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The distribution of PS integrins, laminin A and F-actin during key stages in *Drosophila* wing development

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†Michael Wilcox died 21st February, 1992. This paper is dedicated to his memory

SUMMARY

We first summarize wing development during metamorphosis of *Drosophila* and identify four critical steps in the conversion of a folded single layered wing disc to a flat bilayered wing. Each step occurs twice, once during the 12 hour prepupal period and again during the 84 hour pupal period. (1) *Apposition* in which basal surfaces of dorsal and ventral epithelia come close together. (2) *Adhesion* in which basal junctions form between the apposed basal surfaces. (3) *Expansion* in which wing area increases as a result of cells flattening. (4) *Separation* in which dorsal and ventral epithelia are separated by a bulky extracellular matrix but remain connected by slender cytoplasmic processes containing the microtubules and microfilaments of the transalar cytoskeleton.

Disc ultrastructure is correlated with the distribution of the β chain of integrin, laminin A, and filamentous actin for each key stage of pupal development. Integrin and laminin exhibit a mutually exclusive distribution from the adhesion stage onwards. Integrin is present on the basal surface of intervein cells but not on vein cells whereas laminin A is absent from the basal surfaces of intervein cells but is present on vein cells. We conclude that laminin is not a ligand for integrin in this context. During apposition and adhesion stages integrin is broadly distributed over the basal and lateral surfaces of intervein cells but subseqently becomes localized to small basal foci. These foci correspond to basal contact zones between transalar processes. The distribution of filamentous actin is dynamic, changing from an apical distribution during hair morphogenesis to a basal distribution as the transalar cytoskeleton develops. Basal adherens-type junctions are first evident during the adhesion stage and become closely associated with the transalar cytoskeleton during the separation stage. Thus, basal junction formation occurs in two discrete steps; intercellular connections are established first and junction/cytoskeletal connections are formed about 20 hours later. These observations provide a basis for future investigations of integrin mediated adhesion in vivo.

Key words: PS integrin, laminin A, *Drosophila*, wing morphogenesis

INTRODUCTION

The development of wings in *Drosophila* is an excellent model for the study of epithelial morphogenesis. Wing development is not only amenable to genetic analyses, as witnessed by the vast array of viable wing mutations, but it is also attractive from developmental and cell biological perspectives. Wings are relatively simple tissues consisting of two large flat epithelial sheets that undergo a clearly defined sequence of developmental events in vivo (Waddington, 1941) and in vitro (Milner and Muir, 1987). Except for narrow channels or veins, the two sheets of a differentiated pupal wing adhere to each other via slender basal cytoplasmic extensions that meet in basal junctions. The basal junctions connect an elaborate array of microtubules and microfilaments spanning all intervein (non-vein) cells from apex to base (Mogensen and Tucker, 1987; Mogensen and Tucker, 1988; Mogensenet al., 1989; Tucker et al., 1986). Because of their wing spanning nature, these cytoskeletal units are referred to as transalar arrays or the transalar cytoskeleton (Tucker et al., 1986). Here we will refer to the entire complex of transalar cytoskeleton plus basal junction as the transalar apparatus. The differentiation of the transalar apparatus occurs synchronously in all intervein cells across the wing (see below), facilitating molecular and cell biological studies. Despite its potential value for developmental studies, an overview of wing metamorphosis has not been published since Waddington's (1941) detailed light microscopic study. Thus, we first briefly summarize wing development during metamorphosis by combining our current observations with those of previous investigators. We then focus on the development of the transalar apparatus as a model for the development of an integrin-based adhesion system.

Genetic and immunological evidence demonstrate that integrins (transmembrane adhesion proteins) are involved in the formation and/or maintenance of the wing bilayer. The two integrins expressed in the wing, were originally identified as position specific (PS) antigens based on their restricted and complementary distributions in larval discs (Brower et al., 1984; Wilcox et al., 1981). PS antigens, like vertebrate integrins, are αβ heterodimers. They share a common β subunit (PSβ) that is distributed over most of the basal cell surface of wing discs. One α subunit (PS1α) is expressed on the future dorsal wing epithelium and the other (PS2α) is expressed on the future ventral wing epithelium. These expression patterns are seen in larval discs long before dorsal and ventral surfaces are apposed (Brower et al., 1985). The PS2α subunit is encoded by the *inflated* locus and the β subunit by the *myospheroid (mys)* locus on the X chromosome. (The α1 subunit is also encoded on the X chromosome but α1 mutations have not been identified.) A defect in either gene product, among other phenotypes, can result in a wing 'blister' i.e. a region of the adult wing where the two surfaces are not apposed (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990). The complementary integrin distribution in larval wing discs and the phenotype of the integrin defective mutants points to the basal junctions of wings as likely sites of integrin function. This view is further supported by the similarities between wing junctions and the junctions formed between muscle cells and epithelial tendon cells. Microtubular arrays, like the transalar arrays in wings, traverse the tendon cell from apex to base and insert into a basal junction that is ultrastructurally similar to the wing junction (Lai-Fook, 1967). Also like wings, there is a complementary distribution of integrins with PS1α expressed on the tendon cell and PS2α on the muscle cell (Leptin et al., 1989).

In vertebrates, integrins have been shown to act both as mechanical connectors and as signal transducers between extracellular molecules and the actin cytoskeleton (reviewed by Hynes, 1992; Singer, 1992). In many cases this interaction occurs via a specialized junction such as a focal adhesion between cell and matrix (Burridge et al., 1988). Cell to cell junctions of the adherens type also associate with the actin cytoskeleton. In both cell/matrix and cell/cell junctions, a subplasmalemmal plaque or undercoat containing α-actinn, vinculin and actin links the cytoplasmic terminus of the transmembrane adhesion molecule to the actin cytoskeleton. Although the PS integrins of *Drosophila* have been well characterized molecularly (Bogaert et al., 1987; Brown et al., 1989; Mackrell et al., 1988) the molecular links to the cytoskeleton have not been identified in *Drosophila*. Nor has an extracellular ligand/s been identified, although *Drosophila* homologs for laminin and collagen, two common ligands for vertebrate integrins, have been described (reviewed by Fessler and Fessler, 1989; Hortsch and Goodman, 1991). Investigations of genes that interact with integrin mutants to produce wing blisters may well provide one or more of these missing links in the *Drosophila* integrin complex (Wessendorf et al., 1992; Wilcox, 1990).

In this paper we show for the first time that PS integrins localize to the basal junctions of developing wings during metamorphosis. We describe the formation of these junctions and related morphogenetic events ultrastructurally and immunologically to provide a basis for future genetic and cell biological analyses. We also document dynamic changes in the distribution of PSβ integrin, laminin A and F-actin that are correlated with the differentiation of basal junctions, the secretion of a voluminous extracellular matrix and the differentiation of the transalar apparatus.

MATERIALS AND METHODS

Development of Oregon R prepupae and pupae was staged from puparium formation at 25°C (0 hour). Because of individual variability in rates of development, times given are approximate. Morphological criteria were also used to assess developmental stage (Bainbridge and Bownes, 1981; Mitchell et al., 1990).

Immunostaining and confocal microscopy

Immunostaining was carried out on frozen transverse sections of whole animals. Pupae were typically fixed before sectioning in 4% formaldehyde in PBS. Pupae were immersed in fixative and a mid-dorsal incision made through the pupal case and body wall. Fixation was for at least 1 hour at room temperature with agitation. The details of preparation varied with the stage of development and the antisera used. (Detailed protocols are available on request). The following antisera were used: a PSβ-specific monoclonal (CF6G11) a PS2α-specific monoclonal (CF2C7; Brower et al., 1984), a rat polyclonal against *Drosophila* laminin A (kindly provided by Claire Henchcliffe and Corey Goodman) and a monoclonal α-tubulin antibody (Amersham). Second antibodies conjugated with fluorescein or CYIII were obtained from Jackson Laboratories. F-actin was detected with rhodamine-phalloidin (Molecular Probes). Immunostained sections were observed by conventional immunofluorescence (Zeiss axiophot) and confocal microscopy (BIORAD 6000). Note that all the observations made by confocal microscopy were also made by conventional fluorescence microscopy.

Light and electron microscopy

Wings were fixed for electron microscopy in 1.5% glutaraldehyde, 0.5% formaldehyde in 0.1 M sodium cacodylate. The whole animal was fixed as above, then the wings dissected and processed by standard procedures into Epon 812 (Polysciences). 1 μm sections were stained with toluidine blue. EM sections were stained with uranyl acetate and lead citrate and examined on a JEOL 1200 electron microscope.

RESULTS

Critical stages of wing development

Metamorphosis occurs in two discrete periods associated with two major peaks and troughs of the molting hormone, 20-hydroxyecdysone (20-HE). The prepupal period lasts for 12 hours, from the formation of the puparium (white prepupa) until pupation (head eversion). The 84 hour pupal period extends from pupation to eclosion of the adult. Many of the cellular events in wing development occur twice, first in a brief and attenuated fashion in the prepupal period and again during the pupal period and are briefly described with reference to Fig. 1.

Apposition 1 (Fig. 1A)

During the first four hours of the prepupal period, in response to the metamorphic rise in 20-HE titer, the con-

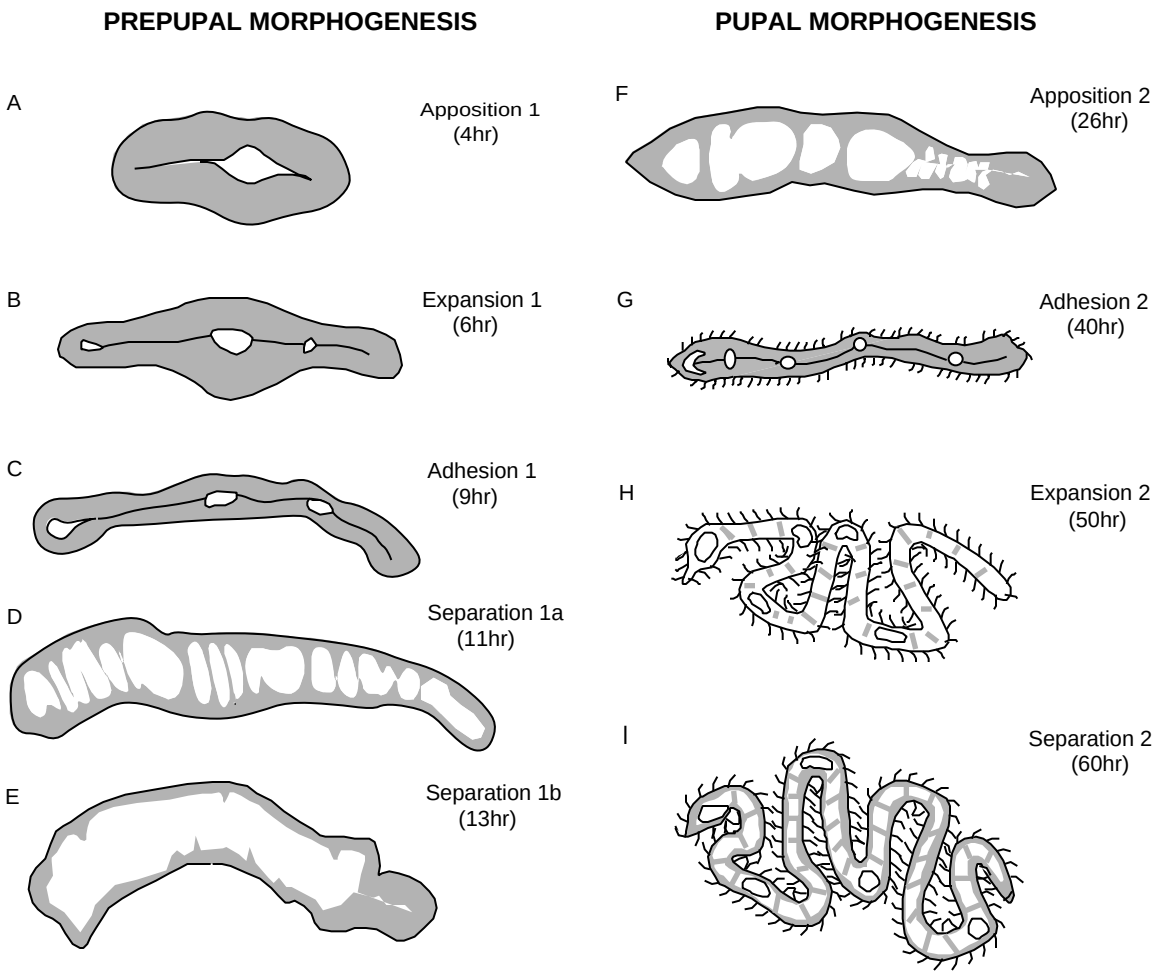

Fig. 1. Diagrams based on representative transverse sections of wings at successive stages of prepupal and pupal morphogenesis. Times shown are hours after puparium formation at 25°C. Note that expansion occurs before adhesion during prepupal development but after adhesion during pupal development.

centrically folded monolayered epithelium of the third instar wing disc is converted into a bilayered wing. This process involves the apposition of dorsal and ventral epithelia along with elongation and eversion of the wing blade and is accomplished by a series of localized cell shape changes (reviewed by Fristrom and Fristrom, 1992). At the end of the apposition period the basal surfaces of dorsal and ventral wing epithelia are in close proximity except for a prominent channel in the center of the tongue-shaped wing. The epithelium remains columnar. Following the final 'larval' mitoses that occur shortly after pupariation, most cells are in G2 arrest for the remainder of the prepupal period (Schubiger and Palka, 1987).

Expansion 1 (Fig. 1B)

From 4 to 7 hours the wing epithelium flattens and increases in surface area, taking on a more wing-like appearance. Expansion begins at the wing margins and extends inwards. Lacunae or preveins corresponding in position to adult longitudinal veins L3, L4 and sometimes L5 are now evident in whole mounts (Murray et al., 1984). In sections a crescent-shaped channel corresponding to L1 can be seen along the anterior margin. Two of these channels (L1 and L3) provide pathways for the migration of sensory nerve bundles from the periphery of the wing to the CNS, a process that begins during this stage and is completed by 16 hours (Murray et al., 1984). By 4 hours the pupal cuticle begins to be deposited at the apical cell surface in response to a fall in the 20HE titer and by 7 hours the apical surface is highly convoluted and a continuous cuticulin layer surrounds the disc (Fristrom and Liebrich, 1986). The basolateral ends of the cells are also convoluted and embedded in an amorphous extracellular matrix apparently secreted by the disc cells (see below). Basal junctions have not yet appeared.

Adhesion 1 (Fig. 1C)

From 8 to 11 hours there is little or no change in wing shape but the epicuticle and part of the procuticle is deposited (Fristrom and Liebrich, 1986). The presence of impermeable cuticle surrounding very flat tissue makes good ultrastructural preservation difficult. However, because basal junctions are absent in the previous stage and present in the next stage (see below), we infer that basal

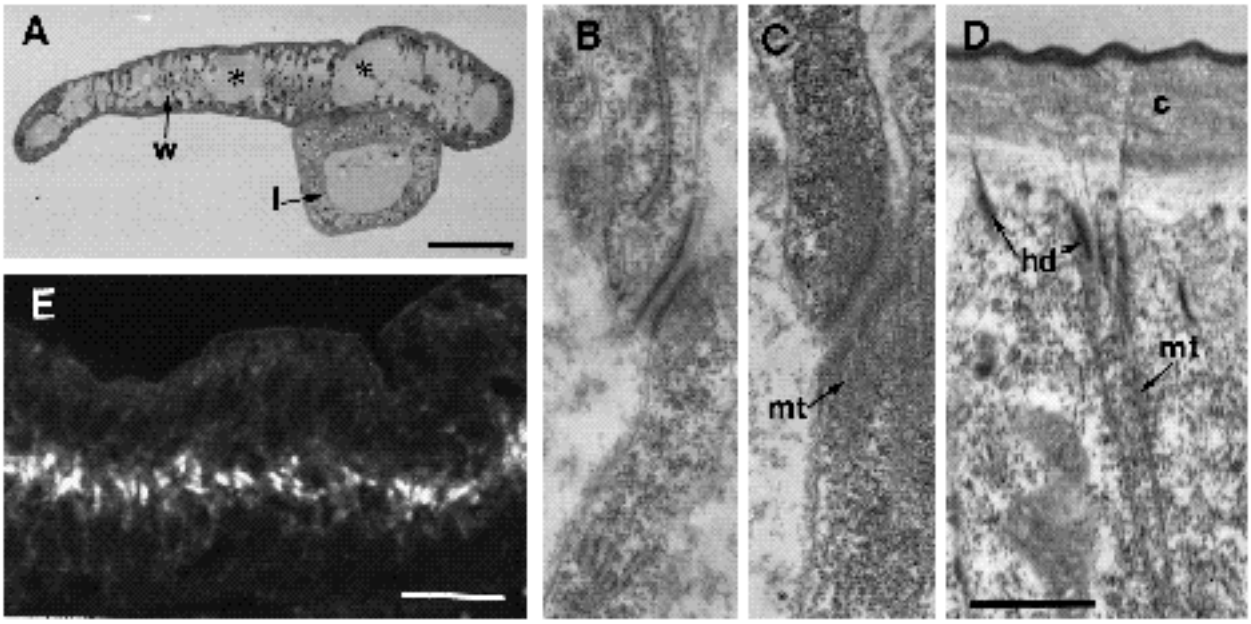

Fig 2. The formation of basal junctions and transalar arrays during prepupal development. (A-D) Light and electron micrographs of an 11 hour wing. (A) A transverse section of a wing (w) shows the transalar connections between dorsal and ventral epithelia. No connections are present in the preveins (*). The third leg (l) is also present in the section. (B, C) Two examples of basal junctions between transalar processes from the same wing. Microtubule arrays (mt) are absent in B but present in C. (D) Apical surface showing microtubules (mt) of the transalar arrays associated with hemi-desmosomes (hd). Note the strands of material connecting the hemi-desmosomes to the cuticle (c). (E). A frozen section of an 8 hour wing stained with a monoclonal antibody against β integrin. Integrin staining is localized to basal foci, presumably corresponding to the sites of junctions seen at 11hours. Scale bar in A, 50 μm; in B-D, 500 nm; in E, 10 μm.

junctions develop between the apposed basal surfaces during this period.

Separation - transalar connections 1 (Fig. 1D-E)

The apposed wing epithelia begin to separate around 11 hours permitting fixative penetration. The two surfaces are connected by cytoplasmic (transalar) processes stretching across the inflated wing cavity (Figs 1D, 2A). Microtubule arrays arise at apical hemidesmosomes and in some instances insert into basal junctions (see also Mogensen and Tucker, 1987; Tucker et al., 1986) thus forming the first transalar apparatus (Fig. 2C, D). However, in the center of the wing these arrays do not extend to the basal junctions and thus are not yet truly 'transalar' (Fig. 2B).

Pupation occurs at 12 hours when contractions of the abdominal muscles evert the head (reviewed by Fristrom and Fristrom, 1992). At the same time an influx of hemolymph forces the dorsal and ventral wing surfaces further apart until the wing is a bloated sac. This extreme separation disrupts most of the transalar processes, except for those at the periphery of the wing (see also Waddington, 1941). In wings cultured in vitro the separation is less extreme and transalar connections evidently remain intact (Tucker et al., 1986).

The final mitoses of wing development occur between 15 and 24 hours with the mitotic peak at 17-18 hours (Schubiger and Palka, 1987). In order to divide, cells round up and tubulin is organized into microtubules of the spindle apparatus. Thus, disruption of the transalar apparatus may be necessary to permit the final rounds of mitoses to take place. Deposition of pupal cuticle continues into the pupal period. The pupal cuticle is apolysed (i.e. separated from the epithelium) and loosely encases the wing for the remainder of development. Apolysis is triggered by a pronounced rise in 20-HE titer at 18 hours (Bainbridge and Bownes, 1988) that also heralds the morphogenetic processes of the next developmental stage.

Apposition 2 (Fig. 1F)

Between 20 and 35 hours the 20-HE level remains elevated and no cuticle deposition occurs. This period is largely devoted to reapposition of dorsal and ventral epithelia. Cytoplasmic processes extend from the basal surfaces of intervein cells through the matrix filled cavity of the wing until they meet processes extending from the opposite side. This process occurs in a precise and complex sequence (Waddington, 1941). The wing veins emerge progressively as regions that do not extend basal processes and thus remain as open spaces. Numerous hemocytes dispersed throughout the wing cavity after pupation eventually become restricted to the wing veins. During the process of reapposition large extracellular spaces between intervein cells give the wing a 'spongy' texture (Waddington, 1941). This extracellular material gradually disappears as the epithelium becomes columnar. By the end of this period the wing has the general shape and venation pattern of the adult wing. Basal surfaces of intervein regions have reapposed but extracellular spaces are still evident.

Adhesion 2 (Fig. 1G)

Extracellular spaces continue to diminish until around 40 hours when the wing epithelia are composed of a tidy pal-

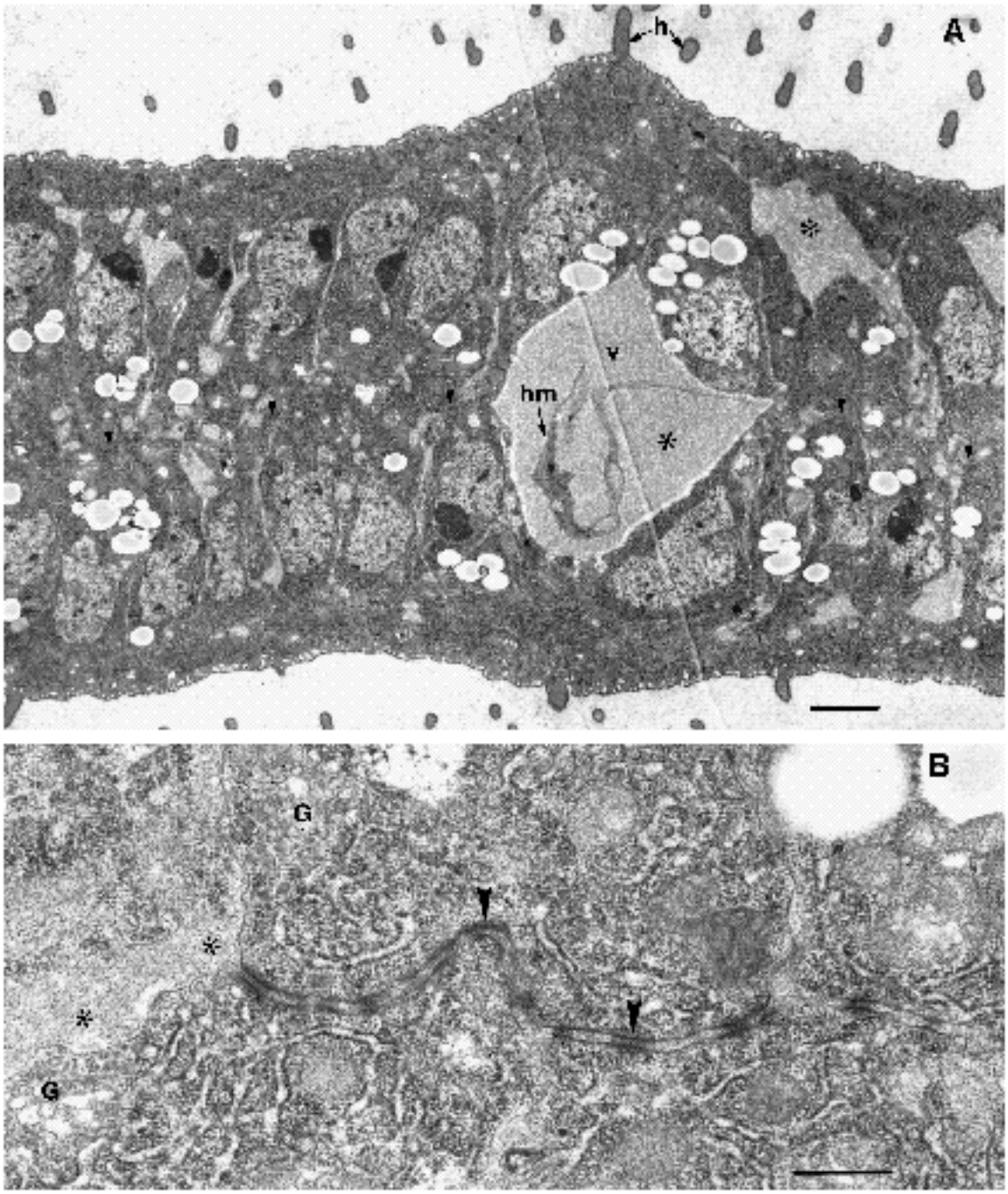

Fig. 3. Electron micrographs of pupal adhesion stage wings. (A) A 40-hour wing with extensive basal contacts between the regular palisade epithelia (arrowheads). Hairs (h) are present on the cuticulin covered apical surfaces. A vein (v) contains extracellular matrix (*) and a hemocyte (hm). The basal surfaces of the vein cells are lined by an electron lucent region (cf. Fig. 5F-H). (B) A higher magnification showing the basal junctions (arrowheads) between the apposed basal surfaces. Note the extensive rough endoplasmic reticulum, Golgi vesicles (G), and the newly secreted matrix (*). Scale bar in A, 2 μm; in B, 500 nm.

isade of columnar cells interrupted only by veins (Fig. 3A). This corresponds to the 'definitive' wing stage of Waddington (1941). Between 35 and 40 hours basal junctions appear between dorsal and ventral intervein cells (Fig. 3B) and cell hairs (actin filled cytoplasmic projections) are rapidly and synchronously extruded from the apical surface (Mitchell et al., 1990; Mitchell et al., 1983). During the latter part of this period the cuticulin layer of the adult cuticle is deposited. This is a brief but critical developmental period and is described more fully below.

Expansion 2 (Fig. 1H)

Beginning about 45 hours the wings begin to expand laterally and by 50 hours they are folded in a characteristic

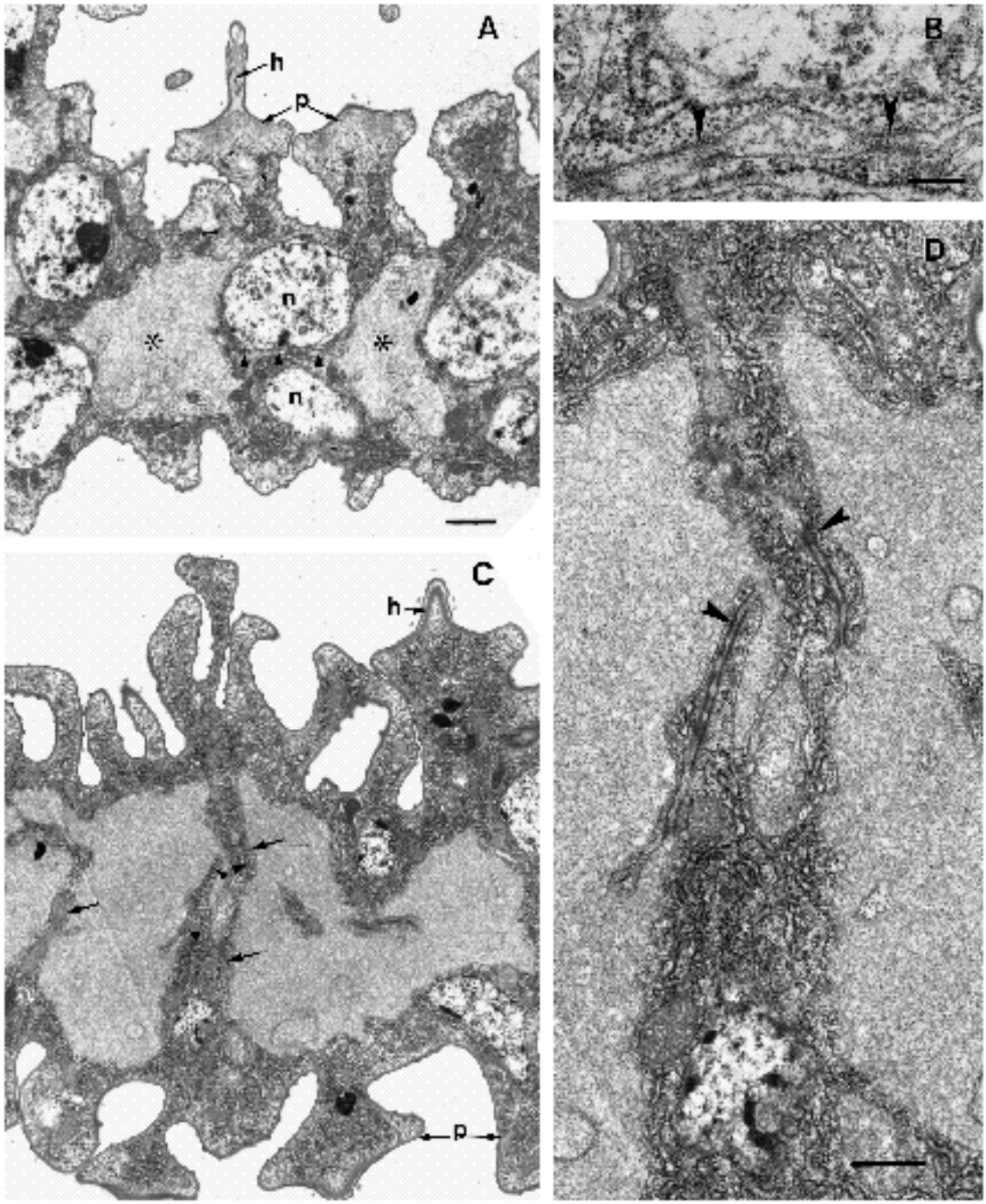

Fig. 4. Electron micrographs of pupal wings at expansion stage (A, B) and at separation stage (C, D). (A) A 50-hour wing with large matrix-filled extracellular spaces (*). Basal contact zones (small arrowheads) between the basally located nuclei (n) are straight and parallel to the wing plane. Hairs (h) and hair pedestals (p) are filled with filamentous actin (cf. Fig. 5L). (B) A high magnification of the basal contact zone from the wing shown in (A). Arrowheads indicate basal junctions. (C) A 60-hour wing with transalar processes (arrows) bridging the matrix filled wing cavity. Basal contact zones (small arrowheads) are folded so that the junctions are oriented obliquely to the wing plane. Hairs (h) are covered by a dense fibrous layer and the pedestals (p) are filled with rough ER. (D) A high magnification of a basal contact zone from the wing shown in (C). Arrowheads indicate basal junctions. Scale bar in A (for A, B), 1 μm; in C, 200 nm; in D, 500 nm.

manner within the apolysed pupal cuticle. Expansion results in a 2-3 fold increase in surface area of the wing. (The presence of pale yellow eye color (Bainbridge and Bownes, 1981) is a particularly useful marker for the early expansion stage). Hair pedestals (apical surface bulges at the base of the hairs) appear (Mitchell et al., 1983). At this stage both

hairs and their pedestals are filled with microfilaments (Fig. 4A). Pairs of coupled dorsal and ventral intervein cells separate from their neighbors basolaterally creating a new set of extracellular spaces. These spaces are filled with extracellular matrix secreted by the intervein cells (see below).

Separation - transalar connections 2 (Fig. 1I)

By 60 hours, expansion and folding of the wing is complete and the extracellular spaces have enlarged. Although this is referred to as the period of separation (Tucker et al., 1986), the increase in distance between dorsal and ventral surfaces is small compared to the first round of separation. This stage is largely devoted to the elaboration of the second transalar cytoskeleton, described in a series of elegant papers by Tucker and his colleagues (Mogensen and Tucker, 1987; Mogensen and Tucker, 1988; Mogensen et al., 1989; Tucker et al., 1986). Transalar microtubules begin to appear at 60 hours and by 84 hours they form an extensive parallel array stretching from their nucleating sites at apical hemidesmosomes to the basal junctions. The plus (growing) ends of the tubules are directed basally (Mogensen et al., 1989) and there is some ultrastructural evidence of bridges connecting the tubules to the cytoplasmic face of the basal junction. This second set of transalar tubules are unusual in consisting of 15 rather than 13 protofilaments. The transalar cytoskeleton also contains parallel arrays of actin filaments (Mogensen and Tucker, 1988) that also appear to be oriented unidirectionally.

At 60 hours deposition of endocuticle has begun with the cuticle covering the hairs being thicker than elsewhere (Fig. 4C). Cytoplasm including microfilaments is gradually excluded from the hairs (Mitchell et al., 1983) while the hair pedestals become very large and contain most of the cytoplasmic organelles (Fig. 4C). Although adjacent cells have separated basolaterally with the secretion of matrix material, the remaining apicolateral cell contacts are highly interdigitated and connected by extensive zonulae adherens junctions and septate junctions.

The 20-HE titer falls to basal levels between 60-65 hours; the deposition of the adult cuticle is completed and bristles and hairs become darkly pigmented.

Eclosion

The final stages of wing development occur after eclosion at 96 hours. The wings unfold and expand, hair pedestals disappear and the intervein cells degenerate so that the dorsal cuticle is directly apposed to ventral cuticle (Johnson and Milner, 1987). The cells surrounding the wing veins persist presumably to keep vein lacunae open.

The differentiation of the transalar apparatus and the distribution of integrin, laminin and actin

As outlined above, a transalar apparatus consisting of basal junctions associated with the transalar cytoskeleton develops in both prepupal and pupal periods. In this section we correlate changes in distribution of β integrin, laminin A and F-actin with the differentiation of these structures. The pupal transalar apparatus is much more pronounced than the prepupal structure. It is also more amenable to study because it develops over a period of some 30 hours as compared to 3 hours for the prepupal structure. We describe the formation of the prepupal transalar apparatus very briefly and then focus our attention on the formation of the pupal transalar apparatus.

(a) The prepupal period

At 11 hours, just prior to pupation, the two wing surfaces have separated but remain connected by transalar processes (Fig. 2A). Basal junctions between processes from opposite surfaces are evident (Fig. 2B,C) as are microtubules of the transalar arrays (Fig. 2C,D). The spaces between the transalar processes are filled with an extracellular matrix.

At 8 hours PSβ localizes to small discrete foci in the center of the wing blade (Fig. 2E) that are comparable in size, position and orientation to the distribution of basal junctions seen in EMs three hours later. We infer that PS integrins become restricted to sites of basal contact, basal junction precursors, at around 8 hours. This represents a pronounced change in distribution from the preceding stage when the distribution is over the entire basolateral surface (Wilcox et al., 1989). F-actin co-localizes with PSβ at junction sites at 11 hours but not at 8 hours (not shown). A similar association of PSβ and F-actin during basal junction formation is seen in the pupal period (below).

(b) Pupal period

Basal apposition

Towards the end of the period of apposition the basal surfaces of dorsal and ventral epithelia have reapposed so that the wing again forms an epithelial bilayer in which the major wing veins are evident (Fig. 5A). Despite the 'finished' appearance of whole mounted wings, sections show that there are still extensive extracellular spaces containing electron dense material. The apical cell surface is devoid of cuticle and hairs. The apposed basal surfaces of the intervein epithelium are highly convoluted and interdigitated but basal junctions have not yet formed.

An antibody to *Drosophila* laminin A exhibits two distinct staining patterns in wings: (1) intense staining of the basal surfaces of wing veins and (2) weaker, diffuse staining of extracellular spaces. Wing veins stain intensely for laminin A at all stages observed (Fig. 5B,F,J,N), and is characteristic of basal lamina staining seen elsewhere in the organism with this antibody (not shown). However, instead of an ultrastructurally identifiable basal lamina, an electron lucent space lines the wing veins (Fig. 3A).

Staining of the extracellular spaces was seen only in wings and suggests that these regions contain specialized matrix material rather than simply being hemocoelic spaces. We do not know whether this staining results from cross reactivity of the antiserum with the matrix or whether the wing ECM actually contains diffusely distributed laminin A. In either case this antibody is a useful marker for the extent and location of extracellular material. For example, at the end of the apposition stage, the large extracellular spaces are not readily seen in light micrographs (Fig. 5A) but are obvious in laminin stained preparations (Fig. 5B). A thin band of laminin staining persists between the apposed basal surfaces.

Staining for β integrin occurs along the lateral as well as the basal surfaces at 30 hours but is much more intense

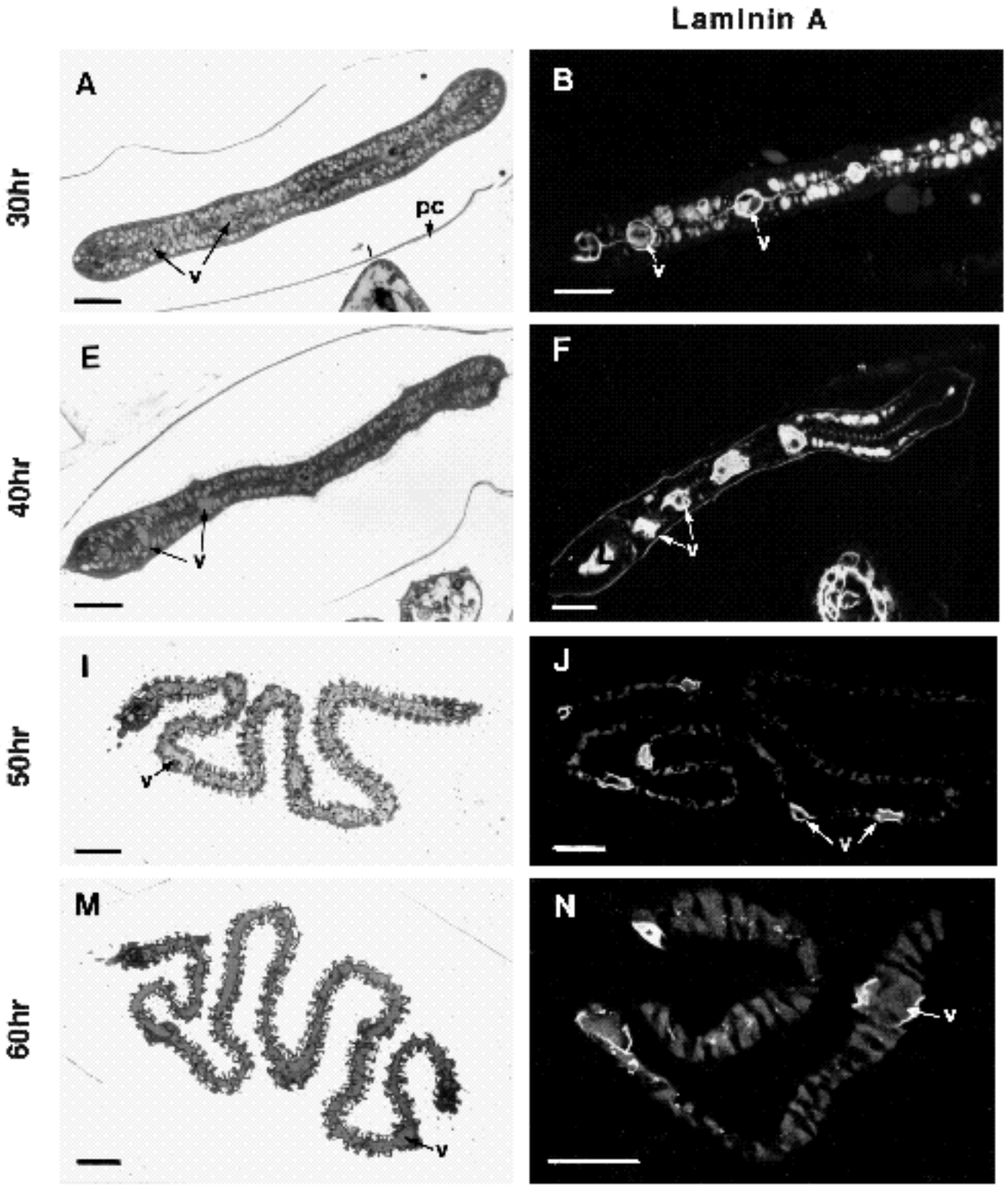

Fig. 5. Transverse sections of wings from four successive stages in pupal development. Developmental times indicated on left are approximate. (A-D) Late apposition stage. (E-H) Adhesion stage. (I-L) Expansion stage. (M-P) Separation stage. For each stage plastic embedded sections stained with toluidine blue (A,E,I,M) and frozen sections stained for laminin A (B,F,J,N); PSβ integrin (C,G,K,O) and F-actin (D,H,L,P) are shown. Anterior is to the left. Wing veins (v); pupal cuticle (pc). See text for further explanation. Scale bars on left page, 25 μm; on right page, 10 μm.

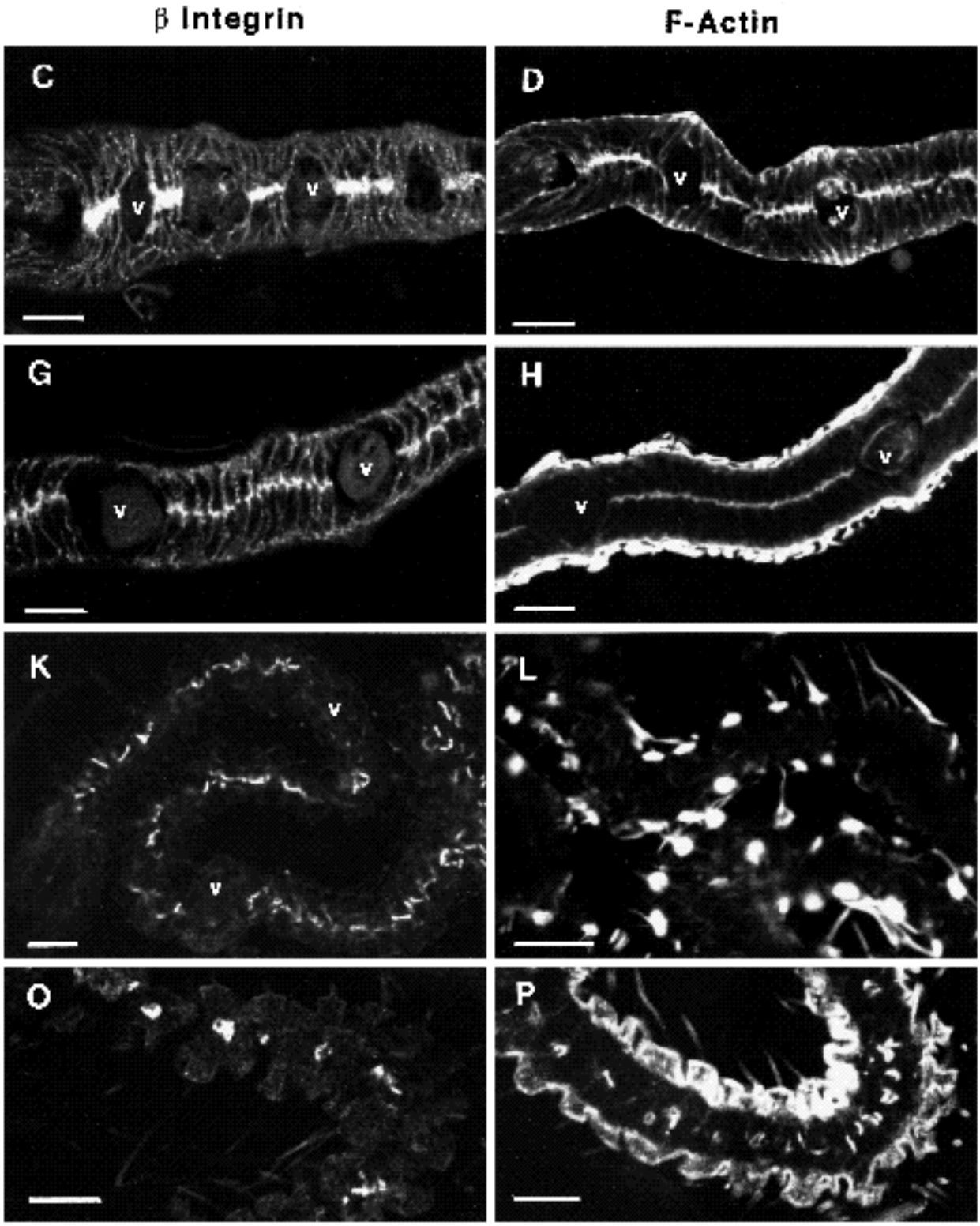

Fig. 5

basally than laterally (Fig. 5C). The relatively broad band of basal integrin staining presumably reflects the highly convoluted state of the basal surface. Integrin staining is absent from the basal surface of the wing veins (Fig. 5C). Phalloidin-staining for F-actin is associated with all cell surfaces but is most pronounced at the basal surface of intervein cells and at the apical surface of vein cells (Fig. 5D). The apical ends of the vein cells are narrower than their basal ends suggesting that they may be actively constricted by apical actin filaments.

Basal adhesion

The wing epithelium is now a tidy palisade of columnar cells (Figs 3A, 5A). The basal surfaces of intervein cells are smooth and closely apposed, forming a *basal contact zone* dotted with intercellular junctions. The junctions are characterized by electron dense material in the extracellular space and a thin fringe of fibers on the cytoplasmic face typical of adherens junctions (Fig. 3B). Junctions differentiate rapidly basally at about 35 hours, the time when hairs are extruded apically (Mitchell et al., 1990). (Six independent examples of 35-hour wings were studied. In three, extensive basal junctions were observed and hairs were present. In three others, neither basal junctions nor hairs were seen.)

At 35-40 hours most of the ECM staining seen with the anti-laminin antiserum has disappeared from the intervein regions (anterior half of wing shown in Fig. 5F). It is notably absent between the apposed basal surfaces, eliminating laminin A as a likely ligand for integrins present in the basal contact zones of pupal wings. Wing veins are now the major laminin-staining structures (Fig. 5F). An intense band of signal lines the vein and diffuse staining appears in the vein lumen (cf. Fig. 3A).

The distribution of β integrin remains essentially the same as in the preceding stage, although the basal staining is in a narrower, sharper band than before, reflecting the smoother basal cell surface (cf. Figs 3B, 5G). Integrin staining is conspicuously absent from the basal surface of the wing vein cells (where laminin staining persists). F-actin is concentrated in the newly extruded wing hairs and is also seen along the basal surface and very weakly along the lateral cell surfaces (Fig. 5H).

Adhesion-stage wings show clear ultrastructural evidence of 'gearing up' for the massive exocytosis of ECM to follow. There is an enormous proliferation of rough ER. Numerous basally located Golgi bodies (Fig. 3B) in addition to the usual apical Golgi are present. The basal Golgi are very close to the cell surface and secretory vesicles are only rarely seen. These ultrastructural features are characteristic of systems displaying rapid constitutive exocytosis (Moore et al., 1988). Note, however, that the Golgi of discs (small clusters of smooth surfaced vesicles of various sizes) lack the highly structured organization of vertebrate Golgi. Towards the end of this stage, the beginning of 'new' matrix accumulation between intervein cells can be seen ultrastructurally (Fig. 3B) and in laminin-stained preparations (posterior half of wing shown in Fig. 5F).

Expansion

As intervein cells flatten, the wing blade expands and folds (Fig. 5I). Large matrix-filled extracellular spaces appear (Fig. 4A). The basal secretory machinery (Golgi and rough ER) present in the preceding stage has largely disappeared leaving the basal ends of the cells occupied by nuclei (Fig. 4A). Basal contact zones and their junctions have not changed appreciably from the preceding stage. There is a broad, straight area of contact with three to four basal junctions per pair of connected cells (Fig. 4B).

The newly formed extracellular spaces stain lightly and diffusely with the anti-laminin antibody and are readily distinguished from the intensely stained veins (Fig. 5J). Integrin staining has disappeared entirely from the lateral surfaces. Short discontinuous stretches of basal staining corresponds to basal contact zones between pairs of cells separated by extracellular spaces (Fig. 5K). Wings double stained for integrin and laminin showed integrin staining alternating with patches of laminin stained ECM (not shown). F-actin appears to be restricted to the developing hairs, particularly the newly formed pedestals (Fig. 5L). In sharp contrast to every other stage examined, no F-actin staining was seen at the basal cell surface.

Transalar connections

The overall morphology of the wing has not changed markedly from the previous stage (Fig. 5N). The nuclei have moved apically so that the transalar processes are now slender cytoplasmic threads connecting the flattened dorsal and ventral epithelia (Fig. 4C,D). As the diameter of the transalar processes decrease, the basal contact zones become folded or interdigitated so that each basal junction is oriented obliquely to the plane of the wing (Fig. 4D). The junctions are longer than in the preceding stage and have conspicuous undercoats (Fig. 4D). In some areas basal surfaces between junctions loop apart. These changes in the topography of the basal contact zone are reflected in the pattern of integrin staining (Fig. 5O).

By 60 hours the first of the transalar microtubules can be detected ultrastructurally (see also Mogensen and Tucker, 1987; Tucker et al., 1986) and by anti-tubulin staining (not shown). Also by 60 hours F-actin has reappeared in the basal contact zones (Fig. 5P) where it presumably occurs in the junction undercoats (Fig. 4D). Actin is diffusely distributed apically, rather than concentrated in hairs and pedestals (Fig. 5P).

(c) The differentiated wing

By 84 hours wing differentiation is essentially complete. Transalar microtubules form an extensive parallel array stretching from their apical nucleating sites to the basal junctions (Fig. 6A) and thus stain intensely with anti-α tubulin except for a thin band corresponding to the basal contact zone (Fig. 6B). The basal junctions are very well developed with pronounced undercoats and a distinct line of dense material in the intercellular space (Fig. 6A; see also Fig. 4 in Mogensen and Tucker, 1987). Each basal contact zone forms a characteristic zigzag with the sides occupied by junctions and the apices by non-junctional membrane. PSβ localizes to the basal contact zones in 84-hour wings (not shown) but as in earlier stages does not appear to be localized to junctions per se.

Actin staining is now confined to the transalar cytoskeleton; it is particularly intense in the region of the basal junctions and tapers off apically (Fig. 6C). This is in agreement with the observations of Mogensen and Tucker (1988) who demonstrated that actin filaments were interspersed among the microtubules by decoration with S1 subfragments of myosin.

DISCUSSION

Coordination of morphogenetic processes during develop-

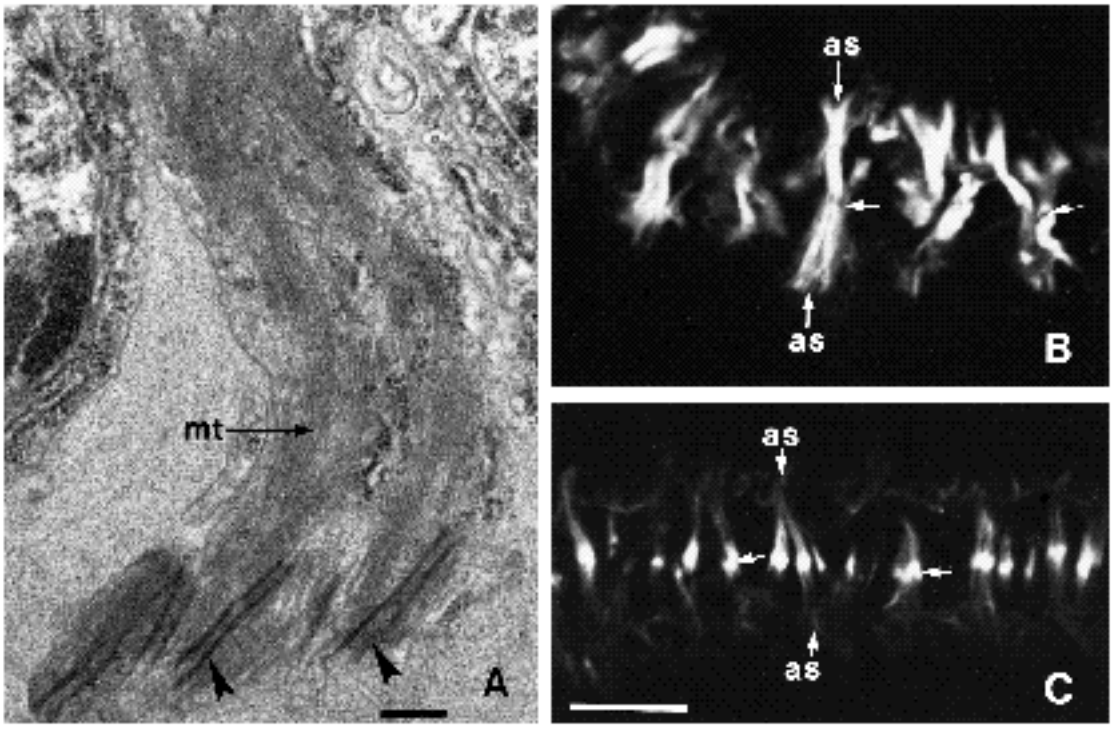

Fig. 6. The transalar apparatus of late (84 hours) pupal wings. (A) An electron micrograph showing the prominent bundles of transalar microtubules (mt) ending at the zigzag basal junctions (arrowheads). (B) A frozen section stained for α-tubulin. Note that the tubulin staining extends uniformly from apical surface to apical surface (as) except for a narrow band in the region of the basal junctions (arrows). (C) A frozen section stained for F-actin. Note that F-actin is concentrated near the basal junctions (arrows) and tapers off towards the apical surface (as). Scale bar in A, 200 nm; in C (for B, C), 10 μm.

ment depends on interactions between the cytoskeleton (the effector of cell movements and cell shape changes) and the immediate environment (adjacent cells or extracellular matrix). One system in which such interactions have been intensively studied is integrin-mediated adhesion, where some of the molecular links between the cytoskeleton, the transmembrane integrins and the external milieu have been identified (reviewed by Hynes, 1987; Hynes, 1992). During wing development in *Drosophila*, integrins appear to provide a linchpin in the transalar apparatus that stretches from one wing surface to the other. Wing development thus provides a rare opportunity to study the morphogenetic role of integrins from genetic, molecular and cellular perspectives. Our current descriptive paper begins to assess the nature and roles of the multicomponent integrin-based adhesion system in wing development to provide a basis for its further study.

During metamorphosis, wing morphogenesis depends on a series of four key steps: 1. *Apposition* (the basal surfaces of dorsal and ventral epithelia come together). 2. *Adhesion* (junctions form between the apposed basal surfaces). 3. *Expansion* (the wing expands in area as cells flatten). 4. *Separation* (the transalar apparatus differentiates). Each of these steps occurs during both the prepupal period and the pupal period although not in exactly the same order (Fig. 1). Apposition involves different cellular mechanisms in prepupal and pupal development and will be the subject of a separate paper. The remaining steps appear to involve similar cellular processes each time they occur, with the pupal events being much more pronounced than the corresponding prepupal events; compare, for example, the extent of basal junctions and transalar microtubules formed in the prepupal period (Fig. 2B) with those formed in the pupal period (Fig. 6A). Although our studies focus on the pupal period, the repetition of these cellular processes has a useful implication. Gene products that effect these morphogenetic processes should be expressed during both prepupal and pupal development.

We have correlated changes in the distribution of β integrin, laminin A and F-actin with ultrastructural observations on the development of basal junctions, secretion of extracellular matrix and differentiation of the transalar cytoskeleton during the pupal period. These events, occurring between 30 and 60 hours are summarized in Fig. 7. Developmental implications of these observations are discussed below.

Integrin distribution

(a) Apposition during pupal development involves the growth of cellular extensions across the matrix-filled wing cavity until they meet extensions from the opposite wing surface (Fristrom et al., unpublished data). We find that β integrin staining occurs along lateral and basal surfaces throughout the apposition stage (Fig. 5C). This is true even before the basal extensions have reached their destination (not shown) and raises the intriguing possibility that integrin-matrix interactions may be involved in the extension of basal cell processes through the wing matrix. A migrat-

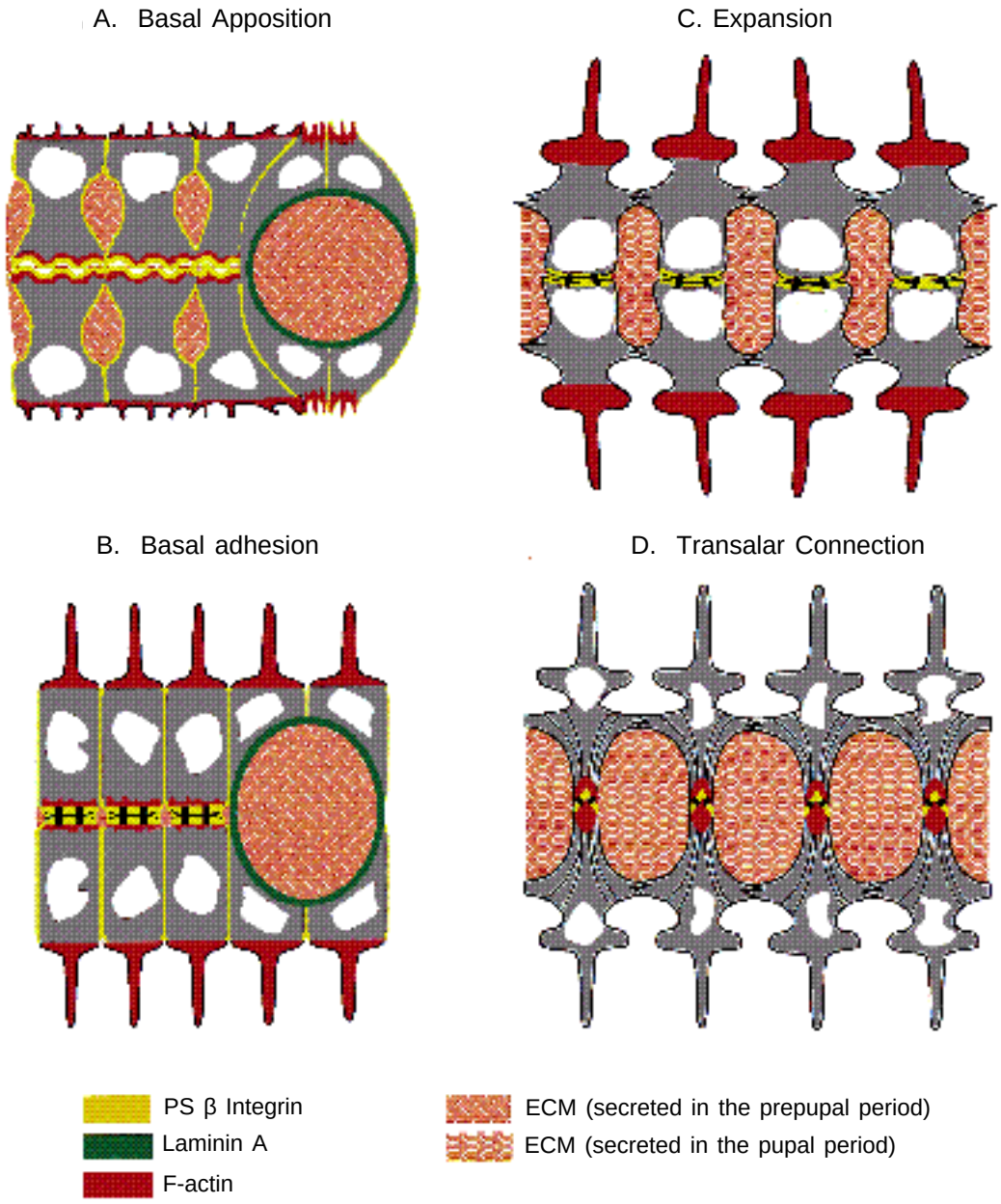

Fig. 7. A schematic summary of the localization of PSβ integrin, laminin A and F-actin at four key stages in the differentiation of pupal wings. White areas represent nuclei. Black bars connecting cells in B, C and D represent basal junctions. Wing veins (green) are shown only in A and B but persist unchanged for the remainder of development. Extracellular spaces containing matrix (ECM) of unknown composition in (A), disappear (B) and reappear (C, D).

ing cell or cell process gains traction by forming transient adhesions with its substrate, a process in which vertebrate integrins have been implicated (reviewed by Hynes and Lander, 1992). A role for PSβ in cell-substrate adhesion was recently demonstrated for a *Drosophila* cell line (Hirano et al., 1991) although the vertebrate substrate, vitronectin, used in these experiments has not yet been found in *Drosophila*. Further characterization of *Drosophila* wing mutants should address the possibility of an in vivo role for integrins in the extension of basal processes during reapposition. Blisters in adult wings can result from either a failure in the apposition of the wing epithelia or a failure in the maintenance of apposition (see below). A blister that results from a failure in apposition should be detectable in 30- to 40-hour wings, i.e. before the basal junctions appear.

(b) β integrin is distributed on lateral cell surfaces when those surfaces are in close contact, i.e., during apposition and adhesion stages. It is absent from the lateral surfaces of cells that are not in close contact i.e. during expansion when the cells become separated by large extracellular spaces. Thus, integrins may have a role in maintaining contact between adjacent cells. Lateral distribution of β integrin is seen on other tissues during development, including leg discs, myotubes, salivary glands, gut epithelia and

regions of columnar epidermis. Even before the product of the *mys* locus was identified as β integrin, Newman and Wright (1981) observed that the midgut epithelium became extremely flattened in *mys* embryos and proposed that the product of this locus was required for proper lateral cell-cell adhesions to maintain the columnar cell state.

(c) PSβ localizes to the basal contact zones between dorsal and ventral cells and, based on genetic evidence (see introduction), has a role in adhesion between the apposed basal surfaces. Importantly, no obvious change in the distribution of β integrin occurs at the time when basal junctions first appear. Integrins are present on closely apposed basal surfaces long before the junctions differentiate. After junctions appear, integrin staining apparently remains generally distributed over the basal contact zone. If PSβ became restricted to the junctions per se, one would expect to see a punctate pattern rather than the observed pattern of unbroken lines and loops (where basal surfaces between junctions have presumably separated). It would be desirable to confirm these confocal observations by immunostaining at the ultrastructural level. We conclude that the formation of basal junctions is far more complex than the simple juxtaposition of cell surfaces expressing complementary integrins. The integrins may become modified at the time of junction formation (an alternately spliced form of PS2α has been identified; Brown et al., 1989) or a new or modified ligand may be present at junctional sites. Most likely, other transmembrane component/s (e.g. a cadherin, often associated with adherens junctions) may be present in and restricted to the junctions.

Thus, based on distribution alone, we have at least three possible roles for integrin in wing metamorphosis, one involving a cell/matrix interaction and two involving cell/cell interactions. Furthermore, integrins are distributed over the basal surface of wing discs long before metamorphosis takes place, where they may have a role in adhesion to the disc basal lamina. In vertebrates mutiple adhesion tasks are performed by a wide variety of integrins. In contrast, in *Drosophila*, only a few integrins have been identified. Perhaps here the multiplicity of tasks might be served by variations in ligands.

Laminin distribution

Laminin, a widespread component of basal laminae in vertebrates has a well characterized *Drosophila* homolog (Fessler et al., 1987; Hortsch and Goodman, 1991). Vertebrate laminin is used as a substrate by six identified integrins (Hynes, 1992). Here we examine the distribution of *Drosophila* laminin A (the largest of the three subunits comprising this heterotrimer). Surprisingly, laminin A and PSβ appear to have mutually exclusive distribution patterns. From 40 hours onward, laminin staining is absent from the basal contact zones where integrin is localized and is strongly expressed on the basal surface of vein cells where integrin staining is absent (Fig. 5). Thus, at least two adhesion systems function in pupal wing development; in veins an unknown receptor presumably binds to laminin and in intervein cells integrins bind to an unknown ligand(s). Our conclusion that laminin is not a likely ligand for integrin in basal junctions is consistent with those of other laboratories. Volk et al. (1990) observed that adhesion of cultured *Drosophila* myotubes to laminin substrates was independent of PSβ. Bunch and Brower (1992) showed that a *Drosophila* cell line transfected with PS2 integrin, adheres to vertebrate vitronectin and to RGD peptide but not to *Drosophila* laminin.

Actin distribution

F-actin shows particularly dramatic changes in distribution during wing metamorphosis. For example, at 50 hours F-actin is almost exclusively apical and at 84 hours it is almost exclusively basal. The apical concentration of F-actin at 40 and 50 hours (Fig. 5) correlates with the formation of the microfilament filled hairs and hair pedestals. Once the shape of the hairs and pedestals is established, actin staining and microfilaments disappear from these structures.

Actin is a ubiquitous component of subplasmalemmal plaques or undercoats associated with junctions of the adherens type (Tsukita et al., 1990). Short actin polymers are just one of many components that localize to junctional undercoats in vertebrates. Others include vinculin and α-actinin that have been directly implicated in connecting integrins to the cytoskeleton (Burridge et al., 1988; Otey et al., 1990; Tsukita et al., 1990). Although an α-actinin gene has been isolated in *Drosophila*, the non-muscle isoform does not appear to be essential (Roulier et al., 1992). The basal junctions of 60-hour+ wings is one site where such linking molecules might be expected to appear. In wing development there appears to be a temporal separation in the formation of junction (intercellular connection) and its undercoat (cytoskeletal connection). No detectable F-actin was associated with the basal junctions at 50 hours but F-actin reappeared basally at 60 hours, when microtubules of the transalar cytoskeleton are first seen (Mogensen and Tucker, 1987) and when subplasmalemmal plaques associated with the junctions become prominent (Fig. 4D).

Function of the transalar apparatus

The transalar apparatus is a mechanically continuous structure consisting of parallel arrays of microtubules and microfilaments anchored apically to the cuticle via hemi-desmosomes and basally to the opposite epithelial layer via the basal junction. Equivalent structural organization is seen in epidermal cells that are attached to muscles (Lai-Fook, 1967). In these myoepidermal junctions, muscle contraction exerts stress on the junction. Indeed, in severe alleles of the PSβ gene, *mys*, the muscles pull away from the embryonic epithelium when they begin to contract (Newman and Wright, 1981). Similarly in wing mutants such as *inflated*, the basal surfaces separate to form blisters. In wings, the source of tension on the basal junctions is less obvious than for myoepidermal junctions. It has been assumed that the transalar arrays prevent the wing surfaces from coming apart as they unfold and expand after eclosion. However, Johnson and Milner (1987) report that intervein cells are degenerating before unfolding starts. Degeneration is complete in the newly expanded wing so that dorsal cuticle is in direct contact with ventral cuticle. Thus, a function for the transalar apparatus after eclosion is moot. We have described the accumulation of a voluminous extracellular matrix in the wing cavity beginning around 50 hours, coincident with wing expansion and folding. The accumulation

(and possible hydration) of this material would tend to separate the two wing layers and thereby exert tension on the basal junctions. We therefore propose that the transalar apparatus functions as a tensility structure during the latter part of pupal development.

The integrin/ligand interactions in myoepidermal junctions and the basal junctions of wings may have unique properties that enable the junctions to withstand tension. One unusual properity of these junctions is the complementary distribution of integrins (PS1 on one surface and PS2 on the other). In myoepidermal junctions such complementarity might reflect a requirement for different cytoplasmic domains in integrins of muscle versus epidermal cells. However, this cannot be the case in wings where epidermal cells occupy both sides of the junction. The width of the junctional space in EM sections is 30-50 nm and the extracellular domain of a typical integrin molecule has been estimated as 20-23 nm (Nermut et al., 1988) Thus, a direct heterophilic interaction between the external domains of PS1 and PS2 appears physically possible. Another, perhaps more likely, possibility is that PS1 and PS2 bind to different sites on a shared ligand. The vertebrate integrin ligands, laminin, fibronectin and fibrinogen, have all evolved multiple recognition sites (reviewed by Hynes, 1992) creating the opportunity for simultaneous binding of two different integrins.

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