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Requirement for integrins during *Drosophila* wing development

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THE position-specific (PS) integrins of *Drosophila*[1,2] are highly homologous to vertebrate integrins[3–5], most of which are cell-surface receptors for extracellular matrix components[6,7]. Integrins are heterodimers, each consisting of noncovalently associated α- and β-subunits. As for the subfamilies of vertebrate integrins, the same β-subunit is found in both *Drosophila* PS integrins, combined with a specific α-subunit to generate either a complete functional PS1 or PS2 integrin[1,5,8]. Both α- and β-subunits are large transmembrane proteins (relative molecular masses >100,000). Either one or both of these two PS integrins are expressed in most fly tissues during development. A particularly intriguing pattern of expression is found in the mature wing imaginal disc, where the PS1 integrin is expressed primarily on the presumptive dorsal wing epithelium, and the PS2 integrin is found almost exclusively on the ventral epithelium[1]. Immediately after pupariation, the central wing pouch evaginates, folding along its centre to appose the epithelia that will secrete the dorsal and ventral surfaces of the adult wing blade[9]. Here we report the results of a genetic analysis indicating that both of the PS integrins are required to maintain the close apposition of the dorsal and ventral wing epithelia during morphogenesis. Also, we conclude that the integrins are not necessary for the maintenance of the cell lineage restriction between the two presumptive wing surfaces in the developing imaginal disc[10–12].

The α-subunit of the PS2 integrin seems to be encoded by the *inflated* (*if*) locus on the X chromosome[13], and null mutations at *if* cause embryonic lethality (M. Wilcox, A. DiAntonio and M. Leptin, manuscript submitted; see also Fig. 2 legend). We examined if^3 mutant larval tissues by using monoclonal antibodies directed against the PS integrins (anti-PS antibodies), and our results suggest that the if^3 allele is a regulatory mutation at the locus. Homozygous if^3 late third-instar larvae (just before pupariation and disc evagination) had apparently normal levels of PS2 integrin on most tissues (for example, muscles, salivary glands), but displayed greatly reduced levels of integrin on the surfaces of some imaginal disc cells (Fig. 1*b*). Specifically, there was relatively little PS2 integrin α-subunit in the ventral region of the wing pouch; these cells normally express the protein at very high levels. PS2 integrin expression was not so severely reduced in other regions of the disc, such as the peripodial membrane. The expression of the PS1 integrin in the wing disc seemed to be unaffected by if^3 (not shown).

Although all of the mutant wing discs examined were clearly affected, there was variability in the extent of PS2 integrin reduction (for example, Fig. 1*b*, *c*). In the wing pouch, mutant discs typically stained at higher levels anteriorly, and often displayed significant levels of staining with anti-PS2 antibody dorsally (Fig. 1*c*). Similar antero-posterior asymmetry and dorsal staining are characteristic of wild-type PS2 integrin expression in mid-third instar discs[14], about 24 h before pupariation. It is interesting that the pattern of PS2 integrin expression in if^3 discs seemed relatively normal at the mid-third larval instar (not shown), and it is possible that this allele simply does not allow the cells of the pouch to progress to the more mature pattern of expression.

Adult flies bearing the if^3 allele display wing blisters, in which the dorsal and ventral surfaces of the wing blade are separated[13]. We found that the penetrance and expressivity of the if^3 blisters were variable (Fig. 2); this is not surprising in light of the variability of the immunofluorescence results. Nonetheless, these data indicate that the PS2 integrin is important for maintaining the close apposition of the dorsal and ventral epithelia during morphogenesis of the adult wing.

That this is the case was confirmed by clonal analysis studies[15] of flies with mutations in the *myospheroid* gene (*mys*). The *mys* locus encodes the common PS integrin β-subunit[4,5], so null mutations at this locus would be expected to eliminate both PS1 and PS2 integrins[5]. Flies homozygous for null mutations of *mys* die as embryos (see refs 16 and 17 for descriptions of the *mys* lethal phenotype), so to examine the role of integrins in wing morphogenesis, we made clones of cells homozygous for a null mutation of *mys*, along with the flanking markers *yellow* (*y*) and

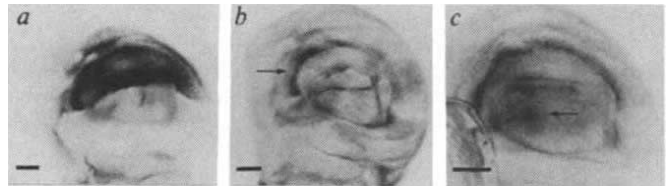

FIG. 1 Anti-PS2-antibody immunofluorescence of the basal surfaces of late third-instar wing imaginal discs. At this stage, the disc consists primarily of a single layered but highly folded columnar epithelium of ~50,000 cells[22]. All micrographs are centred on the 'pouch' region, which evaginates into a flat sac before secreting the cuticle of the adult wing blade[9]. Images are reverse-contrast, so staining is dark. *a*, Wild type. PS2 integrin is found at high concentration throughout the ventral (upper) region of the wing pouch. The sharp horizontal boundary of staining across the pouch marks the line along which the epithelium will fold during evagination (that is, the presumptive wing margin). *b*, Mutant for if^3. PS2 expression is greatly reduced in the pouch. Relatively high levels of staining remain in more peripheral areas, especially along the anterior edge of the pouch (arrow). *c*, Mutant for if^3. Higher magnification of another disc with a less extreme phenotype, showing residual PS2 in the pouch. Typically, expression is greater in the anterior (left) half of the pouch, and is often seen dorsally (arrow) as well as ventrally. Scale bar, 50 μm.
METHODS. Mutants for if^3 were grown as a homozygous stock at 25 °C. Immunofluorescence of wing discs was performed with an anti-PS2 (CF.2C7) monoclonal antibody and fluorescein-conjugated goat anti-mouse antibody (Antibodies Inc.) as previously described[1]. Images were detected with an ISIT video camera, and stored on video tape.

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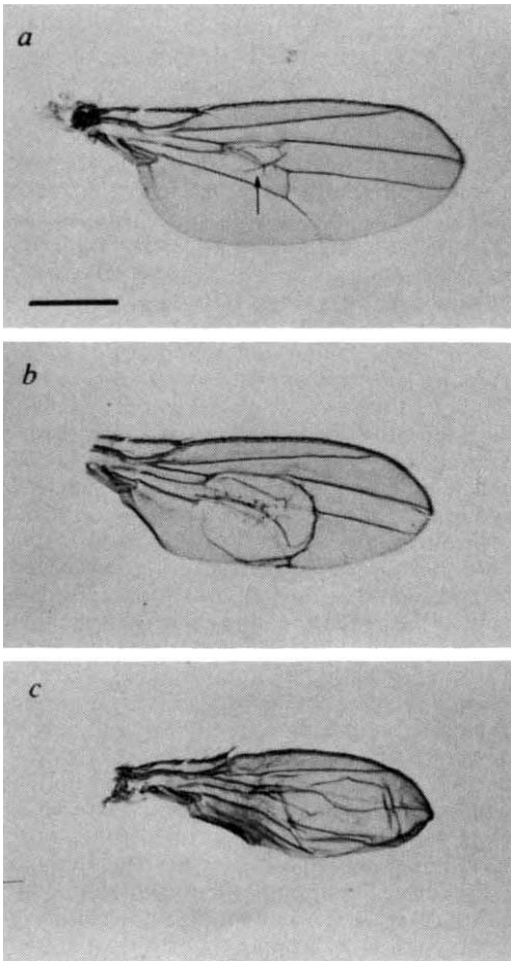

FIG. 2 Adult wings from if^3/if^{k27e} flies. *a*, A small blister. This phenotype is relatively rare, but when seen, such minor defects are generally found distal to the anterior crossvein. *b*, A large round blister. This is the most common phenotype seen in if^3 homozygotes. *c*, The most extreme phenotype, in which there is a general defect in wing flattening. This phenotype is especially common in if^3/if^{k27e} flies. Overall, the penetrance of the blister phenotype was variable, but typically at least one wing blister was observed in 15–20% of if^3 homozygotes or hemizygotes (males). The penetrance increased to 60–70% for if^3/if^{k27e} flies. The penetrance was decreased by low temperature and crowding, and we have noted in most experiments that the first progeny from a cross were much more likely to have blisters than the last flies to eclose. Scale bar, 500 μm.

METHODS. Males ($y\,w\,if^3$) were crossed to females ($r^{54c}if^{k27e}f/FM7$) to generate if^3/if^{k27e} flies (identifiable as adults or larvae by their y^+ phenotype). Adult wings were stored in ethanol–glycerol (7:3), dehydrated in ethanol, and mounted in Euparal (ASCO Labs) for microscopy. For descriptions of mutations see refs 13, 23 and 24. The alleles if^3 and if^{k27e} are inferred to be alleles of the locus encoding the PS2 integrin α-subunit on the basis of the following observations: within the limits of resolution, the genetic map positions of if^3 and if^{k27e} correspond to the chromosomal location of the gene for the α-subunit of PS2, the latter based on *in situ* hybridization of molecular probes[3,13,24]. Flies bearing the if^3 allele express reduced amounts of PS2 integrin in some disc locations (as reported here), and no PS2 integrin is detectable on immunoblots from if^{k27e} embryos (M. Wilcox, A. DiAntonio and M. Leptin, manuscript submitted). The if^3 blister phenotype, and the reduction in PS2 expression, are enhanced when if^3 is in *trans* with if^{k27e}. Although improbable, it remains a formal possibility that the *if* locus defined by one or both of these alleles encodes a factor that regulates a closely linked gene for the α-subunit of PS2; in any case, this distinction is probably unimportant for the purposes of this study.

forked (*f*), in a phenotypically wild-type background. (see Fig. 3 legend) Because essentially every cell of the wing blade epithelium gives rise to a trichome or bristle which is affected by the marker mutations, the entire structure can be scored for *y mys f* mutant clones with high spatial resolution.

Blisters were the most common phenotype associated with mutant wing clones (Fig. 3*a–c*), and were almost always found wherever there was a large clone (>100 cells). Other phenotypes included folds in one or both surfaces of the wing (more common for long thin clones; Fig. 3*d*), or minor disruptions in the planarity of the wing. The latter were mostly associated with small clones of fewer than 50 cells, and probably represented blisters that simply were too small to allow for a large separation of the epithelia. Blisters were usually much larger than the associated clones, and although the clones generally were elongated in the proximo-distal axis, the blisters tended to be grossly circular in form, like the moderately sized *if* blisters. Ventral and dorsal clones had similar phenotypes (for example, see Fig. 3*a*, *b*). In general, there was no indication that the *mys* mutation affected the ability of cells to differentiate cuticular structures such as bristles, sensillae, or the thickened cuticle characteristic of veins.

The wing margin was generally unaffected by the absence of PS integrins. Normal morphology was seen around *mys* clones that included many (20 or more) bristles of the anterior triple row (Fig. 3*e*), or hairs of the posterior row (Fig. 3*f*), and the only large wing clones that failed to produce blisters were those confined to the margin region.

The above data demonstrate that the PS integrins are required on both dorsal and ventral epithelia to maintain the close apposition of the wing surfaces. These surfaces first come into contact as a result of the evagination of the wing pouch in the puparium[9]. During the subsequent morphogenesis of the wing, the dorsal and ventral surfaces separate and rejoin at least twice[9,18]. The first separation is so great that the wing becomes almost circular in cross section; but the two surfaces apparently remain connected by thin basal processes[9,18]. Considering the role of integrins in focal adhesion sites in vertebrate cells[19], as well as in muscle attachment sites in *Drosophila*[3,5], it seems reasonable to hypothesize that the PS integrins are components of the basal attachment sites that connect the pupal wing surfaces[18]. The expression of different PS integrins on the two sides of the attachment is reminiscent of the molecular polarity of the muscle attachment sites in the embryo[5].

It is interesting that the blisters generally were much larger than the associated *mys* clones, and in contrast to the clones, the blisters were roughly circular in shape. This indicates that the physical forces pushing the epithelia apart (probably hydrostatic pressure of the haemolymph) are substantial, and an individual wild-type cell is likely to be torn from its counterpart on the opposite surface if its neighbours are not also joined. We did not detect any consistent spatial relationship between the clones and blisters. That is, clones were found on the inside and outside edges or in the middle of blisters. (The potential interactions of specific mutant effects and the physical constraints inherent in the developing wing are discussed in detail by Waddington[9].)

The dorso-ventral specificity of PS1 and PS2 integrin expression in the wing disc arises at about the time that a cell lineage restriction is established between these two domains[14,20]. This correlation indicated that the integrins might be causally related to the lineage restriction. Our data do not support such a relationship. Some *mys* clones did cross the wing margin, although this is to be expected of any clone generated as early as 48 h of development[20]. More informative are the clones that defined the wing margin for large distances, without crossing to the other surface. For example, the large clone partially illustrated in Fig. 3*f* included 69 dorsal hairs of the marginal posterior row, but no ventral hairs. Similarly, we found clones that included 20 or more bristles of the ventral row or the dorsally

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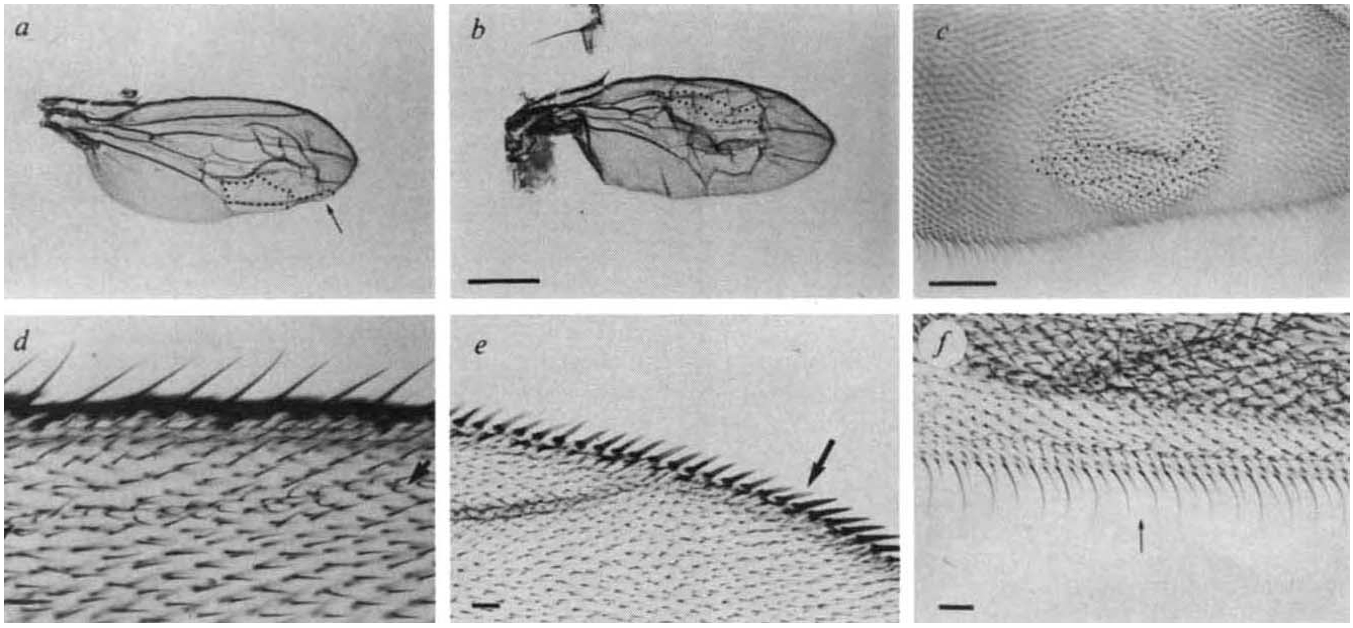

FIG. 3 Homozygous *mys*^{XG43} clones in adult wings. All clones illustrated were generated by irradiation at 48 ± 4 h after egg laying, except for that shown in *c*, which was generated at 72 ± 4 h. *a*, *b*, Blisters caused by large dorsal (*a*) or ventral (*b*) clones. The approximate extent of the clones is indicated by the dotted outlines; the clone in (*a*) abuts the wing margin distally (arrow). Note that the blisters are much larger than the clones, and are roughly circular. *c*, Blister resulting from small clone. The smooth oval shape of the blister contrasts with the irregular shape of the clone (dotted line). *d*, Long thin clone, which results in a discrete fold in the wing (between arrows), in contrast to the blisters. *e*, Clone along the anterior margin. The arrow indicates the last *y f* marginal bristle of the clone; the darker bristles to the right are wild type. This clone includes 35 bristles of the central row, yet has no obvious effect on the morphology of the margin. Overall, the only margin phenotype that was sometimes observed was that of folding near the wing edge, but this seemed to correlate with the presence of nearby blisters, and was probably an artefact of the more general disruption. *f*, Part of a large dorsal clone that abuts the posterior margin. Long ventrally derived wild-type marginal hairs alternate with shorter dorsally derived *y f* hairs (arrow). This clone includes 69 marginal hairs, all dorsal, in addition to its causing a large wing blister. Scale bars: *a* and *b*, 500 μm; *c*, 100 μm; *d*–*f*, 10 μm.

METHODS. For clonal analysis, *y mys*^{XG43} *f*^{36a}/*FM7* females were crossed to wild-type males. The progeny were grown in uncrowded, well-yeasted vials, and irradiated with X-rays (1,500 rads) at 48 or 72 ± 4 h after egg laying, all at 25 °C. As a control, the same cross was performed with the markers but without the *mys*^{XG43} allele. Embryos homozygous for the *mys*^{XG43} allele[25] make no immunologically detectable β-subunit (ref. 5, and R. Salatino, unpublished results), and *mys*^{XG43} behaves as a typical strong allele in various complementation tests (R. Salatino and D.B., unpublished results); we therefore infer that it is a null allele of *mys*. On the basis of sizes and frequencies of *mys*^{XG43} and control clones, there was no indication that *mys*^{XG43} reduced cell proliferation. Initially, 133 wings were scored for marked clones without regard to overall wing morphology. These wings yielded 44 *mys*^{XG43} clones of greater than 50 cells, and virtually all of these clones were associated with clear morphological defects. Subsequently, we examined additional wings selected on the basis of abnormal morphology. Ultimately, a total of 135 wing clones were scored, about half of which included more than 100 cells. Although all results reported here are for the *mys*^{XG43} allele, similar wing blisters have been observed in clones of another putative null allele, *mys*^{XB87} (our unpublished observation).

derived central row of the anterior margin, but which did not cross to the other wing surface. These clones apparently reached the presumptive margin after the lineage restriction was established; even so, clones that define the margin over such distances would not be expected if the integrins are important for the maintenance of the lineage restriction.

Although most third instar imaginal disc cells express some surface PS integrin, these proteins are found in particularly high concentrations on the basal surface of the wing pouch epithelium[14]. Also, the spatial specificity of the PS1 and PS2 integrins in this disc region indicates that they are likely to be involved in a specific morphogenetic process there, and we have shown that the integrins are indeed critically important for the proper joining of the dorsal and ventral wing epithelia. It is interesting that our *mys* clones in the leg (15–30 bristles) or abdomen (7–10 bristles) displayed no obvious morphological phenotype (unpublished; see also ref. 21). It seems that the integrins serve a general function in discs, perhaps related to epithelium–matrix connections, but that they additionally perform a much more specialized morphogenetic function in the developing wing. □

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