Drosophila miniature and *dusky* encode ZP proteins required for cytoskeletal reorganisation during wing morphogenesis

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Summary

We have characterised the function of two *Drosophila* genes, *miniature* and *dusky*, that are required for the morphological reorganisation of the apical membrane during wing epidermis differentiation. These genes encode transmembrane proteins containing a ZP (zona pellucida) domain and are homologous to several vertebrate and invertebrate apical matrix components. *miniature* and *dusky* are only expressed in tissues secreting a cuticle, and the Min protein localises to the apical membrane during the early stages of cuticle formation. We propose that Min and Dusky form a novel subfamily within the ZP domain proteins and are specifically involved in the interactions between the apical membrane, the cytoskeleton and the forming cuticle.

Key words: ZP proteins, Cuticulin envelope, Epithelial morphogenesis, Cuticule formation, *Drosophila*

Introduction

The epithelium of the *Drosophila* wing undergoes a conspicuous cytoskeletal reorganisation during differentiation. Between 32 hours and 60 hours APF (after puparium formation) a columnar epithelium of roughly hexagonal cells expands in surface area four-fold, as each cell flattens and interdigitates with its neighbours. By 60 hours, each cell has also elaborated a complex apical structure – the hair pedestal that supports the growth of actin-filled apical extensions termed wing hairs (Mitchell et al., 1983; Fristrom et al., 1993). Remarkably, this entire morphological transformation occurs after the first layer of cuticle has already been assembled above the epidermal cells (Mitchell et al., 1983).

This first cuticle layer – known as the cuticulin envelope – is only 120-175 Å deep. It is structurally different from other cuticle layers, being mainly composed of crosslinked proteins and lipids (see Locke, 2001). In *Drosophila* wings, this protective envelope is secreted at about 32-34 hours APF, it covers the epidermis while changes in cell shape are taking place and eventually forms the outer-most surface of the mature cuticle, once the bulk of the chitinous cuticle has been secreted (Mitchell et al., 1983). The cuticulin envelope defines an enclosed extracellular compartment above the apical membrane, where the assembly of the cuticle can proceed (Locke, 1998). The formation of a cuticulin envelope is a common feature of all arthropods cuticles and has long been recognised as critical for defining the final form of arthropod epithelia (Locke, 2001).

Nothing is known about the genes required to specify the form and function of the cuticulin envelope in *Drosophila*. We have studied mutants in two genes, *dusky* (*dy*) and *miniature* (*m*), that have been known for a long time to affect the morphology of adult wing cells [*m* was isolated in 1910 by T. H. Morgan and *dy* in 1916 by C. Bridges (reviewed in Lindsley and Zimm, 1992)]. In mutants for either gene, the size of the whole wing is significantly reduced and the cuticle is darker than in the wildtype (hence the names of the genes). Dobzhansky showed that the reduced size of *m* wings is because of a reduction in the size of individual wing epidermal cells (Dobzhansky, 1929): these wings have a normal number of cells and are correctly patterned (see also Newby et al., 1991).

One of these two genes, *dusky,* has recently been shown to encode a transmembrane protein containing a ZP (zona pellucida) domain (DiBartolomeis et al., 2002), a motif common to a large family of vertebrate and invertebrate extracellular matrix components (Bork and Sander, 1992; Wassarman et al., 2001). One member of this family, the product of the *C. elegans cuticulin-1* gene (*cut-1*), has been identified as a structural component of the most external part of the worm cuticle (Sebastiano et al., 1991). Here we show that the *m* gene also encodes a ZP protein. Min and Dy proteins, together with one other encoded by a previously unidentified *Drosophila* gene revealed by the genome sequence, define a novel subfamily of *dusky*-related ZP proteins. We show that all three genes of this subfamily are expressed in cuticle-secreting epithelia. We also describe the cellular behaviour of mutants of both *m* and *dy* during wing morphogenesis. We argue that these proteins may be components of the cuticulin envelope itself or of a specialised

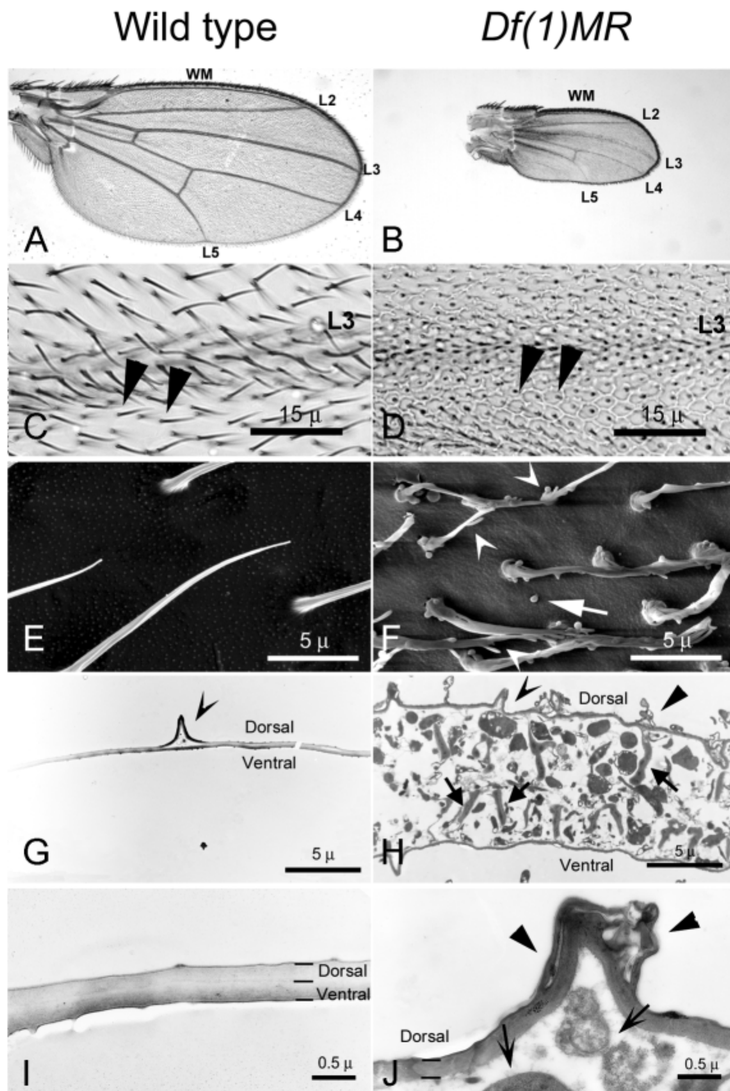

Fig. 1. Cuticle morphology is disrupted in *Df(1)MR* adult wings. Wild-type and *Df(1)MR* wings dissected 2 hours after adult eclosion. (A-D) Light microscopy. The mutant wing in B is reduced in size, although veins (L2-L5) and wing margin (WM) are correctly patterned. (C,D) A closer view of the same wings reveals that hairs are much closer in the mutant than in the wildtype (arrowheads). (D) The picture of the mutant corresponds to a slightly lower focus plane, showing the cell outlines. Note the ring of cuticle surrounding each hair in the mutant. (E,F) SEM micrographs. The mutant hairs appear twisted and branched (open arrowheads), in contrast to the long and slender wild-type epidermal hairs (E). Some cuticle blobs appear bulging out of the main wing surface in the mutant (arrow). (G-J) TEM sections. The two wing surfaces (dorsal and ventral) are separated in the mutant (H,J), and the resulting space contains cuticle invaginations (small arrows) and cell debris (arrows). The presence of cuticle defects is indicated by arrowheads and at a higher magnification in the lower panels (I,J). The position of some epidermal hairs is also indicated (open arrowheads in G,H).

apical matrix that is necessary for the organisation of the apical membrane and its interaction with cytoskeletal components during cell shape reorganisation.

Materials and Methods

Strains and characterisation of m^1

We used the following strains: Oregon-R, *Df(1)MR* (Roberts and Jackson, 1997) and m^1 and dy^1 (Lindsley and Zimm, 1992). *UASα-cateninGFP* (Oda and Tsukida, 1999) expression was driven in pupal wings in the posterior compartment by means of the *engrailedGAL4* driver (Brand and Perrimon, 1994). The entire *CG9369* coding region (*miniature*) from wild-type and m^1 adult flies was sequenced from three independent clones after PCR amplification of genomic DNA.

Microscopy

For scanning electron microscopy (SEM), adult whole animals were dehydrated in absolute ethanol and then in a critical point dryer and subsequently coated with gold in a Polaron sputter coater. They were visualised with a Philips XL30 FEG scanning electron microscope. Samples for transmission electron microscopy (TEM) were fixed overnight at 4°C by immersion in 4% glutaraldehyde in 0.1 M PIPES buffer at pH 7.4 containing 2 mM $CaCl_2$ and 0.3% H_2O_2. After fixation, samples were rinsed twice in PIPES buffer, treated with 1% osmium ferricyanide for 1 hour at 4°C, rinsed in distilled water, bulk stained in 2% uranyl acetate, dehydrated in ethanol and finally embedded in Spur's epoxy resin. Thin sections (50 nm) were cut with a Leica Ultracut-UCT microtome, double stained with uranyl acetate and lead citrate and viewed in a Philips CM100 transmission electron microscope operated at 80 KV. For confocal microscopy, pupae were collected at puparium formation (0 hours APF), aged until the desired stage and fixed overnight in 4% formaldehyde in PEM (0.1 M PIPES, 1 mM EGTA, 2 mM $MgSO_4$, pH 6.9) after removal of the operculum. The following day, the pupal case was removed and the wings hand peeled before staining with 2 mM rhodamin phalloidin (Molecular Probes) in PBT. The wings were mounted in Vectashield (Vector) for visualisation on a Leica confocal microscope.

In situ hybridisation

Exonic fragments of 1.5 kb and 1.8 kb corresponding respectively to *CG9369* and *CG15013* were cloned from genomic DNA by PCR in the pGEMT vector (Promega). Sense and antisense DIG-labelled riboprobes were synthesised using the Boehringer kit from these vectors and from the *dy* complete cDNA cloned in Bluescript (a gift of R. Jackson). In situ hybridisation in embryos and pupae were

performed following standard protocols (Sturtevant et al., 1993) with some modifications, all available from F.R. upon request.

Immunostainings and generation of the Min antibody

The fragment coding for amino acids 346 to 547 of the CG9369 predicted protein was amplified by PCR from wild-type genomic DNA and cloned in-frame in the pRSETB vector (Invitrogen). The resulting 6× His-tagged protein was purified with the Pharmacia kit from the soluble fraction obtained after bacterial sonication. The fraction containing the purified protein was loaded in a SDS-PAGE gel, and the resulting band was cut and used to immunise rabbits following standard protocols (Eurogentec, Belgium). The rabbit anti-Min serum fails to recognise any specific signal in immunostainings performed on m^1 mutant embryos and is capable of specifically recognising the Min protein produced in vivo with a *UAS-miniature* transgene (F.R. and C.R.A., unpublished). Stainings were carried out using rabbit anti-Min (1:100) and rat anti-E-Cadherin (1:50) (a gift from O. Renaud). Secondaries were 1:200 FITC anti-rabbit and 1:200 Cy5 anti-rat (Jackson Labs). Cell nuclei were labelled with the TOPRO nuclear dye (Molecular Probes).

Results

All *miniature* and *dusky* mutations map very close to one another in the 10E1-2 region of the X chromosome (Dorn and Burdick, 1962). Although both classes of mutation affect cell morphology in a similar way, the fact that they fall into two distinct complementation groups suggests that they affect distinct genes (Newby et al., 1991).

In this work we have studied the phenotypes caused by *Df(1)MR*, a small deletion that fails to complement both *dy* and *m* mutations (Roberts and Jackson, 1997). Males hemizygous for this deficiency are viable, but the size of their wings is significantly smaller than in either wild-type (Fig. 1A,B), or in *dy* or *m* single mutants (data not shown). *Df(1)MR* removes the *dy* coding sequence and is thus a *dy*-null allele (DiBartolomeis et al., 2002). Comparison of the molecular maps of the *m-dy* region (DiBartolomeis et al., 2002) with the genome sequence (Adams et al., 2000) reveals that *Df(1)MR* also removes most of the coding region of an adjacent predicted gene, *CG9369*. This gene, which we have identified unequivocally as *miniature* (see below), encodes a transmembrane protein clearly related to that encoded by the *dy* locus. This, and the fact that *dy* and *m* point alleles have similar phenotypes, suggests that these two proteins may have a similar function.

miniature and *dusky* mutant wings secrete abnormal cuticles

To understand the cellular basis for the observed reduction in cell size, we characterised in detail the phenotype of adult males hemizygous for *Df(1)MR* and hence lacking both *m* and *dy* functions. Light and scanning electron microscopy (SEM) show that *Df(1)MR* wings dissected 2 hours after adult eclosion have pronounced defects in their cuticle. In both wild-type and *Df(1)MR* wings, each epidermal cell makes a hair on its apical surface. However, in *Df(1)MR*, wings hairs are much closer to

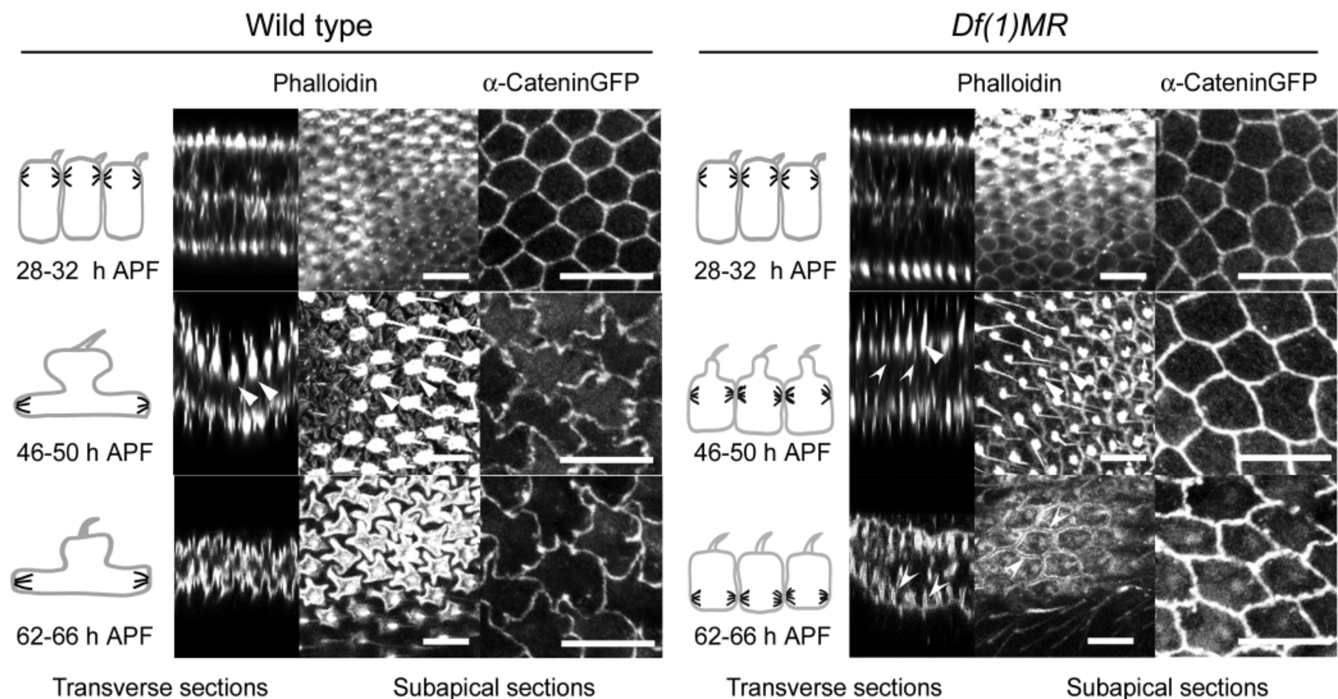

Fig. 2. Apical membrane reorganisation fails to occur in Df*(1)MR* mutants. Confocal images of wild-type and *Df(1)MR* pupal wings of different stages stained as indicated. Cartoons show cell outlines (grey) and the position of the apical junctions (black) in a transverse section of the epithelium at each stage. At 28-32 hours APF, wild-type and mutant epithelia are indistinguishable. By 46-50 hours APF, the formation of hair pedestals accumulating large amounts of actin (arrowheads) is hindered in the mutant. Note also the presence of an actin-ring around each cell in the mutant, visible also in the transverse sections (open arrowheads). The mutant cells also fail to acquire a star-like cell contour at the level of their apical junctions. At 62-66 hours APF, the rhodamin-phalloidin binds the apical membrane that appears folded inside the wing. Note that in the mutant a space between the apical membrane folds is present, where cuticle is secreted (open arrowheads). The aberrant apical junctions of the mutants also accumulate large amounts of α-catenin–GFP. Bar, 5 μm.

each other than in the wildtype, suggesting that the size of individual cells is significantly reduced (Fig. 1C-F). *Df(1)MR* hairs are also shorter than in the wildtype and their structure is abnormal: they are twisted and often branched (Fig. 1F). We also observe localised distensions of the cuticle surface that bulge out of the main wing surface, a phenotype that indicates defects in cuticle formation (Fig. 1F). Similar defects are also observed in both *dy* and *m* single mutants, but they are less severe (data not shown).

Transmission electron microscopy (TEM) of thin sections of *Df(1)MR* adult wings confirms that cuticle structure is severely affected in the mutants. In wild-type animals, wing epidermal cells have undergone apoptosis by 2 hours after eclosion, and the dorsal and ventral cuticle surfaces forming the wing are closely apposed by their basal sides (Johnson and Milner, 1987) (Fig. 1G,I). In the mutants, cuticular material present inside the wing prevents the apposition of dorsal and ventral surfaces. These indentations of cuticle form a honeycomb-like pattern, marking the cell outlines that are visible in adult mutant wings under light microscopy (Fig. 1D). The indentations appear to obstruct clearance of the cellular debris produced after apoptosis of the epidermal cells (Johnson and Milner, 1987), as we observe degenerating cell fragments between the two wing surfaces (Fig. 1H,J). In contrast, wild-type wings of similar age are totally clean of cell debris (Fig. 1G,I).

The effects of *dusky* and *miniature* mutations are obvious only in the wings: the cuticle covering the epidermis of other parts of the body (haltere, notum, legs and abdominal plates) appears completely normal. To determine if subtle defects may be present elsewhere, we also performed TEM studies on the cuticle covering the adult haltere, a dorsal appendage that is homologous to the wing. Despite the high resolution of this analysis, we could detect no defects in the haltere cuticle (data not shown).

miniature and *dusky* mutants disrupt cell morphogenesis during differentiation

To investigate the origin of the defects observed in *Df(1)MR* adult wing cells, we compared the morphology of wild-type and mutant cells at different stages during pupal development using confocal microscopy. We employed two cytoskeleton markers: rhodamine-phalloidin, a fluorescent compound that binds specifically to actin filaments and stains cell outlines and epidermal hairs (Fristrom et al., 1993) and an α-catenin–GFP fusion protein (Oda and Tsukida, 1999). This GFP-tagged protein localises in the apical junctions between epidermal cells where the apical and basolateral membrane compartments meet.

We detect no obvious abnormalities in the morphology of *Df(1)MR* mutant cells at 28-32 hours APF, a time when wing cells are columnar and still hexagonal in outline (Fig. 2). Between 42 and 48 hours APF, wild-type cells flatten, expand and become star shaped (Fristrom et al., 1993). They also develop prominent actin-filled extensions of their apical surface, called hair pedestals, that can be visualised in wings staged 46-50 hours APF (Fig. 2, arrowheads). In mutants of the same age the apical cell contours remain hexagonal, and the accumulation of actin at the hair pedestals is impaired (Fig. 2). They also accumulate actin abnormally, forming a ring in the apical part of the mutant cells (Fig. 2, open arrowheads).

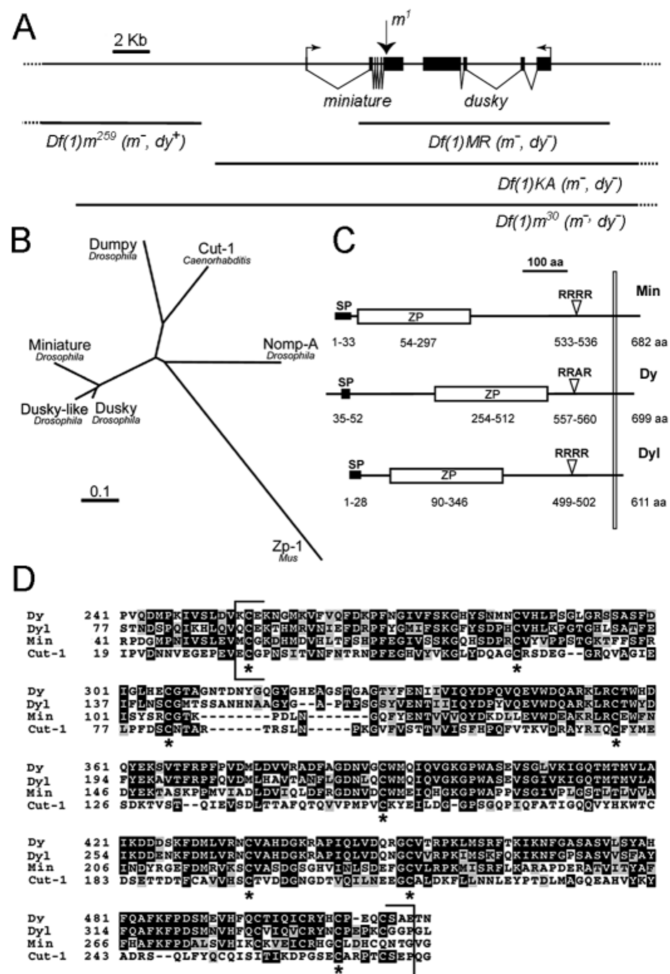

Fig. 3. *m* and *dy* encode transmembrane proteins with a ZP domain. (A) Genomic organisation of the *m/dy* region. The position of exons is indicated by black boxes. The regions deleted in the different deficiencies are shown below. The position of the small rearrangement present in the m^1 mutant is indicated (arrow). (B) Similarity diagrams based on a ClustalW multiple sequence alignment of the ZP domains from the indicated *Drosophila*, nematode and mammal proteins. (C) Predicted modular structure of Min, Dy and Dyl proteins (protein accession numbers are AAF48088, AAF48089 and AAF47884, respectively). The position of the transmembrane domain is highlighted by a vertical box. Tetrabasic RRR/AR motifs are indicated by triangles. Signal peptides are indicated by black boxes (SP). (D) Alignment of Dy, Dyl, Min and Cut-1 ZP domains. Black and shaded boxes indicate identical and similar residues, respectively. The position of the ZP structural domain is shown between brackets, and the eight conserved cysteines characteristic of this domain are marked with asterisks (see text for details).

In wild-type wings staged 62-66 hours APF, phalloidin strongly stains the apical cell membrane, which by then is secreting the adult chitinous cuticle (Fristrom et al., 1993) (Fig. 2). In *Df(1)MR* mutants, the apical membrane folds deeply into the cleft between adjacent cells, almost forming a capsule around the apical side of each cell (Fig. 2). The walls of these capsules are apposed to those of the neighbouring cells, leaving a narrow space in between (Fig. 2, open arrowheads). Below these structures, the cell maintains its hexagonal contour at the

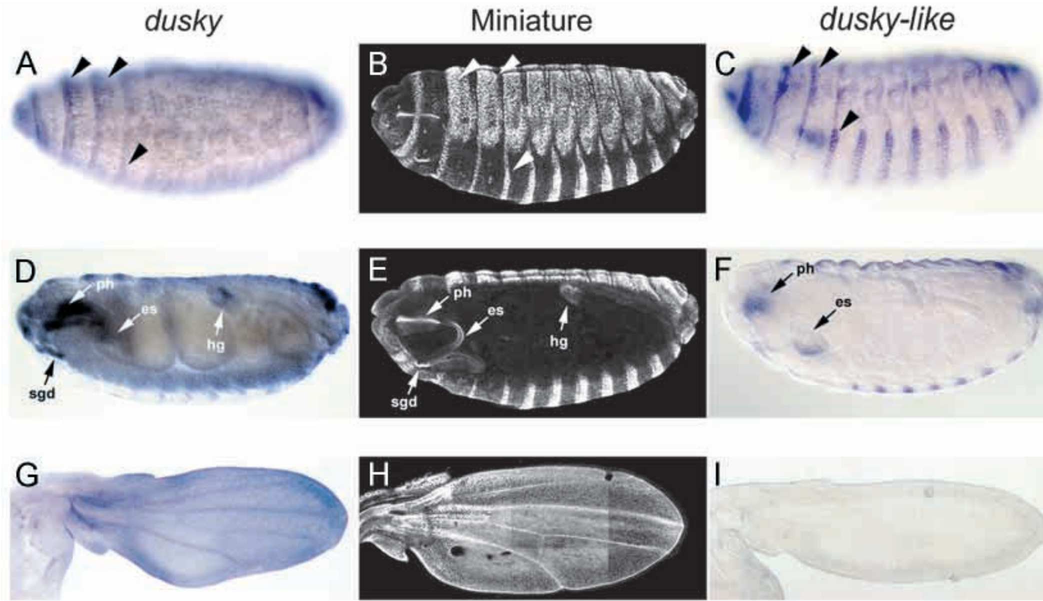

Fig. 4. *dy*, Min and *dyl* are expressed in cuticle-forming tissues. *dy* (A,D) and *dyl* (C,F) RNA in situ hybridisation and anti-Min immunostaining (B,E) in wild-type embryos of stage 16-17. (A-C) A view of the embryonic epidermis. By stage 16, the three genes are upregulated in regions corresponding to the dorsal and ventral denticle belts (arrowheads). (D-F) View of embryonic internal structures. *dy* and Min are expressed in the pharynx (ph), oesophagus (es), the duct of the salivary glands (sgd) and in the hindgut (hg). *dyl* is present in the epidermis but also in the pharynx and oesophagus (arrowheads in F). (G-I) Pupal wings dissected at 28 hours APF and stained as above. At this stage all wing cells express both *dy* (G) and Min (H), but not *dyl* (I).

level of the apical junctions, which accumulate larger amounts of α-catenin–GFP than in the wildtype (Fig. 2).

miniature, like *dusky*, encodes a transmembrane protein with a ZP domain

The *dy* gene has recently been identified (DiBartolomeis et al., 2002). It encodes a putative transmembrane protein with an extracellular motif called a ZP domain, a conserved domain present in transmembrane proteins described in both vertebrates and invertebrates as components of various extracellular matrices (Bork and Sander, 1992; Wassarman et al., 2001; Wilkin et al., 2000; Chung et al., 2001).

Genetic data indicate that *m* mutations lie just to the left (i.e. telomeric) of *dy* (Dorn and Burdick, 1962). A survey of the *Drosophila* genome sequence (Adams et al., 2000) reveals that the gene immediately to the left of *dy* in the chromosome (*CG9369*) also encodes a transmembrane protein with a ZP domain. We sequenced the complete predicted coding region of this gene in the m^1 mutant and found a deletion of 33 nucleotides associated with an insertion of 13 nucleotides, 370 amino acids downstream of the CG9369 predicted start codon (Fig. 3A). This small rearrangement causes a frameshift and a premature stop codon within the coding sequence, indicating that *CG9369* is the *m* gene and that m^1 is probably a null allele for *m*. This is consistent with deficiency mapping data published by the F. R. Jackson group (DiBartolomeis et al., 2002). $Df(1)m^{259}$, which fails to complement *m* alleles but not *dy* alleles, removes only sequences upstream of *m* (presumably regulatory sequences necessary for *m* expression), whereas *Df(1)MR*, $Df(1)m^{30}$ and *Df(1)KA6*, which fail to complement both *m* and *dy* alleles, delete sequences from both coding regions (DiBartolomeis et al., 2002) (Fig. 3A).

A BLAST search of the whole genome of *Drosophila,* using either the *dy* or *m* ZP domains as probes, reveals that there are more than a dozen putative proteins encoded by the fly genome that contain a ZP domain. Sequence comparison of the *Drosophila* ZP domains among themselves and with other non-fly homologues indicates that three of these *Drosophila* ZP genes encode a distinct subfamily of ZP proteins (Fig. 3B and data not shown). These three genes are *dy*, *m* and *CG15013*, which is a predicted open reading frame located at 64B1 (Adams et al., 2000) (Fig. 3B). The ZP domains of Dy and CG15013 are most similar (70% identity), with that of Min being more divergent (45% identity with Dy, Fig. 3D). Their similarity to other *Drosophila* ZP domains and *C. elegans* Cut-1 is largely confined to eight key cysteine residues, landmarks of a ZP domain (Fig. 3D and data not shown).

We have studied the structure of the Dy, Min and CG15013 proteins using SMART software to predict the position of architectural domains from protein primary sequences (Schultz et al., 1998). The three proteins include a putative transmembrane domain separating a short intracellular C-terminus and a large extracellular N-terminus containing the ZP domain (Fig. 3C). In addition, Dy, Min and CG15013 contain an ER import signal peptide in their N-terminus (DiBartolomeis et al., 2002) (Fig. 3C), which is consistent with them being single-pass transmembrane proteins.

They also have a basic tetrapeptide RRRR (RRAR in the case of Dy) located in between the ZP domain and the transmembrane domain, within the extracellular part of the protein. This small motif is common to many protein precursors cleaved by endopeptidases of the secretory pathway

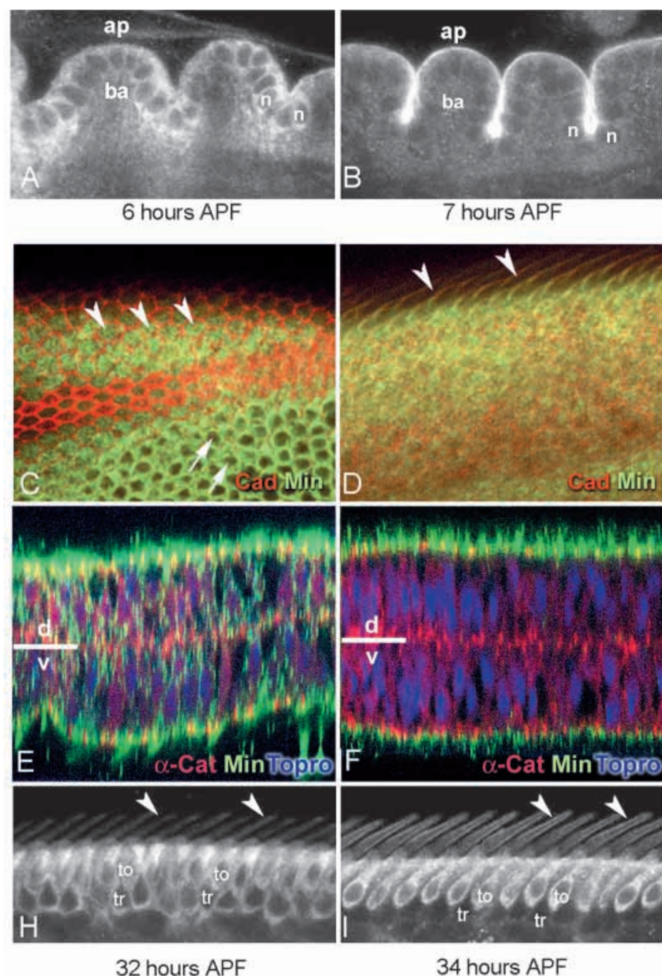

Fig. 5. The Min protein localises to the apical membrane at the time of cuticulin envelope assembly. (A,B) Confocal image of a transverse section taken from wild-type pupal legs stained for Min protein. (A) At 6 hours APF, Min protein accumulates in the cytoplasm (n, cell nuclei), whereas the staining accumulates in the cells apical surface 1 hour later (B). The position of the basal and apical surfaces is indicated. (C,D) Confocal images corresponding to an apical plane of a slightly tilted pupal wild-type wings. (C) At 32 hours APF, Min (green) accumulates in the cytoplasm, both apically (arrowheads, upper part of the picture) or in a more basolateral focal plane (arrows, bottom of the picture). (D) At 34 hours APF, a uniform layer stained with the anti-Min antibody covers the whole wing and the epidermal hairs (arrowheads). Anti E-cadherin antibodies (red) mark the position of apical junctions. (E-F) Transverse section of similar wings to those shown in B. Strong expression of α-catenin–GFP (red) marks both apical junctions and the basal part of the cells (white line). (E) At 32 hours APF, Min protein accumulates in the apical side of the cell and in the cytoplasm (green). Nuclei position is marked by the TOPRO dye (blue). The dorsal and ventral cell layers composing the wing are evident; the basal membrane is now in the middle of the wing (white line). (F) At 34 hours APF, Min protein is detected only in the apical side of the cells. (H,I) Confocal view of the wing margin row of stout bristles stained with anti-Min. (H) By 32 hours APF, most of the staining accumulates in the cell body of the trichogen cells (tr), whereas the bristle shafts are not stained (arrowheads). (I) 2 hours later the staining has disappeared from the cytoplasm of the trichogen cells (tr) and accumulates in the periphery of the growing shaft (arrowheads). The tormogen cells (to) of each bristle still accumulate Min in their cytoplasm at this stage.

(Hosaka et al., 1997) and could be a target in *Drosophila* for a furin type endopeptidase, which releases the ZP-domain-containing region. Because of the similarities between CG15013 and Dy proteins (see also below), we name the gene *CG15013 dusky-like* (*dyl*).

miniature, *dusky* and *dusky-like* are expressed in tissues involved in cuticle secretion

To determine how *dy*, *m* and *dyl* are expressed, we carried out RNA in situ hybridisations at different stages with probes for these genes. We also generated an antibody against the Min protein to study its subcellular localisation.

The three gene products are expressed in partially overlapping domains during embryogenesis and pupal development. In the embryo, they are only expressed in tissues that will secrete cuticle, including the epidermis, foregut and hindgut (Hillman and Lesnick, 1970) (Fig. 4A-F). In the epidermis, expression starts at about stage 14, and RNA levels increase until at least stage 17, when formation of the cuticle prevents the penetration of our probes. In each segment, transcripts of all three genes are more abundant in cells forming the dorsal and ventral denticle belts than in other parts of the epidermis (Fig. 4A,C). These cells also contain high levels of Min protein (Fig. 4B). *dy* and Min are also expressed in the cells forming the duct linking the salivary glands with the oesophagus (Fig. 4D,E), and Min protein is detected at low levels in the cells forming the embryonic tracheae (data not shown).

RNA in situ hybridisation and antibody staining show that *dy* and Min are also expressed in pupal wings by 28 hours APF, which is consistent with their genetic requirement in this tissue (Fig. 4G,H). However, we could not detect expression of *dyl* in pupal wings of the same stage (Fig. 4I), indicating that this gene could have an embryo-specific role.

We have characterised in detail the expression and localisation of the Min protein in imaginal tissues from late third larval instar onwards. During this period the imaginal epidermis secretes two different types of cuticle. The first, the pupal cuticle, is a thin unpigmented sheet that starts forming at 7 hours APF, and it is finally shed at about 18 hours APF. The second – the future adult cuticle – begins to form by 32-34 hours APF. In both cases, the first layer to be secreted is the cuticulin envelope. We find that Min is expressed in two separate phases that occur prior to the assembly of these cuticulin envelopes.

During the first phase, Min protein accumulates in the cytoplasm of all epidermal cells from late in the third larval instar until 6 hours APF (Fig. 5A). Between 6 and 7 hours APF, an abrupt transition occurs; most of the Min protein now localises to the apical side of the cell, where it forms a continuous layer (Fig. 5B). After 7 hours APF, the specific

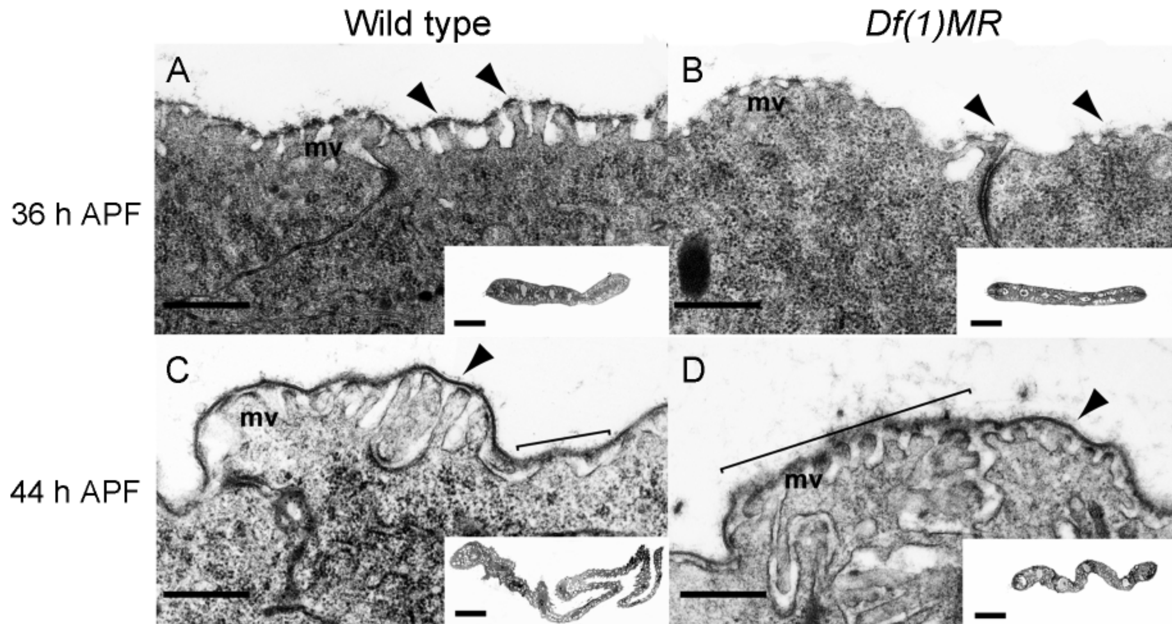

Fig. 6. TEM sections of wing epidermis of wildtype (A,C) and *Df(1)MR* (B,D) at 36 hours APF (A,B) and 44 hours APF (C,D) (bar, 500 nm). The insets correspond to micrographs of thick sections of the same specimens, stained with toloudine blue and showing the whole wing (bar, 50 μm). (A) At 36 hours APF, the small patches of cuticulin envelope (arrowheads) appear at the tip of microvilli (mv) in the wildtype. (B) In the mutant, microvilli are shorter, and the process of cuticle deposition is less advanced (arrowheads). (C) At 44 hours APF, the cuticulin envelope forms an almost continuous layer over the epidermis in both wild-type and mutant wings. In some regions (arrowheads), the characteristic trilayer can be observed, whereas in other regions (brackets) the cuticulin layer seems less organised. (D) The structure of the apical microvilli is disorganised in the mutant wings (mv). The process of wing extension is prevented in the *Df(1)MR* mutant (compare insets in C and D).

staining disappears, probably because Min protein has become chemically crosslinked to itself or to other components of the forming cuticle.

We observe a similar pattern in late pupal wings, before the secretion of the adult cuticle. Min protein first appears in the wing at about 28 hours APF (Fig. 5C-F). It accumulates to high levels in the cell cytoplasm until, by 32-34 hours APF, it localises to the apical membrane, forming a continuous layer covering the epidermal cells (Fig. 5D,F) and the developing bristles (Fig. 5H,I).

The pattern of accumulation of Min protein in the haltere epidermis is similar to that in the wing (data not shown), even though no mutant phenotype is visible in this tissue.

Formation of the cuticulin envelope is not prevented in *Df(1)MR* wings

The data above show that Min protein localises to the apical membrane at the same time or slightly before the cuticulin envelope is forming in the pupal and adult wings (Mitchell et al., 1983). To determine whether the formation of the cuticulin envelope is affected in the absence of *m* and *dy* activities, we examined the ultrastructure of the forming cuticle in *Df(1)MR* mutants at 36 and 44 hours APF using TEM. In wild-type animals staged 36 hours APF, discontinuous patches of cuticulin are observed associated with the tips of microvilli (Fig. 6A); by 44 hours APF the cuticulin envelope is essentially complete, except above cell boundaries, and cell expansion has begun (Mitchell et al., 1983) (Fig. 6B). In *Df(1)MR* mutants staged 36 hours APF, formation of the cuticulin envelope appears to be less complete and microvilli shorter than in the wildtype (Fig. 6B), but by 44 hours APF, an envelope is clearly visible and not obviously different from that seen in wildtype (Fig. 6C,D). However, at this stage, membrane invaginations beneath the forming cuticle appear to be more extensive in the mutant and less well organised than in the wildtype (Fig. 6C,D). As the wild-type cells have begun to expand by 44 hours APF, it is not clear whether this difference in the apical cell organisation is a cause or a consequence of the altered cell behaviour seen in the *Df(1)MR* wings, which fail to expand (Fig. 6C,D, insets).

Discussion

We have studied two adjacent *Drosophila* genes, *m* and *dy*, whose products are required for morphogenesis in the wing epidermis. We have analysed the phenotypes caused by a mutation that simultaneously impairs the activity of both genes and found that they are required for the reorganisation of the apical membrane during cell differentiation in pupal wings.

The *m* and *dy* loci encode transmembrane proteins that include a ZP motif in their extracellular domain. Searching for other related proteins in *Drosophila* we have identified a third protein containing a ZP domain, the product of the *dyl* gene, that is highly similar to both Min and Dy. Our analysis shows that *m*, *dy* and *dyl* are more related to one another than to any other ZP domain protein and are expressed only in tissues secreting a cuticle. Preliminary analysis of the genome sequence of the mosquito *Anopheles gambiae* (Holt et al., 2002) reveals the existence of two closely linked genes

mapping in the X chromosome coding for proteins highly similar to Min and Dy. This suggests that this specific gene family has a long history within the Dipterans. Outside this group, no specific orthologues of the *min/dy/dyl* family have yet been defined. However, there are suggestions that the function of ZP proteins in cuticle synthesis might be widely conserved. One of the *C. elegans* proteins containing a ZP domain, Cut-1, has been identified as a structural component of the nematode cuticle. Cut-1 protein is apically secreted and localises in the most external cuticle layer, the cuticulin (Sebastiano et al., 1991). In the *Drosophila* wing, the epidermal cells secrete the cuticulin envelope prior to the reorganisation of the apical membrane that takes place during differentiation. One attractive hypothesis is that *m*, *dy* and *dyl*, like the Cut-1 protein, could be structural components of the fly cuticulin envelope. We observe that Min accumulates in the cytoplasm and is then transported to the apical membrane by the time of cuticulin envelope assembly. Min protein then forms a continuous layer covering the whole epidermal surface, consistent with the idea that Min is an envelope component. Unfortunately, our antibodies fail to detect Min protein shortly after secretion, so we cannot determine whether it becomes integrated into the cuticle or not. Nevertheless, we observe that in *Df(1)MR* mutants the formation of the cuticulin envelope is not prevented, indicating that neither Min nor Dy are essential for the formation of this structure. Instead, we see that the formation of the cuticulin envelope is delayed and that the apical membrane is disorganised. Whether this is the primary cause or is simply correlated with the failure of epidermal wing cells to undergo changes in cell shape is difficult to establish.

A second possibility consistent with our observations is that *m*, *dy* and *dyl* are components of a specialised apical matrix secreted at the time of the cuticulin envelope formation and necessary for the reorganisation of apical membranes during differentiation. ZP-domain-containing proteins characterised in flies and vertebrates seem to participate in the formation of apical extracellular matrices in different cellular contexts. For instance ZP1, ZP2 and ZP3 proteins are the main components of the mammalian oocyte zona pellucida and form a specialised apical matrix that is required for oocyte maturation (Rankin and Dean, 2000). Another example is the *Drosophila* NompA protein, which is a component of an extracellular structure called the dendritic cap of mechanosensory receptors (Chung et al., 2001). This structure mediates the interaction between the neuronal sensory processes and the external components of the sensory organ, where mechanical stimuli are transduced into neuronal membrane potentials (Chung et al., 2001). However, the NompA protein contains other conserved domains in its extracellular portion (Chung et al., 2001), and the role of its ZP domain remains to be elucidated. Perhaps proteins of the *m/dy* family, containing only a conserved ZP extracellular domain, are specialised in mediating interactions between the forming cuticle, the cell membrane and cytoskeleton components involved in cell shape reorganisation.

We have shown that *m*, *dy* and *dyl* are expressed throughout embryogenesis in many cuticle-forming tissues. It is thus paradoxical that the phenotype we observe in the *m/dy* mutant is specific to the wings. Indeed, the *Df(1)MR* mutant is viable, and we have not detected defects elsewhere in the adult or in the embryonic cuticle. One possible explanation for this specificity is that loss of the ZP proteins activity is most deleterious in tissues undergoing extensive rearrangement of their apical membranes, like the wing cells. We note that normal haltere cells do not undergo major changes in shape after the assembly of the cuticulin envelope (Roch and Akam, 2000), and even though Min is expressed in this tissue in a similar way to the wing, the haltere cells differentiate normally.

Functional redundancy is another possible explanation for the wing specificity of the phenotypes. The product of the *dyl* gene is a good candidate for fulfilling the roles of *dy* and *m* in their absence. All three proteins are closely related, and all are expressed in the embryonic epidermis during differentiation. Interestingly, *dyl* is not detectably expressed in the wing, providing a simple explanation for the sensitivity of this tissue to *m* and *dy* mutations. Unfortunately, there are at present no specific mutations affecting the *dyl* gene. Other more distantly related *Drosophila* ZP proteins may also contribute to the formation of the cuticulin envelope or to the apical matrix. In regard to the functional redundancy, a clear parallelism can be established with the proteins forming the mammalian zona pellucida. In mice, the presence of ZP3 and ZP2 is absolutely required for the formation of this matrix, whereas loss-of-function mutants for ZP1 present milder defects in its structure (Rankin et al., 2001).

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