and JAM-1 positions were strongly altered (Fig. 7B, blue bars, and F,I). HMP-1 was detected at the top, middle and bottom sections in similar percentages of membranes, and JAM-1 was also found at each membrane section but detected at the middle section in most membranes. Interestingly, this distribution of JAM-1 along the membrane in *let-413(RNAi)* embryos is particularly similar to its localisation in immature epidermal cells (Fig. 7B, yellow bars).

In order to determine whether DLG-1 function is necessary for LET-413 localisation, or vice versa, we carried out *dlg-1(RNAi)* in *let-413::gfp* transgenic embryos and *let-413(RNAi)* in *dlg-1::gfp* transgenic embryos. In *let-413(RNAi)* embryos, both HMP-1 and JAM-1 positions were strongly altered (Fig. 7B, blue bars, and F,I). HMP-1 was detected at the top, middle and bottom sections in similar percentages of membranes, and JAM-1 was also found at each membrane section but detected at the middle section in most membranes. Interestingly, this distribution of JAM-1 along the membrane in *let-413(RNAi)* embryos is particularly similar to its localisation in immature epidermal cells (Fig. 7B, yellow bars).

In conclusion, LET-413 is required to position CeAJ proteins subapically and it is striking that the distribution of JAM-1 in *let-413(RNAi)* embryos resembles the situation in the early
wild-type embryo. DLG-1 has a role in forming and maintaining a continuous JAM-1 belt at the CeAJ, but does not appear to be necessary for HMP-1 or LET-413 localisation.

**DISCUSSION**

This work has identified a second locus, *dlg-1*, required for the assembly of *C. elegans* apical junctions (CeAJs) in addition to *let-413* (Legouis et al., 2000). We show that the MAGUK protein DLG-1 colocalises with JAM-1, a marker of the CeAJ electron-dense structure (Mohler et al., 1998; D. Hall, personal communication), but not with HMP-1 or LET-413. DLG-1 is also required for JAM-1, but not HMP-1 or LET-413, localisation. Our data suggest that DLG-1 probably acts downstream of LET-413 in junction assembly and is not essential in maintaining epithelial polarity or adhesion.

**Few homologues of tight junction (TJ) or septate junction (SJ) components are essential for *C. elegans* embryogenesis**

Among the 16 predicted genes that we tested encoding homologues of proteins involved in TJ/SJ formation, only two proved essential for embryogenesis, one of which, *dlg-1*, was required for normal epithelial development and morphogenesis of the embryo. There are several possible reasons to explain why silencing most of these genes produced no obvious effect. The genes may function in the nervous system, which is known to be partially resistant to RNAi (for a review, see Bosher and Labouesse, 2000). In addition, redundancy between proteins may mask further phenotypes. Our findings are consistent, however, with a deficiency screen covering at least 75% of the *C. elegans* genome (Labouesse, 1997), not including the *dlg-1* locus (data not shown). This deficiency screen uncovered only two deficiencies which disrupted the JAM-1 staining pattern, one that deletes *let-413*, and the other that deletes the *jam-1* locus.

**CeAJ function and the role of DLG-1 in CeAJ formation**

Our observations provide an assessment of the role and probable molecular composition of CeAJs, and possible functions of DLG-1. We will deal in turn with the CeAJ structure and with DLG-1 itself. The CeAJ constitutes the single subapical junction visible in the epithelia of *C. elegans* embryos, which has ultrastructural characteristics of a belt desmosome or adherens junction (AJ) (Pries and Hirsh, 1986; Leung et al., 1999) and yet is associated with a protein (DLG-1) homologous to a septate junction (SJ)-associated protein (this work). Our most striking, and yet unexpected, results can be summarised as follows. Firstly, HMP-1/β-catenin defines a membrane domain which remains distinct from that defined by JAM-1 and DLG-1, both in wild-type and LET-413- or DLG-1-deficient embryos. Secondly, in the absence of *dlg-1* function, the electron-dense junction disappears and JAM-1 localisation becomes very punctate and slightly more basal, whereas HMP-1 localisation remains largely unaffected. Finally, despite the disappearance of the electron-dense junction, *dlg-1*(RNAi) embryos can elongate to the twofold stage and do not rupture. If the electron-dense structure were essential for adhesion, one would expect embryos lacking this structure to rupture and fail to elongate beyond the 1.5-fold stage. For comparison, *let-413* embryos elongate to around the 1.7-fold stage or rupture, and *hmp-1* mutants do not elongate at all. From this, we infer that CeAJs comprise at least two components, one which includes JAM-1/DLG-1 and probably other proteins (the JAM-1/DLG-1 unit), and a second that includes HMP-1, presumably HMR-1 (cadherin) and HMP-2 (β-catenin), and possibly other proteins (the HMP-1 unit). Both these components may contribute to the electron-dense structure, although in the absence of DLG-1, when the HMP-1 unit remains at the CeAJ, the dense structure is absent. Ultrastructural analysis has not been reported for *hmr-1*, *hmp-1* or *hmp-2* mutants. One could formulate several hypotheses concerning the respective functions of these components. One possibility is that the HMP-1 unit mainly contributes to cell adhesion while other CeAJ components play only a minor role. Alternatively, the JAM-1/DLG-1 unit could contain the main cell adhesion proteins, which may function adequately in the absence of DLG-1. A third possibility could be that there are several partially redundant adhesion systems. The phenotypes of *hmp-1*, *hmp-2* and *hmr-1* mutant embryos lend support to this third model. When the maternal and zygotic functions of these genes is absent, only the cells that drive ventral enclosure fail to attach to each other, while other cells can still adhere at the ventral midline, general epithelial integrity is intact, and lateral cells maintain a continuous JAM-1 belt (Costa et al., 1998). This suggests that the HMP-1 unit is dispensable for adhesion and cell polarity (Costa et al., 1998; Raich et al., 1999). Similarly, the JAM-1/DLG-1 unit alone does not appear to play a major role in establishing or maintaining membrane polarity as the removal of DLG-1 does not result in a mislocalisation of apical proteins. The localisation of JAM-1 is affected, but this is more likely to be due to the absence of an aggregating protein rather than a loss of polarity.

MAGUK proteins are linked with a role in organising various proteins at cell membranes and facilitating signal transduction, although these functions have been confirmed by mutational analysis in only a few cases (Garner et al., 2000). In *Drosophila*, Dlg is localised to the SJ and its absence induces the mislocalisation of many proteins (including Scribble, Coracle, Expanded and Fasciclin III) along the entire lateral membrane (Bilder et al., 2000; Woods and Bryant, 1991; Woods et al., 1996). Dlg is also required at the neuromuscular junction to cluster the Shaker potassium channel and the cell adhesion molecule Fasciclin II (Tejedor et al., 1997; Thomas et al., 1997). In mice lacking the MAGUK PSD-95, NR2-NMDA receptors still normally localise to synapses but neuronal transmission is impaired (Migaud et al., 1998). Since our results show that the electron-dense structure of CeAJs is absent in *dlg-1*(RNAi) embryos, we propose that one of the functions of DLG-1 is to cluster JAM-1 and other CeAJ proteins (excluding those in the HMP-1 unit) and in doing so, primarily contributes to the formation of the electron-dense structure. In addition, as numerous vacuoles were observed in epithelial tissues in the absence of DLG-1, we postulate that some of the proteins clustered by DLG-1 are required for signalling, and thus that DLG-1 is required to facilitate cell signalling. *dlg-1* is not essential for the maintenance of epithelial membrane polarity, in contrast to *C. elegans let-413* and *Drosophila* *dlg* and *scrib*. This may be due to several differences between *Drosophila* Dlg/Scrib and *C. elegans*...
**A pathway for the assembly of CeAJs**

Our previous electron microscopic analysis showed that in the absence of LET-413, the electron-dense part of the CeAJ is either missing or extends more basally along the lateral membrane (Legouis et al., 2000). In addition, we now show that JAM-1/DLG-1 and HMP-1 are also positioned or extend along the lateral membrane. Cartoons showing the localisation of HMP-1 (blue), JAM-1 (green), and LET-413 (purple) proteins in epidermal cells. (A) An immature wild-type cell showing that HMP-1 and DLG-1/JAM-1 exhibit a mutually exclusive distribution along the lateral membrane. (B) A mature wild-type cell illustrating a compact subapical junction consisting of a JAM-1/DLG-1 unit associated with the electron-dense component, and a HMP-1 unit, which is generally localised more apically than JAM-1/DLG-1 and is always distinct. Arrows from the CeAJ domains indicate their possible functions. (C) A LET-413-deficient cell showing mislocalisation of CeAJ proteins. This suggests that LET-413 plays a role in compacting and positioning CeAJ components to form mature CeAJs. (D) A DLG-1-deficient cell showing mislocalisation of CeAJ proteins. The electron-dense CeAJ component is also missing in these cells, which suggests that DLG-1 aggregates JAM-1 and other proteins at the CeAJ.

DLG-1/LET-413 including localisation and protein structure (Bilder and Perrimon, 2000; Legouis et al., 2000).

**Note added in proof**

While this work was being reviewed, Bassinger et al. also described the function of *dlg-1*. In agreement with our results, they show that loss of DLG-1 affects AJ assembly but not PAR protein localisation. In addition, they show that DLG-1 is required for proper distribution of the apical marker CRB-1, a Crumbs homologue. (Bassinger, O. et al. (2001). Dev. Biol. 230, 29-42.)

**REFERENCES**


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Assembly of *C. elegans* apical junctions


