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Distinct spatial and temporal functions for PS integrins during *Drosophila* wing morphogenesis

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SUMMARY

At the onset of pupariation in the *Drosophila* wing, the PS1 and PS2 integrins are expressed preferentially on the dorsal and ventral wing epithelia, respectively. Clonal analysis experiments have indicated that integrins are required to maintain the tight association of the wing surfaces. Surprisingly, we find that even in clones of cells lacking integrins the wing layers become apposed early in metamorphosis. However, following the normal period of wing separation, large integrin mutant clones do not become re-apposed in the pupa, and integrins are not organized in basal plaques in cells opposite a mutant clone. Paradoxically, our experiments indicate that at least one integrin function requires different integrins on the dorsal and ventral wing surfaces, however in some cases both α_{PS} subunits can function to some degree on each wing surface. Finally, overexpression of an α_{PS} subunit throughout the wing leads to a dominant wing blister phenotype, and the critical period for this phenotype is the beginning of pupariation. These data indicate that integrin requirements in wing morphogenesis can be separated into early (prepupal) and late (pupal) functions. The late function seems to reflect the traditional view of integrins as cell-matrix adhesion proteins. The early requirement, which probably requires dorsoventral segregation of PS1 and PS2, suggests functions for PS1 and PS2 in signaling events that regulate morphogenesis.

Key words: integrins, *Drosophila*, wing morphogenesis, signaling, adhesion

INTRODUCTION

Integrins are a large, phylogenetically conserved family of cell surface receptors (reviewed by Hynes, 1992). A functional integrin is composed of an α and β subunit, both of which consist of a large extracellular domain and a relatively small cytoplasmic tail. Most integrins are receptors for extracellular matrix components, although some are known to bind directly to other cell surface proteins.

Integrins were first recognized as cell adhesion proteins, and as such have been shown to be important in a variety of morphogenetic and physiological events, including cell migrations during embryogenesis and extravasation of lymphocytes during immune responses (see review by Gumbiner, 1996). Such processes require careful modulation of adhesive functions, and it is known that cells can regulate the activity of integrins at the cell surface, apparently by transmitting a signal from the cytoplasmic domains to the external parts of the protein. Integrins also transmit extracellular signals to the cell interior, and have been shown to modulate the activities of a number of cytoplasmic signal transduction pathways. This outside-in signaling appears to be necessary to organize cytoplasmic complexes required for adhesion and it can also regulate more complex cellular behaviors such as control of cell proliferation and differentiation (see reviews by Juliano and Haskill, 1993; Humphries et al., 1993).

The discovery of the PS integrins of *Drosophila* (reviewed by Brown, 1993, and Gotwals et al., 1994a) provided the opportunity to examine integrin functions in complex morphogenetic processes in situ, using the arsenal of genetic and molecular manipulations for which the fly is famous. The PS integrins, like all integrins, are composed of heterodimers of two large transmembrane polypeptides; PS1 is an $\alpha_{PS1}\beta_{PS}$ dimer, PS2 contains a different α subunit (α_{PS2}) in combination with the same β_{PS} (Brower et al., 1984; Wilcox et al., 1984). Recently, an α_{PS3} subunit that combines with β_{PS} has been identified (see Gotwals et al., 1994a), but relatively little has been reported concerning its structure and function. Like most vertebrate integrins, the PS integrins are receptors for extracellular matrix proteins; PS1 will mediate cell spreading in culture on *Drosophila* laminin, and PS2 binds to tiggrin, another fly matrix protein, for which no vertebrate homologue has been reported (Gotwals et al., 1994b; Fogerty et al., 1994).

The *Drosophila* wing provides an excellent system for the study of morphogenesis in an intact animal. The wing is non-essential, so that mutations or other manipulations affecting it will not necessarily reduce viability. The wing's large size facilitates the process of genetic screening and scoring of mutant phenotypes. Finally, wing morphogenesis is a relatively simple process involving the conversion of a single layered

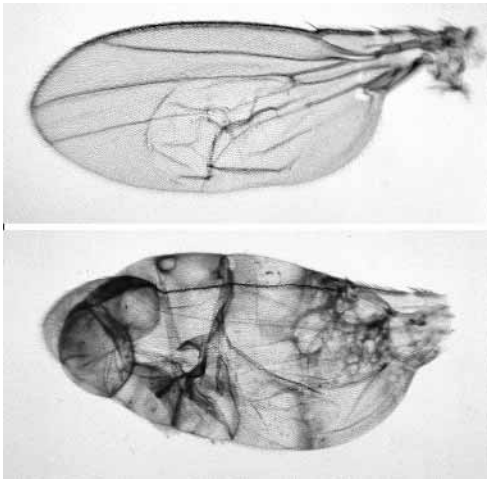

Fig. 1. Blisters associated with *myospheroid* clones. (Top) A typical blister. (Bottom) An extreme example where dorsal and ventral surfaces are completely separated.

columnar epithelium to a flattened bilayer where the basal surfaces of dorsal and ventral epithelia are in close contact.

The PS integrins were first identified (and named) in monoclonal antibody screens that noted their Position Specific expression in wing imaginal discs prior to pupariation (Wilcox et al., 1981; Brower et al., 1984). In the late third larval instar wing disc, PS1 is expressed primarily in cells that will become the dorsal surface of the adult appendage, while PS2 is expressed almost exclusively in ventral cells. This remarkable expression pattern of two cell adhesion proteins suggested a specific role in wing morphogenesis, and the cloning and identification of mutations in the genes encoding the integrin subunits (Wright, 1960; Bogaert et al., 1987; MacKrell et al., 1988; Leptin et al., 1989; Wilcox et al., 1989; Wehrli et al., 1993; Brower et al., 1995) has allowed this role to be defined. In the pupal wing, integrins are localized to adhesive contact sites at the basal surface of the epithelia (Fristrom et al., 1993), and elimination of PS integrins from either wing surface in patches of mutant cells, or in some cases even partial loss of integrin function, leads to the separation of the dorsal and ventral wing surfaces (a wing 'blister,' Fig.1) in the adult (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990). These studies have given rise to the idea that PS integrins function as a 'cell-matrix-cell glue,' maintaining the close apposition of the basal surfaces of the two wing epithelia in the pupa and newly eclosed adult.

The studies to date demonstrate that PS integrins hold the dorsal and ventral wing epithelia together, but they do not explain why this should require the dorsoventral separation of PS1 and PS2 seen at the beginning of metamorphosis. Indeed, it is not even clear that dorsoventral specific expression is necessary. In embryonic muscle attachment sites PS1 is found on tendon cells and PS2 on the somatic muscles (Bogaert et al., 1987; Leptin et al., 1989). While loss of PS2 results in failure of attachments at contraction (Brabant and Brower, 1993; Brown, 1994), PS1 can be removed without an obvious muscle phenotype (Brower et al., 1995). In the developing eye, it appears that although PS1 is normally most critical for morphogenesis, either integrin can support fairly normal development (Roote and Zusman, 1996). We set out to ask if integrins in the wing are involved in something other than simple adhesion. Our results indicate that proper morphogenesis requires different integrins on the two wing epithelia. This specificity is probably related to the finding that early, in the prepupal wing, integrins are not obviously important for the establishment or maintenance of dorsoventral adhesion, but an integrin-dependent regulatory process is required at this time in order to permit subsequent pupal morphogenetic events. Taken together, our data suggest that integrin requirements in the wing include an early signaling function and a later requirement primarily related to adhesion.

MATERIALS AND METHODS

Mutant stocks

Mutant integrin alleles have been described by Bunch et al. (1992; *myospheroid*, or *mys*); Brabant and Brower (1993), and Brown (1994; *inflated*, or *if*); and Brower et al. (1995; *multiple edematous wings*, or *mew*). if^{H18} and if^{K13} are previously undescribed lethal alleles. Properties of marker mutations can be found in Lindsley and Zimm (1992). FLP^{F38} is a P-element insertion of the gene encoding the *S. cerevisiae* FLPase recombination enzyme, under the control of a heat shock promoter, on the second chromosome (Chou and Perrimon, 1992). FRT^{18A} is a P-element insertion, at polytene band 18A of the X chromosome, of the site at which FLPase induces recombination (Xu and Rubin, 1993).

Production and examination of mitotic clones

X-linked mitotic recombination was induced in animals homozygous for FRT^{18A} and heterozygous for marked *myospheroid*, *mew* or *inflated* chromosomes by applying heat shocks to induce the FLPase enzyme encoded by FLP^{F38}. (See Xu and Rubin, 1993, for a description of the method.) Mutant chromosomes contained the X-linked bristle and trichome marker *forked* (f^{36a}) to identify wing clones. Wings were removed from flies in ethanol, mounted in Euparal (Gallard Schlesinger) and viewed using bright-field microscopy.

To examine wing clones during metamorphosis, prepupae and pupae were staged from puparium formation at 25°C for 4-36 hours (hours after pupariation, or AP). Wings were fixed, dissected and stained as previously described (Fristrom et al., 1994). Siblings were allowed to develop to adults to assess blister frequency. Clone frequency and size were a function of time of heat shock, which ranged from the second instar to mid third instar. Integrin mutant stocks used were mys^{XB87}, if^{A7} and mew^{M6}. *myospheroid* clones were identified by the absence of staining with the anti-β_{PS} specific monoclonal CF.6G11 (Brower et al., 1984). *mew* and *inflated* clones were identified by defective f^{36a} hairs (stained with phalloidin) after 36 hours AP. Wings were examined by conventional immunofluorescence (Zeiss Axiophot) and confocal microscopy (BIORAD 600). Two kinds of optical sections were analyzed. (i) Standard optical sections in the x-y axis parallel to the broad wing surface. By stepping up and down in small increments through the z axis one obtains an overview of the wing structure and can select 'sections' of particular interest such as ones passing through the basal cell surface. (ii) 'Line' collections that scan a stack of lines (i.e. a single value for y in the x axis) separated by small increments (usually 0.2 μm) in the z axis to provide cross sectional views of the tissue.

Expression of integrin transgenes

Plasmids pUASPS2m8 and pUASPS2c (containing α_{PS} genes under the control of UAS^{GAL4}) were made by cloning the respective *Sal*I fragments (extending from nucleotides 252 to 4630, Bogaert et al., 1987) from cDNAs which encode α_{PS2m8} and α_{PS2c} (these contain some 5′ and 3′ untranslated sequences) into the *Sal*I site of pBlue-

script. The cDNA was then removed from this vector with *Xho*I and *Xba*I and cloned into the pUAST vector (Brand and Perrimon, 1993) for injection into embryos. α_{PS1} cDNA from nucleotides 315 to 4700 (Wehrli et al., 1993) was excised from pMETα_{PS1} (Gotwals et al., 1994b) using *Kpn*I and *Xba*I, and the resulting fragment cloned into pUAST.

To drive expression of UAS^{GAL4} constructs, GAL4 enhancer traps, from a collection generated in the laboratory of Kim Kaiser, were selected for high levels of expression in the wing pouch. (See Brand et al., 1994, for a general description of the use of GAL4 enhancer traps.) For the experiments reported here, the *P[GAL4^{684} w$^+$]* insert on the third chromosome was used.

Rescue of mutant clones

To express α_{PS} transgenes in animals with integrin mutant clones, a cross was performed between *w* (*mew* or *if*) *f^{36a} FRT^{18A}/FM7; +/+; P[GAL4^{684} w+]/+* females and *w FRT^{18A}; F38, hs-FLPase; P[α_{PS}^+ w$^+$]* males. This cross yields females that are *w* (*mew* or *if*) *f^{36a} FRT^{18A}/w FRT^{18A}; F38, hs-FLPase/+; P[α_{PS}^+ w$^+$]/P[GAL4^{684} w$^+$]*, which can make integrin mutant clones and express an integrin transgene, and also *w* (*mew* or *if*) *f^{36a} FRT^{18A}/w FRT^{18A}; F38, hs-FLPase/+, P[α_{PS}^+ w$^+$]/+* controls, which will make clones but not express the transgene, due to lack of the *P[GAL4^{684} w$^+$]*. The presence of the *P[GAL4^{684} w$^+$]* insert can be scored by eye color, since the *w*$^+$ from this P element results in a much darker color than the *w*$^+$ genes in the *P[α_{PS}^+ w$^+$]* inserts. Alleles used to obtain the data presented in Table 1 are: *mew^{M6}*, *mew^{498}* (numbers for the two *mew* alleles are pooled) and *if^{A7}*. The *PS1Z9* transgene was used to express α_{PS1} (another transgene has given qualitatively similar results under slightly different experimental conditions), and for α_{PS2c} expression, the parental flies were heterozygous *2CF3/2CGG9*, as each of these inserts is lethal or sterile as a homozygote. All crosses were grown at 22°C, and the animals given 2 heat shocks (37°C for 1 hour), spaced 1 day apart, early in larval development. Larvae and pupae were then allowed to develop at 25°C and adult wing blisters were scored using the dissecting microscope.

Overexpression of α_{PS} subunits

Dominant blister phenotypes have been observed with a number of different transgenes expressing α_{PS1}, α_{PS2c} or α_{PS2m8}, driven by various GAL4 enhancer traps. Most of our observations, including the timing data presented here, have used a *P[α_{PS2m8}^+ w$^+$]* transgene *M8K6*, driven by *P[GAL4^{684} w$^+$]*, which has been recombined onto the same third chromosome. Dominant blister phenotypes have also been observed using heat shock-driven α_{PS} transgenes; details of these experiments have been described by Brabant (1995).

RESULTS

Two distinct periods of dorsal-ventral apposition and integrin expression during wing morphogenesis

Wing morphogenesis from 6 to 30 hours after pupariation (AP) is illustrated in Fig. 2. The dorsal and ventral wing epithelia initially appose during the prepupal period (Fig. 2A), separate at pupation (Fig. 2B,C) and then re-appose during the pupal period (Fig. 2D,E). Each period of apposition culminates in the differentiation of a transalar array of microtubules and microfilaments anchored in integrin containing junctions between apposed basal surfaces (Tucker et al., 1986; Fristrom et al.,

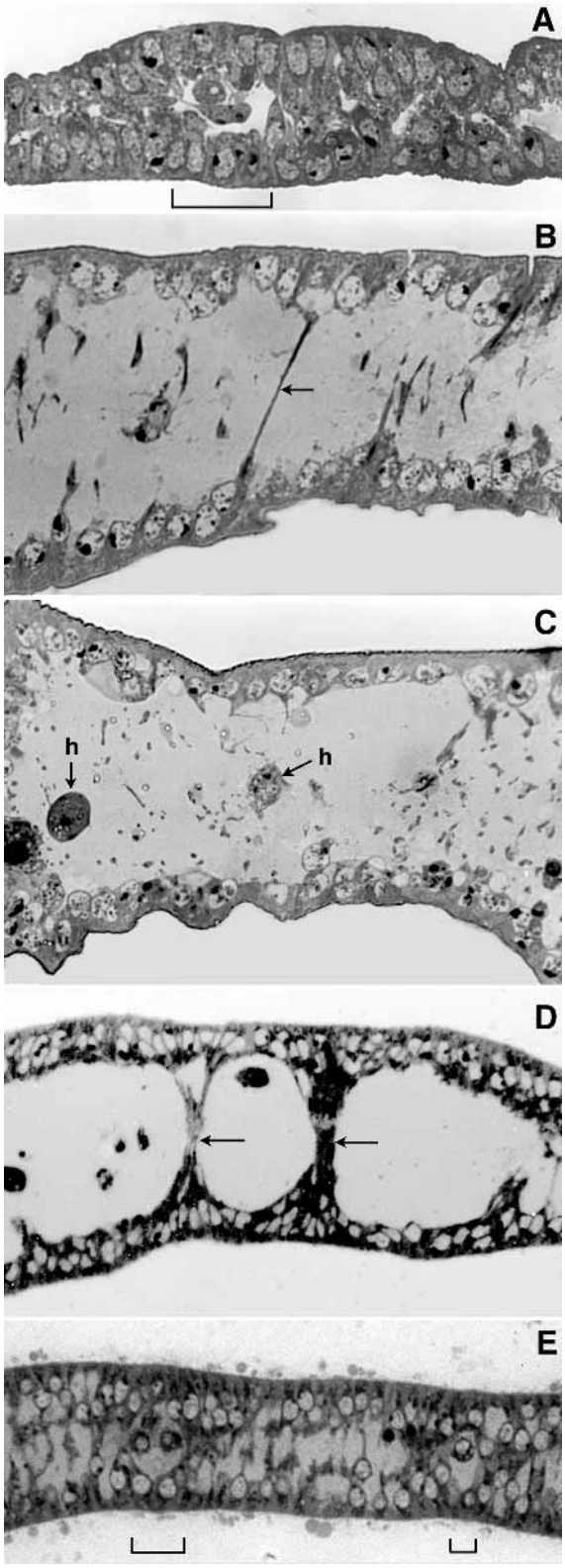

Fig 2. Transverse sections of wings showing the developmental processes of dorsal-ventral apposition, separation and re-apposition. (A) 6 hours AP; surfaces are apposed except for broad prevein areas (bracket). (B) 11 hours AP; cytoplamic processes between the two wing surfaces stretch (arrow) and fragment as the surfaces separate. (C) 12 hours AP; the interior of the wing is filled with cytoplasmic fragments and hemocytes (h). (D) 21 hours AP; the intervein regions begin to re-appose (arrows). (E) 30 hours AP; cytoplasmic connections between intervein cells have reformed and the hemocytes are restricted to vein channels (brackets).

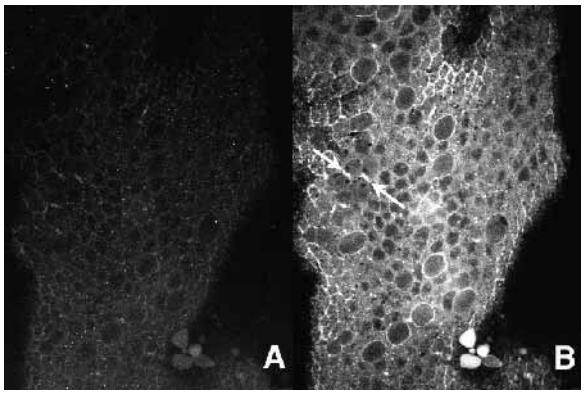

Fig 3. Dual channel confocal image of an 18 hour AP wing (separation stage), double labeled with fluorescein-conjugated anti-β_{PS} (A) and rhodamine-conjugated phalloidin (B) using the same settings for both channels. There is little or no staining for integrin at this stage (cf 30- 36 hours AP; Figs 5, 6). Phalloidin staining shows many large pre-mitotic cells and several post-mitotic cells (arrows).

1993). Prepupal apposition occurs as the larval wing disc folds along the wing margin to bring dorsal and ventral epithelia together (reviewed by Fristrom and Fristrom, 1993). Integrins are initially distributed basolaterally but by 11 hours AP are localized to small basal junctions (Fristrom et. al., 1993). At the time of the pupal molt (12 hours AP) the two wing surfaces separate (except for a narrow band along the anterior margin) and the intervening space becomes filled with extracellular matrix, at least some of which is secreted by hemocytes (e.g., Gullberg et al., 1994; Murray et al., 1995; and L. Fessler, personal communication). As the surfaces move apart, cytoplasmic connections between them are stretched and finally broken (Fig. 2B,C). The resulting cell fragments, including the first set of basal junctions, are presumably removed by hemocytes (Fig. 2C) and the epithelium reverts to a simple, mitotic epithelium (Schubiger and Palka, 1987) that shows little or no integrin staining (Fig. 3). This pupal separation is somewhat surprising since it appears to negate the developmental progress of the preceding 12hrs. However, the separation of wing surfaces is associated with an overall expansion of the tissue (also exhibited by leg discs) that results in a substantial increase in surface area of the appendages. Furthermore, the process is associated with the cycle of apolysis and cuticle deposition during metamorphosis. In contrast to larval molts, the pupal cuticle is not shed at the pupal molt. Thus, the expansion of the wing, and subsequent withdrawal from the pupal cuticle at 18 hours AP, creates an apical space for the differentiation of bristles and hairs.

The second (pupal) apposition begins around 21 hours AP and takes about 15 hours to complete. Basal processes first extend from specific rows of intervein cells to meet similar processes from the opposite cell surface (Fig. 2D and Fristrom et al., 1994). Re-apposition continues systematically until 30 hours AP when most intervein cells are connected to cells of the opposite surface (Fig. 2E). By 36 hours AP the pupal wing resembles a miniature adult wing with the intervein regions fully re-apposed and the veins remaining as narrow open channels. Integrins reappear on basolateral cell surfaces around 21 hours AP and by 36 hours AP are restricted to basal plaques in the intervein regions (Fristrom et al., 1993 and below). These integrin-based connections between the two surfaces persist through the rest of metamorphosis. Note that the 'second period of separation' mentioned in the literature (e.g., Fristrom et al., 1993) refers only to an expansion of intercellular spaces; connections at the basal plaques are maintained.

Development of mutant integrin clones

We examined wings with mutant *myospheroid* clones during prepupal apposition (4-6 hours AP) and during re-apposition (21-36 hours AP). *myospheroid* clones were identified by the absence of β_{PS} (see Methods). *mew* and *inflated* clones were examined only after hairs form at 36 hours AP and were identified by the linked hair marker *forked*. Cell structure was highlighted by labeling actin with rhodamine-conjugated phalloidin and wings were examined by confocal microscopy.

Prepupal apposition

We find no obvious morphological defects in 4 and 6 hour AP wings containing large *myospheroid* clones (Fig. 4). By this stage the basal surfaces of the dorsal and ventral epithelia have apposed, and although the surfaces are in close proximity they are not yet connected by morphologically identifiable junctions. Rather, EM observations suggest that the surfaces adhere via attachment of the convoluted basal membranes to a shared extracellular matrix (D.F., unpublished data). Fig 4 shows the structure of prepupal wings in the region of *myospheroid* clones from two different perspectives. First, a series of optical sections parallel to the basal surface (Fig. 4A-D), shows that the clone cells extend basally as far as adjacent wild-type cells and that the basal meshwork of actin filaments characteristic of this stage of development is present (cf Fig. 4F). Second, in a line series collected perpendicular to the above, a single band of actin staining (arrows in Fig. 4H) indicates that the basal surfaces are apposed in the region of the clone (Fig. 4G). We also find that the two surfaces of fixed wings containing putative clones remain closely apposed when placed in hypotonic buffer (0.5-1× PBS), suggesting that the two surfaces are functionally adherent. We conclude that PS integrins are not essential for the initial apposition of dorsal and ventral surfaces or for the maintenance of apposition at 6 hours AP. Nevertheless, integrins clearly function at this stage of development (see below) and we cannot rule out the possibility that they participate in dorsoventral adhesion.

Pupal apposition

Large *myospheroid*, *mew* or *inflated* clones are associated with pronounced matrix-filled blisters in 30 and 36 hour AP wings (Figs 5, 6). Similar blisters are never observed in wild-type wings. As expected, only dorsal *mew* (Fig. 6E, F) or ventral *inflated* clones (not shown) cause blisters but both dorsal and ventral *myospheroid* clones do so. Blister boundaries generally follow the clone boundaries (Fig. 6). To investigate whether pupal blisters result from a failure in re-apposition per se or a failure to maintain adhesion between transiently re-apposed surfaces we examined *myospheroid* clones in re-apposing wings between 21 and 25 hours AP. Large clones revealed little evidence of basal extensions either in mutant clone cells or in cells on the surface opposite the clone (Fig. 7), although occasionally we found a projection in the approximate position of the AP compartment border (e.g., Fig. 5E). We conclude that pupal blisters result

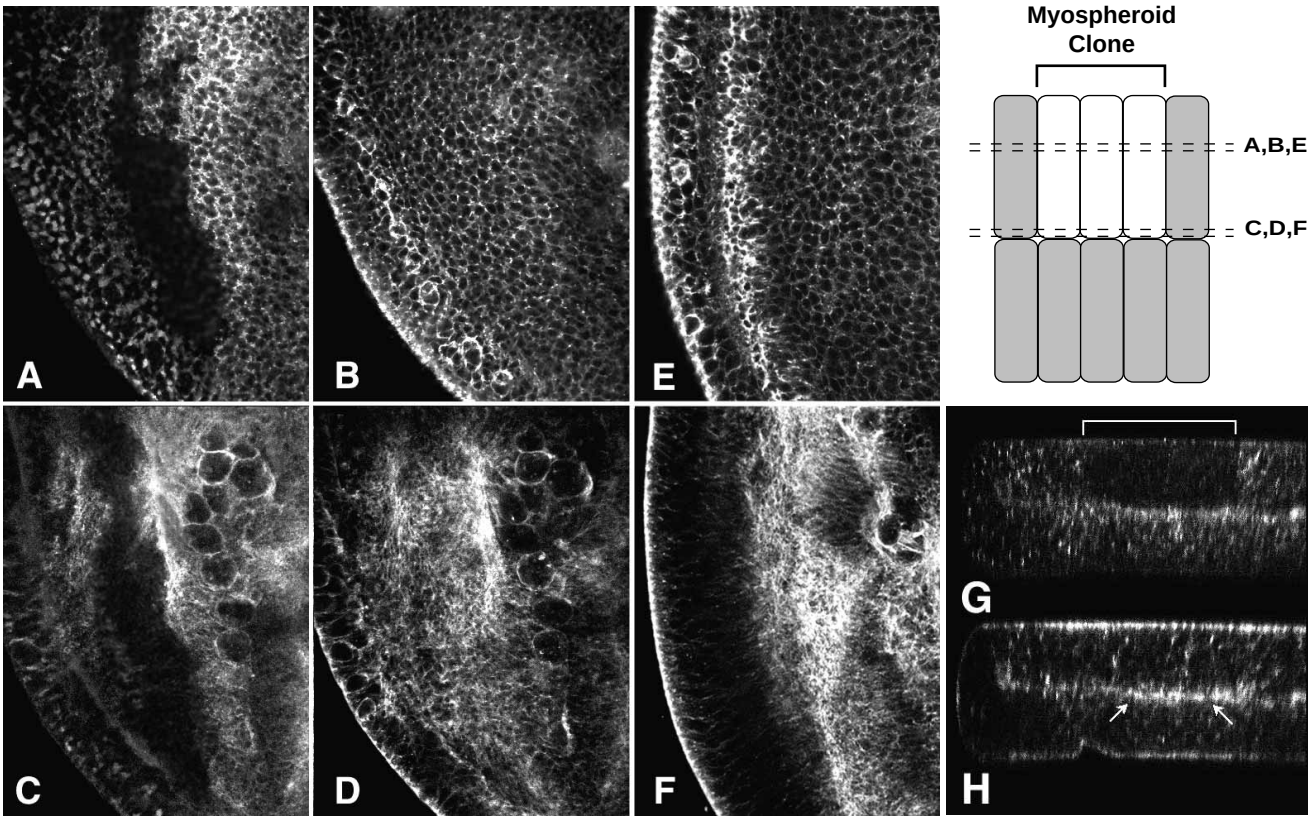

Fig. 4. Prepupal wings at 4 hours AP. Pairs of dual channel confocal images (A,B; C,D; G,H) of wings double labelled for integrin (fluorescein-anti-βPS; A,C,G) and actin (rhodamine-phalloidin; B,D,H). (A-D) A wing containing a *myospheroid* clone optically sectioned in two planes as shown in the schematic at right. The clone is identified in A and C by the absence of integrin staining. Clone cells, outlined by actin staining (B,D) are indistinguishable in shape from non-clone cells. (E,F) A phalloidin stained wild-type wing shows actin distribution at comparable levels to B and D. The basal ends of both clone and wild type cells are characterized by a mat of actin filaments (D,F). (G,H) A wing containing a *myospheroid* clone viewed in optical 'cross section' (see Methods). Note that in the region of the clone (bracket in G), the basal surfaces meet without a detectable gap (arrows in H).

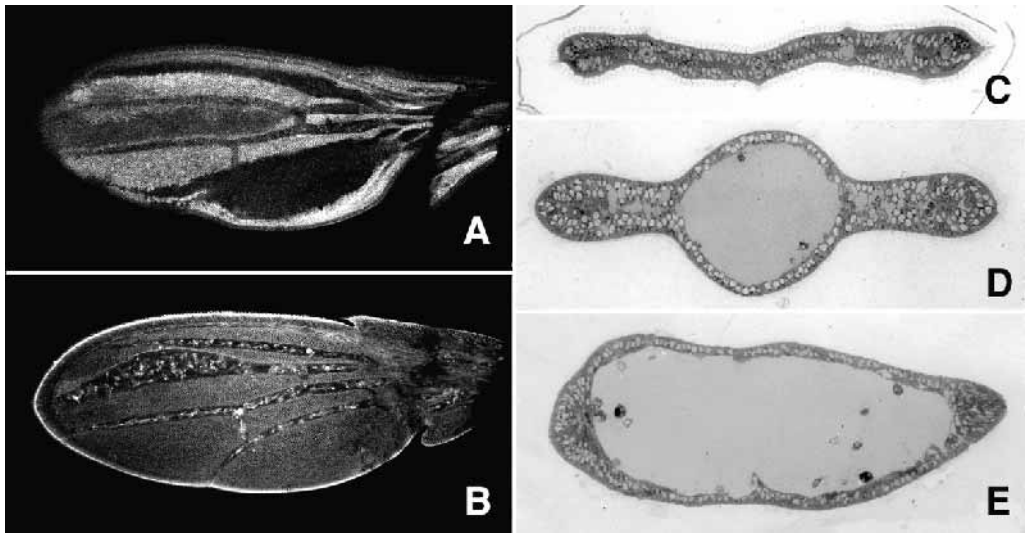

Fig. 5. Pupal wings at 30-36 hours AP showing blisters associated with *myospheroid* clones. (A,B) Low magnification confocal images. (A) A wing stained with anti-βPS alone. The dark areas are blisters associated with two clones in the region of veins L2 and L5. mys^{+}/mys^{+} twin spots, stained more intensely than the mys^{-}/mys^{+} background, are also evident. (B) A wing stained with phalloidin alone has a blister in the region of vein L3. In addition to outlining intervein cells, phalloidin stains hairs along the wing margin and hemocytes in extracellular channels (veins and blisters). (C,D,E) 1 μm sections of wings embedded in plastic. (C) A wild-type wing with dorsal and ventral surfaces re-apposed. (D, E) Wings with large blisters presumably associated with *myospheroid* clones (see text and Figs 4-6). The blister contains scattered hemocytes embedded in extracellular matrix.

from a failure of mutant clone cells to re-appose with normal cells on the opposite surface. Furthermore, both mutant and normal cells generally fail to undergo the cell shape changes required for re-apposition.

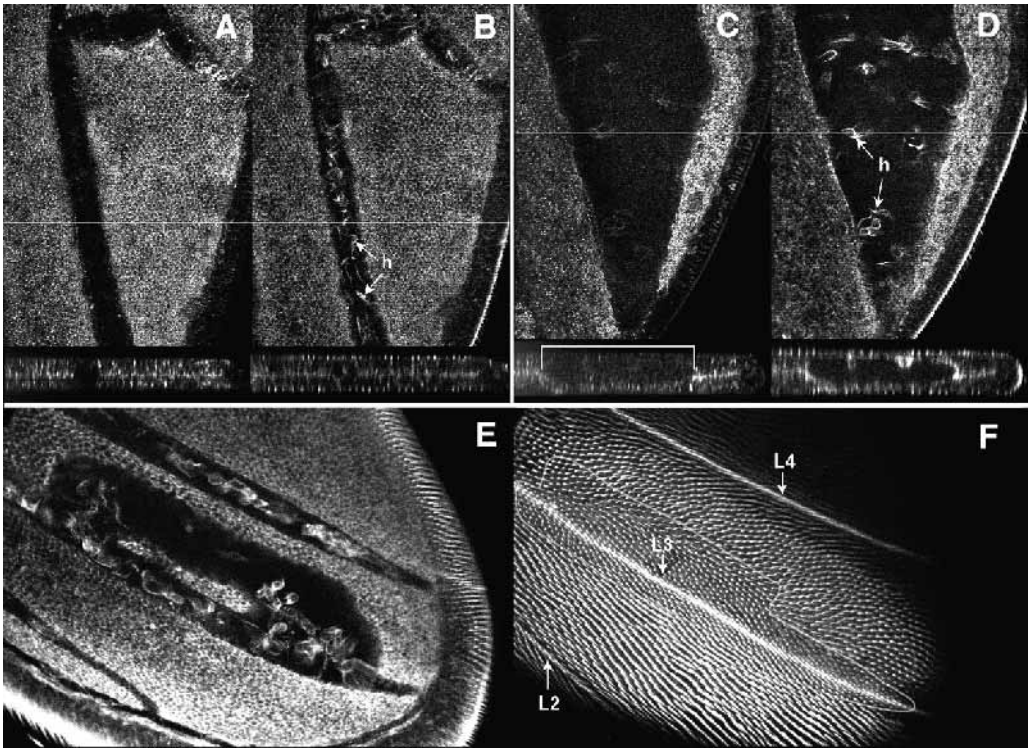

Fig. 6. Pupal wings at 30-36 hours AP. (A, B) Wild type and (C,D) a wing with a *myospheroid* clone, stained and imaged as in Fig. 4; i.e. anti-β_{PS} in A and C, phalloidin in B and D. Insets (bottom) are optical 'cross sections' collected in the regions indicated by the horizontal lines. C illustrates the correlation between the presence of a blister and the absence of integrin staining on one surface (bracketed region in inset). (E,F) A wing with a dorsal *mew* clone, stained with phalloidin. (E) An optical section of the region of apposed basal surfaces shows the extent of the blister. (F) An optical section grazing the dorsal wing surface. The approximate extent of the clone (outlined) is indicated by the distribution of the smaller *forked* hairs. Longitudinal veins 2, 3 and 4 are indicated.

Clones induced relatively late in larval development may be long but only a few cells wide. Most such clones show no evidence of blisters and actin distribution is normal in both clone cells and the cells opposite (Fig. 8). However, even in small *myospheroid* clones in the adult wing the dorsal and ventral surfaces are separated. This implies that defects in small *myospheroid* clones arise after re-apposition (see Discussion).

Finally, we note an unusual distribution of integrins in wild-type cells opposite *myospheroid* clones at 36-40 hours AP (Fig. 9). Instead of being organized into basal plaques, integrins remain concentrated at the basal ends of the lateral cell surfaces. This indicates that matrix alone is insufficient to establish or maintain the basal distribution of integrins; a connection with integrins on the opposite wing surface apparently is required for the localization of integrins into basal plaques.

PS1 and PS2 complementarity is required

While it was clear from previous work that loss of dorsal PS1 or ventral PS2 resulted in separation of the wing surfaces we did not know if complementary expression of these integrins was required for normal wing development. This question can be addressed by eliminating one PS integrin α subunit in a mutant clone and substituting the other α subunit, expressed from a transgene. We generated animals with α_{PS} transgenes, under the control of the GAL4 Upstream Activating Sequence (UAS^{GAL4}). When combined with an appropriate GAL4-expressing enhancer trap (see Brand et al., 1994), these transgenes express α_{PS} subunits in the entire wing pouch. In this background, we generated clones homozygous for strong *mew* (α_{PS1}) or *inflated* (α_{PS2}) alleles, and asked if expression of the transgenic α_{PS} protein could rescue the blister phenotype of the α_{PS}^{-} clones.

As expected, GAL4-driven expression of α_{PS1} rescues wing

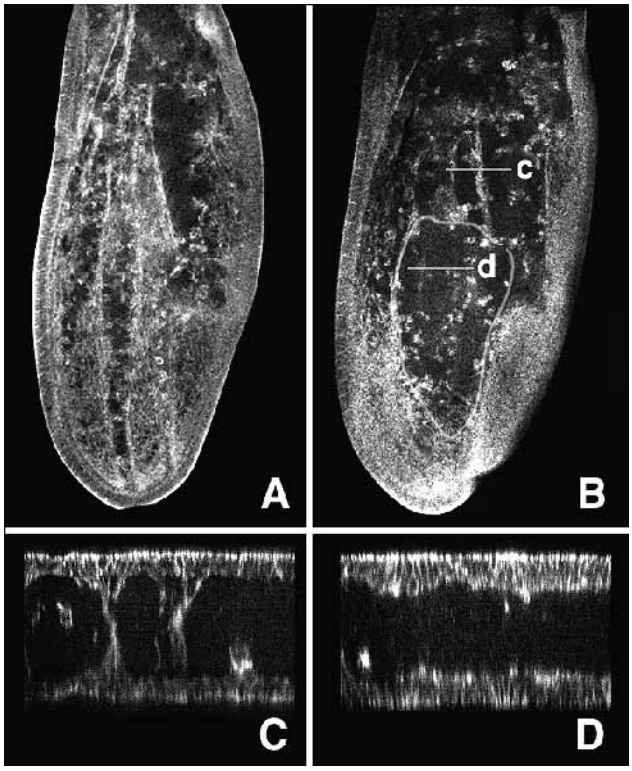

Fig. 7. Pupal wings at 22 hours AP (stained and imaged as in Fig. 4 but only the phalloidin stained panels are shown). (A) A wild-type wing. Stained areas have re-apposed. (B) A wing with a central *myospheroid* clone (outlined). (C,D) Optical cross sections collected at lines c and d in (B). Reapposition has begun in the non-clone regions (C) but not in the clone (D). Note that in D both the clone epithelium (upper) and the opposite epithelium lack basal projections.

mew clones completely, however there is no detectable rescue of *inflated* clones (Table 1). Similarly, α_{PS2c} transgenes rescue the large majority of *inflated* clones, but do not rescue *mew* clones. (α_{PS2c} is one of two splice variants of α_{PS2}, the other being α_{PS2m8}; Brown et al., 1989.) From these data, we conclude that complementary expression of PS1 and PS2 on the two apposed epithelia of the pupal wing is required for proper wing morphogenesis. At this time, we cannot say that α_{PS1} is required dorsally, and α_{PS2} ventrally, since we cannot replace one integrin subunit with the other simultaneously on both wing surfaces; we can only say that different integrins are required on opposite epithelia.

With respect to control animals, it is noteworthy that α_{PS2c} can support wing morphogenesis in the absence of α_{PS2m8}. This has also been observed recently by Roote and Zusman (1996), and we both find that α_{PS2c} can support development from embryogenesis to adult fly. This is consistent with observations that, to date, all PS2 ligands (including tiggrin and heterologous ligands such as vertebrate fibronectin and vitronectin) have been found to bind both splice forms of the α_{PS2} subunit, although there are differences in apparent ligand affinity and cation requirements (Bunch and Brower, 1992; Zavortink et al., 1993; Fogerty et al., 1994).

PS1 and PS2 may substitute for each other in small clones

In our initial clonal analysis study of *mew* mutations, we noticed that small clones (fewer than 150 cells) in the wing were often wild type or had weak phenotypes, even if on the dorsal surface, where *mew* function is presumably required (Brower et al., 1995). The initial analysis of *inflated* wing clones was conducted before the advent of the FRT/FLP system for generating recombination, and focused on large clones made early in development (Brabant and Brower 1993). We generated and analyzed numerous small ventral *inflated* clones and found that, like small *mew* clones, these usually display weak or undetectable phenotypes in the wing blade (Fig. 10). Both of these results are in contrast to similarly sized *myospheroid* (β_{PS}) wing clones, which virtually always show some abnormality in the wing blade.

The fact that clones lacking β_{PS} display a stronger phenotype than similarly sized clones missing α_{PS1} dorsally or α_{PS2} ventrally suggests that more than one alpha subunit is able to function on each surface. Either a third α_{PS} subunit is rescuing the small clones, or some α_{PS1} is acting ventrally and α_{PS2} dorsally. (Formally, β_{PS} alone could function, but this is inconsistent with a large volume of integrin work, and specifically with cell culture experiments which indicate isolated β_{PS} will not mediate cell spreading; T. B. unpublished data.) To test this, we made small clones mutant for both *mew* and *inflated*. As shown in Fig. 10, the double mutant clones are much more likely to display a morphological phenotype than either of the single α_{PS}^{-} clones, indicating that both α_{PS1} and α_{PS2} can function to maintain adhesion on either wing surface.

Table 1. Rescue of mutant clones by expression of α_{PS} subunits

Genotype	Blisters	%
A. α_{PS1} rescues mew but not inflated blisters		
mew / +; α_{PS1} / +	40/230	17
mew / +; α_{PS1} / GAL4^{684}	0/536	0
if / +; α_{PS1} / +	21/54	39
if / +; α_{PS1} / GAL4^{684}	32/72	44
B. α_{PS2c} rescues inflated but not mew blisters		
mew / +; α_{PS2c} / +	20/132	15
mew / +; α_{PS2c} / GAL4^{684}	38/348	11
if / +; α_{PS2c} / +	13/50	26
if / +; α_{PS2c} / GAL4^{684}	3/108	3

One cross was performed to generate each pair of genotypes, so that experimental and control animals (with and without the *GAL4^{684}* enhancer trap, respectively) were reared in the same vials. For clarity, marker mutations, allele names and inserts necessary for clone generation are not included; see Methods for details. Homozygous clones of integrin mutant tissue were induced during larval development, and the resulting adult wing blisters scored. *mew* = gene encoding α_{PS1}; *if* = gene encoding α_{PS2}; *α_{PS1}* and *α_{PS2c}* are GAL4-regulated transgenes expressing the indicated integrin subunit; *GAL4^{684}* = an enhancer trap expressing GAL4 in the wing pouch.

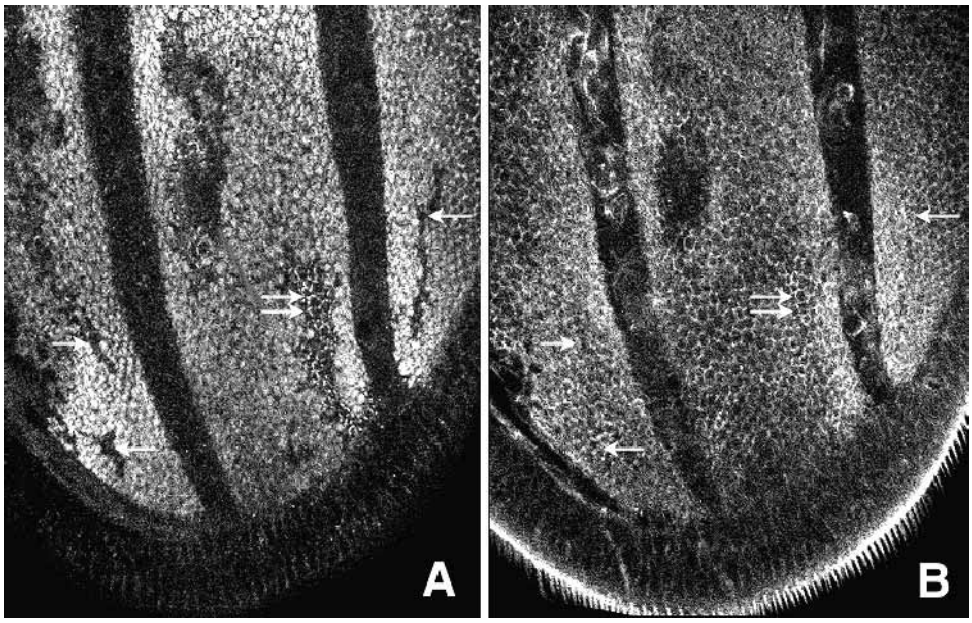

Fig. 8. A pupal wing at 36 hours AP (stained and imaged as in Fig. 4). An optical section through the apposed basal surfaces shows many small (late induced) *myospheroid* clones indicated by the absence of integrin staining (A). Several of these clones are not associated with blisters (arrows), as indicated by the normal distribution of basal actin in B. The absence of separation was confirmed by 'optical cross sections' through the clones (not shown). The integrin staining indicated by the double arrows probably represents cells opposite a clone (see Fig. 9).

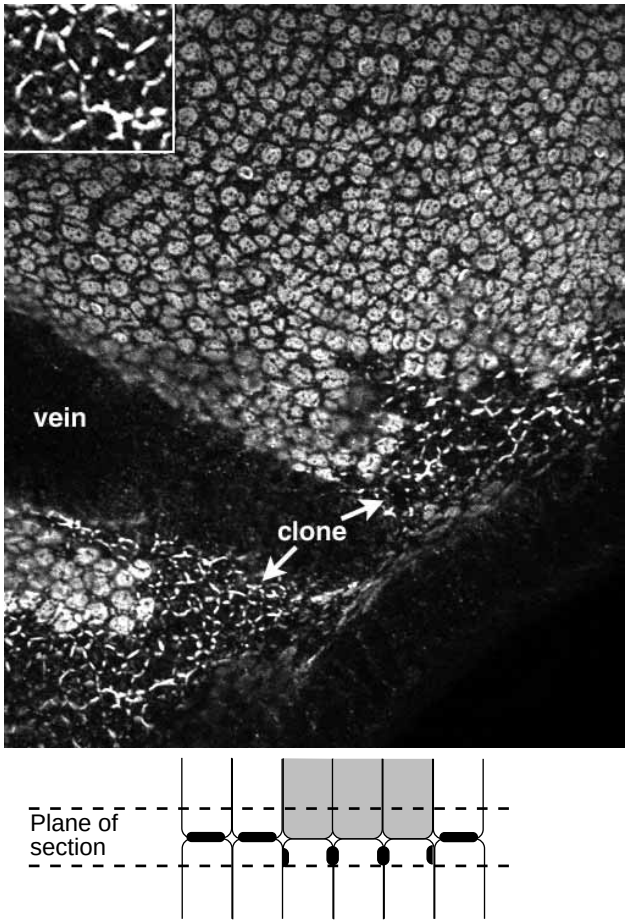

Fig. 9. A pupal wing at 36 hours AP stained with anti-β_{PS}. At this stage integrin is normally concentrated in basal plaques. In a small *myospheroid* clone near the posterior margin the two surfaces are apposed making it possible to view the basal ends of both epithelia in the same optical section as shown schematically below. (The unshaded cells represent the clone and the black areas foci of integrin staining.) The cells on the surface opposite the clone show staining on the basal ends of the lateral cell surfaces (inset) rather than in basal plaques.

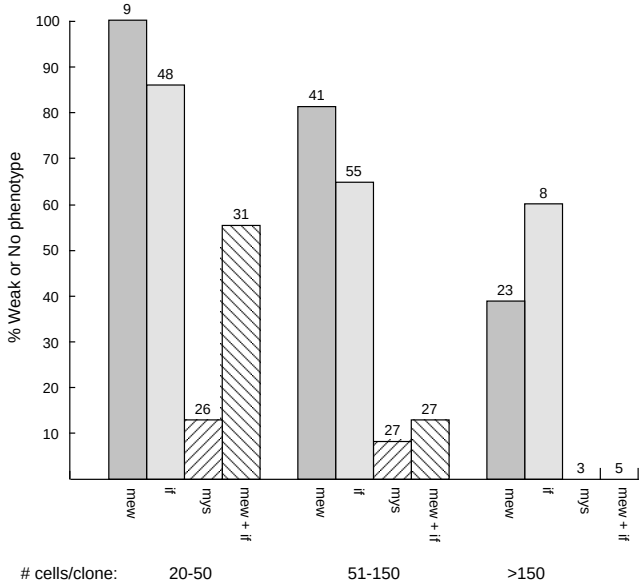

Fig. 10. Phenotypes of small mutant integrin clones in the wing. FRT/FLP clones were generated under similar conditions for each mutant, and wings were examined for clones under high power. Only dorsal clones are included for *mew*, ventral clones for *inflated*. Wings with large blisters, defined as a separation that extended well beyond the clone boundaries, or other major disruptions in dorsoventral adhesion were not scored. This partially accounts for the low number of *myospheroid* and *mew* plus *inflated* clones of greater than 150 cells, as these usually cause blisters. 'Weak phenotype' is defined as a very minor abnormality for smaller clones (<50 cells); for larger clones, much of the clone must be wild type. Data from multiple alleles, including at least one allele known to be a protein null at the molecular level, were pooled for *mew* and *inflated*, as there was no discernable difference for the various alleles. Mutations used were: mew^{M6}, $mew^{H7/H10}$, if^{B2}, if^{B4}, if^{H18}, if^{K13}, mys^{XB87}, and a mew^{H7} if^{B2} double mutant chromosome.

Early α_{PS} overexpression causes blisters

In the course of testing various α_{PS} transgenes, we noticed that overexpression of α_{PS} in the wing can lead to a dominant wing blister phenotype. We have seen this phenotype with α_{PS1}, α_{PS2c} and α_{PS2m8} expressed under the control of UAS^{GAL4}, although α_{PS2m8} inserts generally appear to have the strongest effect. While we have focused primarily on expression using the GAL4 system, we have also generated blisters by expressing α_{PS2m8} using a heat shock promoter (Brabant, 1995) and using a tubulin promoter activated via the 'FLP-out' method of Struhl and Basler (1993).

We do not know why overexpression of integrin subunits causes wing blisters. It does not appear to be due to competition between α_{PS1} and α_{PS2} for β_{PS}, as heterozygosity for *myospheroid* or simultaneous expression of transgenic β_{PS} in the heat shock induction paradigm do not significantly alter the penetrance of blistering. These experiments also argue against the idea that excess cytoplasmic α_{PS} is deleterious. In general, altering the dosage of combinations of transgenes and endogenous genes suggests that it is the total amount of α_{PS} that is most critical.

Whatever the mechanism, this dominant phenotype can be used to demonstrate a temporal difference in sensitivity to integrin function. Expression of transgenes using GAL4 enhancer traps and UAS^{GAL4} typically is sensitive to temperature (Brand et al., 1994) and our genes show this effect. The penetrance and expressivity of the blister phenotype increase with temperature, and this can be used to assess the critical period during development for the generation of blisters. In a number of preliminary experiments, we shifted animals from 22°C to 28°C during larval/pupal development, and assayed for wing morphology when the flies eclosed. In all cases, a large increase in blistering was seen in flies that eclosed 4 days after the temperature shift, and reached a maximum at 5 days. In order to better define the critical period, cultures were grown at 22°C and given a 1 day pulse at 28°C. As shown in Fig. 11, the first sign of increased blistering is seen in flies that emerge 5 days after the beginning of the pulse, and the number of blistered wings reaches a maximum at 6 days. By day 7, the percentage of blistered wings returns to low levels.

If one adjusts for the effects of temperature on the length of pupal development (for wild type, approximately 5.4 days at 22°C, 3.3 days at 28°C; see Ashburner, 1989) these experi-

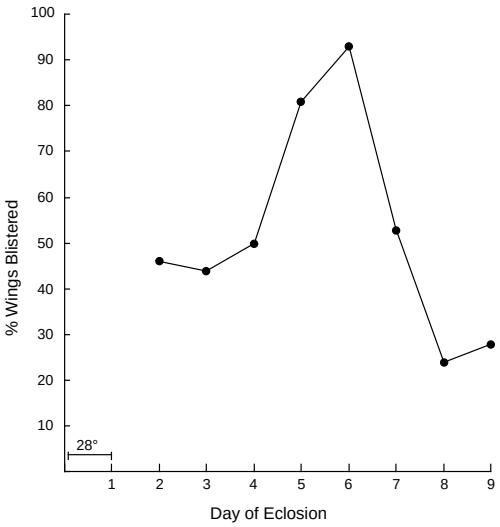

Fig. 11. Critical period for blisters caused by overexpression of α_{PS2m8}. Animals expressing an α_{PS2m8} transgene under the control of the *GAL4*^{684} enhancer trap were grown at 22°C, and the cultures were shifted to 28°C for 24 hours at the time indicated. Flies that eclosed during the previous day were scored for wing blisters. The increase in frequency of blisters due to the higher temperature is first evident at 5 days from the beginning of the 28°C pulse, reaches a maximum at 6 days, and returns to low levels by day 7. Continuous culture at 22°C yields frequencies of 25-50% in this genetic background.

ments indicate that the critical period for the generation of wing blisters by overexpression of α_{PS} subunits is at about the time the dorsal and ventral wing epithelia first become apposed. Significantly, by the time re-apposition is occurring in the pupal period, the wing is relatively insensitive to overexpression. Direct examination of pupal wings expressing transgenic α_{PS2m8} indicates that the dorsal and ventral epithelia do not become re-apposed at 30 hours postpupariation, just as for wings with large integrin mutant clones.

DISCUSSION

We set out to determine if the complementary expression of PS1 and PS2 found at the end of larval development is required for wing morphogenesis, and to further define the roles of PS integrins during the two distinct phases of adhesion between wing epithelia. Our results demonstrate specific requirements for PS1 and PS2, and indicate that an early integrin-dependent process, not obviously required for prepupal adhesion, is essential to permit subsequent wing morphogenesis. These findings lead to a model in which initially, in the prepupal wing, PS integrins function primarily in a regulatory role, becoming critical for strong adhesion only later, in the pupa.

Integrins mediate adhesion in pupal wings

Following re-apposition in pupal wings, the PS integrins appear to function primarily as simple adhesion receptors. In the differentiated pupal wing integrins are localized to junctions between the basal surfaces of apposed epithelia. These junctions have structural similarities to the myotendinous junctions of *Drosophila* embryos and to focal adhesions of vertebrates, both of which are clearly involved in adhesion (e.g. see Brown, 1993).

In adult wings, blisters derived from *myospheroid* clones are typically circular or ovoid, with the long dimension of the clone corresponding to the long dimension of the blister (Brower and Jaffe, 1989; Zusman et al., 1990). This has led to the idea that the epithelia near a clone rip apart, smoothing the contours of the blister boundary. This does not seem to be the case in pupal wings, where the clone and blister outlines correspond closely, and blister edges may be very irregular. Thus, the failure of 'wild-type' connections near mutant clones appears to occur when the wing is expanded at eclosion, prior to drying.

Integrins are not necessary for adhesion in prepupal wings

Large integrin mutant clones do not prevent the initial joining of dorsal and ventral wing epithelia in the prepupa. The blisters associated with clones in pupal wings are not seen during the first apposition, and the basal actin networks typical of this stage are present. In addition, treatment of wings with hypotonic solutions indicates that the epithelia do indeed adhere to one another in the absence of PS integrins.

Although we can see no requirement for PS integrins in maintaining dorsoventral apposition of prepupal wings, there is a requirement for integrins during the prepupal period. Earlier work, in which integrin mutants were rescued by heat shock-induced *myospheroid* transgenes, indicated that integrins expressed after prepupal-pupal separation could not maintain adhesion of wing surfaces that lacked prepupal integrins (Brabant and Brower, 1993; Zusman et al., 1993). This observation is especially intriguing since no connections, via integrins or any other adhesion proteins, are observed in the middle of the 'separated' wing in thick or thin sections, and the morphology of cells during re-apposition does not suggest that they are pulling on pre-existing connections. These early studies scored adult wing blisters to assay integrin function; here we show that large integrin mutant clones do not re-appose in the pupa, and that wild-type cells opposite a clone generally do not attempt to cross the matrix-filled space to join with the corresponding mutant epithelium. Taken together, these findings suggest that integrins must function during the prepupal apposition in order to make cells competent for re-apposition. That is, it would appear that during the prepupal apposition, the primary function of PS integrins is not adhesion per se, but rather the integrins are involved in a signaling event required for subsequent morphogenetic processes.

Two discrete phases of integrin function

Our data suggest a model in which integrin function in the wing is divided into distinct prepupal and pupal phases. The early expression serves primarily a signaling function, triggering or directing subsequent morphogenesis. Later, PS integrins provide a strong link between the epithelia to resist hydrostatic pressure, especially during wing expansion. This model may help to account for the seemingly paradoxical observation that overexpression of an 'adhesion protein' leads to a loss of adhesion; the critical function of PS integrins during the early period, which is most sensitive to overexpression is now postulated to be regulatory, rather than adhesive.

The model also helps to explain the apparent contradictory findings that complementary patterns of integrin expression are required (from the clone rescue experiments) and that α_{PS1} and α_{PS2} function on both dorsal and ventral surfaces (from the analysis of small wing clones). We propose that complementary integrin expression is required only for the prepupal signal, and that the differences in phenotypes in small clones of α_{PS} versus β_{PS} mutants reflect the fact that the α_{PS} subunits can substitute for one another in adhesion. Small clones lacking PS integrins can be brought into close association with opposite cells during re-apposition, probably as a result of mechanical forces provided by the surrounding wild type tissue. (Alternatively, short range interactions between cells at the time of the proposed signaling event could lead to a non-autonomous spread of the ability to re-appose from wild type to neighboring mutant cells.) Whatever the mechanism is that brings small mutant patches into apposition with wild-type cells in the pupa, clones lacking β_{PS}, and therefore all PS integrins, subsequently fail to adhere, while small clones mutant for either α_{PS} subunit often remain tightly associated. Our data indicate that during pupal stages both α_{PS1} and α_{PS2} are likely to be expressed on both surfaces. This late ubiquitous expression allows PS1 and PS2 integrin to substitute for one another to maintain adhesion in *mew* or *inflated* clones. Of course, we cannot rule out a role for α_{PS3} at this time, especially as the *mew inflated* double mutant may be slightly less strong than *myospheroid*, but the comparison of single and double α_{PS}^- clones indicates that α_{PS1} and α_{PS2} each function on both dorsal and ventral epithelia.

Why are two integrins required in the prepupa?

Formally, the simplest hypothesis to explain the requirement for complementary expression of PS1 and PS2 in the wing is that they interact directly with one another during the prepupal apposition. However, direct integrin-integrin binding would be quite unusual. Specifically, there is no evidence from studies in situ that a direct binding of PS1 to PS2 mediates adhesion (see, for example, Brown, 1994, and Brower et al., 1995), and we have seen no indication that they associate with one another in cell culture (T. B., unpublished data). In cell culture experiments, the PS1 and PS2 integrins mediate cell spreading on the extracellular matrix proteins laminin and tiggrin, respectively (Gotwals et al., 1994b; Fogerty et al., 1994), and the available evidence indicates that PS1 and PS2 recognize very different structures. For example, PS2 integrin binds to an RGD-containing site, and only PS2-mediated cell spreading is inhibited by RGD peptides (Bunch and Brower, 1992; Fogerty et al., 1994; T. B., unpublished). Importantly for the studies described here, no ligand has been found that interacts with both PS1 and PS2 integrins. However, the requirement for different integrins does imply that a specific connection is made between the basal surfaces. Such a connection could be via a yet unknown matrix protein that binds both PS1 and PS2, or PS1 and PS2 matrix ligands may form a specific link.

It is possible that PS1 and PS2 transmit information to help direct specific dorsal and ventral fates, but this seems unlikely. Even in integrin mutant clones, there is no consistent inability of cells to differentiate structures specific to one layer, such as sensilla.

A more likely possibility is that integrins are required for a temporally important event. For example, formation of adhesion sites may be involved in telling cells that the two epithelia have made contact, triggering or permitting subsequent differentiation processes. In this scenario, PS1 and PS2 (and perhaps their respective ligands) must be segregated into opposite epithelia in order to prevent premature cross-linking of integrins and signal induction.

Integrins have been associated with a variety of intracellular signaling pathways (reviewed by Clark and Brugge, 1995), and it is possible that PS integrin-ligand associations directly lead to changes in gene expression. However, it is equally likely that the integrin signal functions primarily to modulate signaling from other cell surface receptors. For example, while the approximate locations of wing veins are specified in the late larval disc, signaling between apposed dorsal and ventral layers is required for their final development (reviewed by Garcia-Bellido and de Celis, 1992). Recently, it has become clear that this signal is mediated in part by the EGF receptor, and is modulated by other proteins, including the rhomboid gene product (Sturtevant et al., 1993; Sturtevant and Bier, 1995). The EGF receptor activates the ras-MAP kinase cascade to alter transcription, and our preliminary experiments indicate that the dominant blister phenotype caused by prepupal integrin overexpression can be suppressed by strong loss-of-function mutations in this pathway. Integrin activation of components of the ras-MAP kinase pathway has been reported in other systems (Chen et al., 1994; Morino et al., 1994; Schlaepfer et al., 1994; Zhu and Assoian, 1995; Miyamoto et al., 1995), and an attractive hypothesis is that integrins modulate the EGF-MAP kinase signal. It must be noted, however, that we cannot presently rule out the possibility that the signal pathway is regulating PS integrin function.

It has become clear that integrins generally function as much more than simple adhesion proteins, however separating adhesion and regulation of cellular responses can be difficult in practice, especially in an intact developing system. The *Drosophila* wing appears to provide an excellent system in which these functions can be addressed experimentally, and the challenge now is to elucidate the molecular pathway whereby prepupal integrins regulate subsequent wing morphogenesis.

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