Development 118, 737-750 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Analyses of PS integrin functions during *Drosophila* development

Susan Zusman[1,2,*], Yevgenya Grinblat[3], Gene Yee[1,2], Fotis C. Kafatos[3,4] and Richard O. Hynes[1,2]

[1]Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[2]Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
[3]Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138, USA
[4]Institute of Molecular Biology and Biotechnology, Research Center of Crete and Department of Biology, University of Crete, Heraklion, Crete, Greece

*Present address: Department of Biology, University of Rochester, Rochester, NY 14627, USA

SUMMARY

The *Drosophila* position-specific (PS) antigens are homologues of the vertebrate integrins, a family of transmembrane proteins that function in cell-matrix and cell-cell adhesion. The common β subunit of PS integrins (PSβ) is encoded by the *lethal(l)myospheroid* gene (*mys)* and is required during wing, eye and muscle development. By expressing PSβ protein at defined developmental periods, we have shown that PS integrins are required throughout pupation, but not earlier, for normal development of wings. In contrast, the key requirement for PS integrins in eye development occurs only in the late pupa. Furthermore, PS integrins are apparently not required for the differentiation of the ommatidial cells; only for their organization. These results are consistent with roles for PS integrins in the interactions between the wing epithelia during the two phases of pupal wing expansion and in maintaining the attachment of a fully formed fenestrated membrane to the basement membrane of the retina.

We have also examined the functional significance of alternative splicing of the transcript of the *mys* gene using P element-mediated transformation to introduce transgenes producing only one of the two spliced forms of PSβ. We find that either form is sufficient to rescue postembryonic *mys* phenotypes in the wing, eye and muscle but that both of the two splice forms are necessary to rescue the *mys* embryonic defects. This result indicates a requirement for the alternative splicing of *mys* during embryogenesis. The location of the alternative exons suggests that the two forms of the PSβ integrin subunit may interact with alternative α subunits and/or ligands.

Key words: integrins, alternative splicing, *lethal(1)myospheroid, Drosophila* development

INTRODUCTION

Regulated changes in cell-cell and cell-substratum contact during development affect cell migration, cell shape, inductive signalling and the alignment and integrity of tissues (Hynes and Lander, 1992). Although many cell surface molecules involved in these processes have been identified recently, the mechanisms by which they function in development are still largely unknown. The integrin adhesion receptors are heterodimeric transmembrane proteins consisting of noncovalently linked α and β subunits. Their large extracellular domains bind to molecules of the extracellular matrix, such as fibronectins, laminins and collagens or to members of the immunoglobin superfamily, such as VCAM-1, ICAM-1 and ICAM-2 (Hynes, 1987; 1992; Albelda and Buck, 1990; Hemler, 1990). The small cytoplasmic domains of integrins associate with the cytoskeleton through talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1990). Vertebrate integrins have been shown to play a role in a number of developmental processes including gastrulation, neural crest migration, neuronal pathfinding, and the differentiation of keratinocytes and myoblasts. Integrins are also involved in a variety of signal transduction processes (Shattil and Brugge, 1991; Hynes, 1992).

Studies of vertebrate integrins have provided much data on the potential functions of integrins, but have been limited by the difficulty of detailed *in vivo* analyses of integrin function during development. Such analyses are, however, feasible in *Drosophila melanogaster* because of the ease of genetic manipulation. In *Drosophila*, two groups of integrins have been identified. The best studied of these, the PS integrins, comprise two α subunits (PS1α and PS2α) and a common β subunit, PSβ (Leptin et al., 1987). Through most of embryonic development, the PS2α integrin subunit is expressed mainly in mesodermal derivatives (Bogaert et al., 1987), while PS1α is found primarily on ectodermal and endodermal tissues (Leptin et al., 1989). PSβ is produced in all three germ layers (Leptin et al., 1989; Zusman et al., 1990). In imaginal discs, PS1α and PS2α have spatially

restricted patterns of expression (Brower et al., 1985). For example, in third instar larvae, PS1α and PS2α are expressed in wing imaginal disc cells that give rise to the dorsal and ventral portions of the wing blade, respectively, while in the eye imaginal disc they are expressed on opposite sides of the morphogenetic furrow. The common PSβ subunit is found throughout these disc epithelia. These distributions suggest that PS integrins may play important and diverse roles in developmental processes in *Drosophila*.

Recent studies have shown that PS2α is encoded by the *inflated (if)* gene (Wilcox et al., 1989) and PSβ by the *lethal(l)myospheroid (mys)* gene (Bogaert et al., 1987; MacKrell et al., 1988). Loss-of-function mutations in either *mys* or *if* lead to embryonic lethality. Mutant *mys* embryos have abnormalities in muscle attachments and in germ band retraction and a hole in the dorsal cuticle (Wright, 1960; Newman and Wright, 1981; Wieschaus and Noell, 1986; Leptin et al., 1989). Postembryonic abnormalities caused by partial or localized loss of PS integrins include wing blisters (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990), disorganized photoreceptors in the retina (Zusman et al., 1990) and abnormalities in indirect flight muscles (de la Pompa et al., 1989).

In this paper, we examine in greater detail the functions of PS integrins in the development of wings, eyes and muscles and during embryonic development. First, using PSβ integrin cDNAs under the control of a heat-inducible promoter, we show that PS integrins are required during metamorphosis of the wing and eye discs but not at earlier stages of development of these discs. The results are consistent with roles for PS integrins in maintaining strong attachments between adjoining cell layers during morphogenesis of various tissues. In addition, we have investigated the functional significance of alternative splicing of the PSβ subunit. We have recently found that the fourth exon of the *mys* gene encoding PSβ exists in two mutually exclusive, alternatively spliced forms (4A and 4B), expression of which is developmentally regulated (Yee et al., unpublished data). Here we report that either the 4A or the 4B form of PSβ is sufficient for normal development of wings, eyes and muscles but that both forms appear necessary for embryonic development.

MATERIALS AND METHODS

Fly strains

The *lethal(l)myospheroid* alleles used in these experiments are mys^{XG43}, a null mutation (Wieschaus et al., 1984; Leptin et al., 1989), mys^{XR04}, an antimorphic mutation (Wieschaus et al., 1984; Wilcox et al., 1989; Bunch et al., 1993) and mys^{nj42}, a viable mutation (Costello and Thomas, 1981). Mutant chromosomes carrying the marker mutations *yellow, white, chocolate, forked* or *shavenbaby* (*y w cho f svb*) in various combinations were kept over the balancer chromosome *FM7*. Transformant lines were maintained over balancer chromosomes *CyO* and *TM3, Sb ry*. See Lindsley and Zimm (1985, 1990) for detailed descriptions of the mutations and balancer chromosomes.

Construction of *P[hsp.mys^{4A}]* and *P[hsp.mys^{4B}]*

4A- and 4B-specific cDNAs were isolated from a λgt11 library prepared from 0-16 hour embryos (a gift of Drs K. Zinn and C. Goodman, UC Berkeley). Detailed intron-exon structure of the *mys* gene will be described elsewhere (Yee et al., unpublished data). A P-element vector containing a fusion of the *hsp70* promoter (Ingolia et al., 1980; Pelham, 1982; Lis et al., 1983) and mys^{4A} cDNA, *P[hsp.mys^{4A}]*, was constructed by subcloning an *Eco*RI to *Spe*I fragment of mys^{4A} cDNA previously cloned in the pNB40 vector (Brown and Kafatos, 1988) between the *Eco*RI and *Xba*I sites in the polylinker of Casper hs, a P-element vector marked with $white^{+}$ (a gift of Rick Padgett, Rutgers University). The *P[hsp.mys^{4B}]* construct was produced by replacing the *Sac*II to *Spe*I fragment of this construct with the corresponding fragment of 4B-specific cDNA (Yee et al., unpublished data).

Construction of *P[mys^{+}]4A* and *4B* derivatives

The genomic *P[mys^{+}]* construct contains a 10.5 kb fragment extending from an *Xho*I site upstream of the gene to a point 200 bp downstream of the second *Sma*I site in the 3′ untranslated portion of the *mys* gene. This fragment, which includes the transcribed sequence and 1.5 kb of 5′ flanking sequence, was subcloned between the unique *Kpn*I and *Sal*I sites of the Carnegie 20-based P element vector, HZ50PL, marked with $rosy^{+}$. The 3′ end of the genomic fragment falls just short of the polyadenylation site of the gene. However, polyadenylation sequences are provided by the hsp70 sequences in the HZ50PL vector (Fig. 6, see Hiromi and Gehring, 1987 for further details of the vector). The *P[mys^{4A}]* and *P[mys^{4B}]* derivatives of *P[mys^{+}]* were generated by replacing a 3.4 kb segment between the two *Bam*HI sites of the *mys* gene with 4A- or 4B-specific cDNA. This resulted in the removal of 2.6 kb of intron sequence (see Fig. 6).

Germ-line transformation and functional assays

P[mys^{+}] and its derivatives were injected into preblastoderm *cn*; ry^{506} embryos for P-element transformation (Spradling and Rubin, 1982; Rubin and Spradling, 1982). Genomic incorporation was confirmed with the eye color marker gene $rosy^{+}$, present on the transposons. Similarly, the *P[hsp.mys]* cDNA constructs were stably integrated into *y w* flies. The ability of each integrated construct to rescue *mys* phenotypes (see below and text) was determined by crossing one or two copies into appropriate tester strains as described below.

Genetic crosses used to produce flies with one copy of the transposons

(1) Embryos for assays of larval cuticular phenotypes were derived from the following cross:

$$\frac{w\ svb\ mys^{XG43}}{FM7} \times \frac{+}{Y}; \frac{P[mys*]}{P[mys*]}$$

$$\downarrow$$

$$\frac{w\ svb\ mys^{XG43}}{Y}; \frac{P[mys*]}{+},$$

where *P[mys*]* is either *P[mys^{+}]*, *P[mys^{4A}]*, or *P[mys^{4B}]*, carried on the second or third chromosome.

The hemizygous *mys* progeny were identified by the linked *svb* marker.

(2) Adult mys^{nj42} flies used in assays for heldout wings and wing blisters were produced as follows:

$$\frac{w\ mys^{nj42}\ f}{w\ mys^{nj42}\ f} \times \frac{+}{Y}; \frac{P[mys*]}{P[mys*]}$$

$$\downarrow$$

$$\frac{w\ mys^{nj42}\ f}{Y}; \frac{P[mys*]}{+}.$$

These flies were identified as *w f* males.

(3) Mitotic somatic clones were induced by gamma irradiation (Zusman et al., 1990) of larvae derived from the following crosses:

$$\frac{y\ cho\ mys^{XG43}}{FM7} \times \frac{+}{Y}; \frac{P[mys*]}{Balancer}$$

$$\downarrow$$

$$\frac{y\ cho\ mys^{XG43}}{+}; \frac{P[mys*]}{+}.$$

Balancer chromosomes used were *CyO* or *TM3, Sb ry*. Homozygous *y cho mys*^{XG43} eye clones were identified by the chocolate eye color phenotype. Wing clones were identified by blisters.

Genetic crosses used to produce flies with two copies of the transposons

Embryos with two transposons were assayed for hatching by producing cuticle preparations of first instar larvae derived from a 50:50 mixture of the following two crosses:

(1) $$\frac{w\ svb\ mys^{XG43}}{FM7}; \frac{P[mys*]}{+} \times \frac{FM7}{Y}; \frac{P[mys*]}{+}$$

or

(2) $$\frac{w\ svb\ mys^{XG43}}{FM7}; \frac{+}{+} \times \frac{FM7}{Y}; \frac{P[mys*]}{+}.$$

A mixed cross was used because we were unable to distinguish easily between the two types of females. Since hemizygous *w svb mys*^{XG43} embryos with one or no copies of the transposon die without hatching (see Results), it was possible to score for hatched *w svb mys*^{XG43} larvae. 25% of the progeny of this cross would be expected to be *svb*, but only 1/8 of these would receive two copies of *P[mys*]*. The other 7/8 of the *svb* progeny should receive one or no copies of the transposon. If one copy of *P[mys*]* does not allow hatching but two copies of *P[mys*]* do, then 1/25 (4%) of the hatched larvae should be *svb* and 96% should be *svb*$^{+}$.

Cuticular preparations, whole mounts and sections

Cuticles of embryos were prepared following the procedures of van der Meer (1977) and were examined under phase-contrast microscopy. Heads containing *y cho* or *y cho mys*^{XG43} eye clones were removed and submerged in immersion oil. Eyes were then examined under antidromic illumination (Francescini, 1975). The heads of *mys*^{nj42}*/mys*^{XR04} flies raised at18°C were removed, fixed and embedded in JB4 (Polysciences Inc.) as described in Mahowald et al. (1979); 4 μm sections were cut using a Leitz 1516 microtome, stained with methylene blue and viewed under bright field microscopy. Wings containing *y cho* or *y cho mys*^{XG43} clones, identified by blisters (Zusman et al., 1990), were prepared for photography by embedding in Faure's mountant (Faure, 1910).

RNA isolation and ribonuclease protection experiments

Dechorionated embryos, wing or eye imaginal discs manually removed from early pupae (stages P1-P3), and adult heads or thoraces were washed in 0.1 M $NaPO_4$, pH 7.2 and dissolved in guanidinium thiocyanate followed by acidic phenol/chloroform extraction as described by Chomczynski and Sacchi (1987). Total cellular RNA was then precipitated by adding 1/50 volume of 1 M acetic acid followed by 1/2 volume of ethanol. For ribonuclease protection analyses, RNA samples were hybridized at 37°C with a uniformly labeled RNA probe, encoding the 4A exon as well as upstream constant regions, prepared by in vitro transcription of a *Xho*I to *Bam*HI fragment of *mys*^{4A} cDNA (nucleotides 827-1533, numbered according to MacKrell et al., 1988). Following overnight incubation, the samples were treated with 20 μg/ml ribonuclease A and 2 μg/ml RNase T_1 at 25°C for 30 minutes. The samples were then treated with proteinase K, extracted with phenol/choroform, ethanol, precipitated as described by Norton and Hynes (1990) and analyzed by electrophoresis through 4% polyacrylamide denaturing gels.

Embryonic protein preparation and Western blot analysis

Lysates of 15 embryos were prepared for each sample. Hemizygous *mys* embryos were identified using the linked larval cuticle marker mutation, *shavenbaby* (Wieschaus et al., 1984; Gergen and Wieschaus, 1985). Embryos (between 18 and 28 hours old at 25°C) were hand selected under a dissecting scope, dechorionated in sodium hypochlorite and processed as previously described in Leptin et al. (1989). Embryos with P[*hsp.mys*] transposons were grown at 18°C or were grown at 18°C and shifted to 31°C 16-18 hours after fertilization.

SDS denaturing gel electrophoresis, electroblotting and immunological detection procedures were performed as described in Johnson et al. (1984). PSβ expression was visualized using either (1) anti-PSβ monoclonal antibodies 9A5 and 12A5 (a gift of the late Michael Wilcox, Laboratory of Molecular Biology, Cambridge, England) followed by ^{125}I-labelled anti-rat secondary antibody (Amersham Life Sciences) or (2) a polyclonal rabbit anti-peptide antiserum prepared against a cytoplasmic domain peptide of PSβ followed by ^{125}I-labelled protein A (New England Nuclear). Blots were reprobed with an anti-α-tubulin monoclonal antibody 4A1 (a gift of Margaret Fuller, Stanford University) and ^{125}I-labelled anti-mouse antibody (Amersham Life Sciences) in order to normalize for equal loading of samples. A PhosphorImager Apparatus and its associated ImageQuant computer package (Molecular Dynamics) were used for quantitation of samples.

Temperature-shift experiments

Mitotic recombination was induced in 48-54 hour larvae heterozygous for a *y cho* or *y cho mys*^{XG43} chromosome and heterozygous for a *P[hsp.mys]* transgene. After irradiation, flies were grown at 18°C. Heat-induced expression of the cDNA was promoted by moving the flies to 31°C and was terminated by moving them back to 18°C. Rescue was determined by the absence of wing blisters, normally found as the result of homozygous *y cho mys*^{XG43} clones in approximately 15-20% of irradiated heterozygous *y cho mys*^{XG43} flies (Zusman et al., 1990; see also Results). The *y cho mys*^{XG43} eye clones were scored as rescued if at least one complete group of rhabdomeres in an ommatidium, completely surrounded by *y cho mys*^{XG43} homozygous ommatidia, appeared normal in organization, shape and size under antidromic illumination (Francescini, 1975). Pupal stages were as described in Bainbridge and Bownes (1981) and Cagan and Ready (1989).

P[Rh4.lacZ] histochemical staining

A strain containing the *P[Rh4.1900 lacZ]* transposon (hereafter designated *P[Rh4.lacZ]*) was a gift of M. Fortini (Fortini and Rubin, 1990). The heads of +/+; *P[Rh4.lacZ]* flies or *mys*^{XR04}*/mys*^{nj42}*; P[Rh4.lacZ]* flies raised at 18°C and *y cho mys*^{XG43}*/+*; *P[Rh4.lacZ]* flies containing *y cho mys*^{XG43} homozygous clones were removed and embedded in O.C.T. Compound (Tissue-Tek). Cryostat sections (10-14 μm) were fixed and stained for β-galactosidase activity using the procedures described by Mismer and Rubin (1987) and Fortini and Rubin (1990), and were mounted in 70% glycerol in PBS. Photographs of stained cross sections were traced and the densities of wild-type and mutant R7 photoreceptors were determined with graph paper divided into millimeter squares.

RESULTS

PS integrins are required through most of pupal development for normal wing development

PSβ is expressed throughout the wing disc in association with either PS1α (dorsal epithelium) or PS2α (ventral epithelium) (Brower et al., 1985). Recently Brower and Jaffe (1989) and Zusman et al. (1990) have shown that homozygous *mys* clones induced in the wing disc during larval stages result in wing blisters in which the dorsal and ventral wing epithelia in and around the clones fail to adhere. Similarly, wing blisters were observed in 10-25% of flies hemizygous or homozygous for viable alleles of *mys* (mys^{nj42}) or *inflated* (if^{3}) (Wilcox et al., 1989). In the present study, we find that flies containing one copy of mys^{nj42} and one copy of an antimorphic embryonic lethal mutation, mys^{XR04} (Wilcox et al., 1989), also show wing defects. These flies die at 25°C but survive at 18°C. Of 216 wings of mys^{nj42}/mys^{XR04} flies grown at 18°C, 22% had blisters similar to those observed in homozygous mys^{XG43} clones and 36% were entirely inflated, with the dorsal and ventral epithelial layers completely separated (Fig. 1). Collectively, these results clearly demonstrate a requirement for PS integrins in maintaining the close apposition of the dorsal and ventral surfaces of the developing wing.

During pupation, wing metamorphosis begins with the imaginal disc evaginating and folding along the wing margin, resulting in the apposition of the basal surfaces of the dorsal and ventral epithelia. Subsequently, the epithelial layers separate and rejoin at least twice (Waddington, 1941; Milner and Muir, 1987). The two epithelial sheets are connected via basal cellular processes joined at attachment sites (Mogensen and Tucker, 1988). Subsequently, the wing cuticle is secreted at the apical surfaces of the epithelia (Waddington, 1940). In order to determine when during these morphogenetic events the PS integrins are required, the *Drosophila hsp*70 promoter was fused to a *mys* cDNA and introduced into flies via P-element transformation (see Materials and Methods). Western blot analysis of samples from mys^{XG43}; *P[hsp.mys]* embryos confirmed that significant levels of PSβ (9-35% of the endogenous wild-type levels) could be induced by heat induction at 31°C (Fig. 2).

Mitotic recombination in flies heterozygous for a null mutation in *mys* (mys^{XG43}), with or without a copy of *P[hsp.mys]*, and grown at 18°C (noninduced condition), caused 17-18% of the wings to contain blisters resulting from homozygous mys^{XG43} clones of cells (see Fig. 3A and Zusman et al., 1990). In contrast, when such flies were shifted to 31°C (heat-induced condition) during third instar larval or white prepupal stages, significantly fewer wing blisters were observed (Fig. 3A). Heat induction of similar flies during subsequent pupal stages failed to rescue the blister phenotype, suggesting that mys^{+} expression is not required prior to the onset of pupation but is necessary by early pupal stages (P1-P3), when the first of the two wing expansions begins.

To define further the time of the requirement for mys^{+} during wing morphogenesis, irradiated $mys^{XG43}/+$; *P[hsp.mys]* flies were shifted to heat-induced conditions during the third larval instar and then shifted back to non-induced conditions during subsequent pupal stages (Fig. 3B). Significant reduction in the number of blisters was observed only when the shift down in temperature occurred very late in pupation (after stage P12), when the second wing expansion ends (see Fig. 3). Thus, these experiments suggest that mys^{+} expression is required through much of pupation, including the two wing expansions, to ensure that the dorsal and ventral epithelia of the wing blade remain juxtaposed.

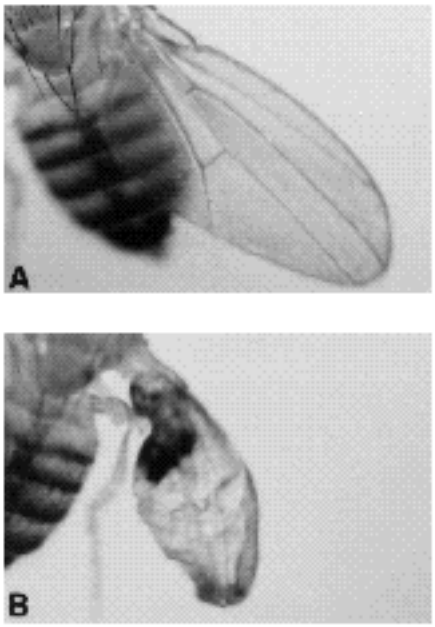

Fig. 1. Wing defects caused by *mys* mutations. (A) Wild-type wing; (B) inflated wing from a mys^{nj42}/mys^{XR04} fly showing separation of the entire dorsal and ventral epithelia. This phenotype is seen in 36% of the wings; a further 22% have smaller blisters.

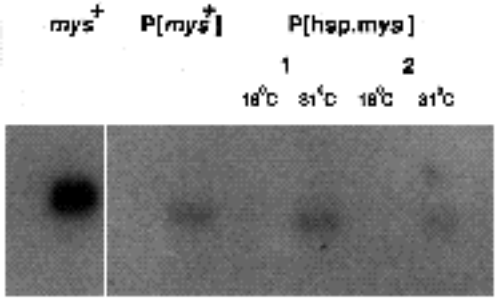

Fig. 2. Western blot analysis of PSβ expression. Embryos with transposons were raised at either 18°C or 31°C. Embryonic protein extracts of 15 embryos/lane were analyzed. The blots were probed with an antiserum against the cytoplasmic tail of PSβ and were subsequently reprobed with an antiserum against α-tubulin (data not shown). Levels of PSβ were quantitated and normalized against those of tubulin. The *P[mys⁺]* construct expressed at 16.5% of endogenous levels and the two *P[hsp.mys]* transposons expressed at 2.6% and 1.1%, at 18°C and 35.0% and 9.3%, respectively, at 31°C, demonstrating good heat induction.

PS integrins are required late during eye morphogenesis

The adult *Drosophila* eye consists of a repetitive array of

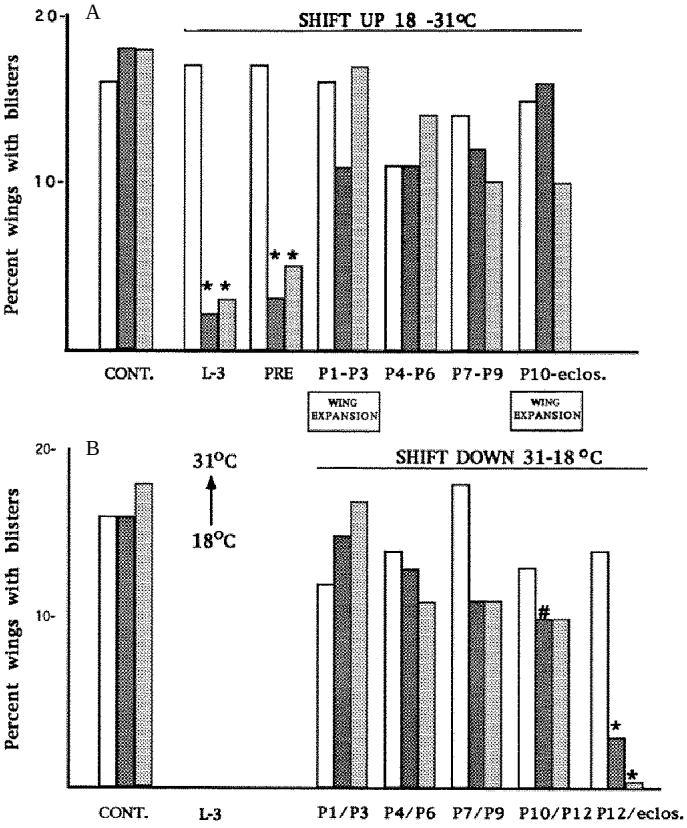

Fig. 3. Rescue of the wing blister phenotype by P[*hsp.mys*]. Figure shows percentage of wing blisters arising in flies irradiated at first larval instar. White columns represent flies heterozygous for *mys*^{XG43} with no *myospheroid* transposons. Such flies typically show 15-20% wing blisters. Shaded columns represent flies which are heterozygous for *mys*^{XG43} and carry one of two transposons; *P[hsp.mys*^{4A}*]*, dark shading, or *P[hsp.mys*^{4B}*]*, light shading. (A) Shift-up experiment. Flies of all three strains showed similar numbers of blisters when maintained at 18°C (control columns). When shifted to 31°C at third instar or prepupal stages, the strains carrying *P[hsp.mys]* showed significant reductions in numbers of blisters. If the shift was delayed to various pupal stages, little or no rescue was observed. (B) Shift-up/shift-down experiment. Flies shifted to 31°C at third instar (cf. panel A) were returned to 18°C at various stages. Significant reductions in numbers of blisters were seen only when the shift back to 18°C occurred late in pupation. The developmental stages were as described in Bainbridge and Bownes (1981) and Cagan and Ready (1989). *Significant decrease in the percent of wing blisters observed ($P \leq 0.05$). #All blisters observed were unusually small.

approximately 800 ommatidia, each including 8 photoreceptors with rhodopsin-containing rhabdomeres, 4 cone cells responsible for secreting the overlying lens and 2 primary pigment cells. These 14 cell units are surrounded by secondary and tertiary pigment cells which form a lattice around the ommatidia. The basal parts of these pigment cells form a fenestrated membrane, which lies against the basement membrane. Photoreceptor axons pierce the fenestrated membrane and synapse in the underlying optic ganglia, the lamina and medulla (Waddington, 1962; Cagan and Ready, 1989). The pigment cell feet forming the fenestrated membrane contain a fibrous network of cytoskeletal stress fibers (Cagan and Ready, 1989).

The complex adult retina develops from a specialized single-layered epithelium, the eye imaginal disc. During the third larval instar, retinal pattern formation occurs in a posterior-to-anterior wave of morphogenesis (Ready et al., 1976). The leading edge of the wave is marked by a dorsoventral morphogenetic furrow. Ahead of this furrow, cells are undifferentiated while, behind it, cell clusters begin to differentiate into the future photoreceptors and other cells of the adult eye (see Tomlinson, 1988 for a review). A striking shift in PS integrin expression, from PS1α to PS2α, occurs at the transition that is marked by the morphogenetic furrow (Brower et al., 1985). Although this may suggest a function for the PS integrins in the initial differentiation of ommatidial cells, mosaic studies argue for a later requirement for PS integrins in eye development. Photoreceptors and other retinal cells appear to differentiate in the absence of PS integrins but are not properly organized (Zusman et al., 1990).

In order to extend these findings and examine further the requirements for PS integrins in eye morphogenesis, we have examined *mys* clones in developing eyes of *mys*^{XG43}*/+; P[hsp.mys]* flies. Flies shifted from 18°C to 31°C during the third larval instar through the P4-P6 pupal stages of development showed rescue of the disorganized photoreceptor phenotype in 76% to 90% of the eye clones scored (Table 1). Rescue was significantly less in eye clones of flies shifted to 31°C after the P6 pupal stage (Table 1). The induction of photoreceptors is completed prior to stage P6. These data are consistent with the notion that *mys*$^{+}$ expression is largely dispensable during larval and early pupal stages, when the morphogenetic furrow moves across the eye disc and the photoreceptors and surrounding cells of the ommatidia initially differentiate, but becomes essential later.

In order to identify specific processes that are affected by

Table 1. Prevention of mutant ommatidia in *mys*$^{-}$ eye clones by *heat shock/mys* P element constructs*,†

	Percent rescue‡ at different stages of shift up					
					Fenestrated membrane is present P8-eclosion	
	No heat shock	Third larval instar	P1-P3	P4-P6	P7-P9	P10-eclosion
Control	0% (29)	0% (20)	0% (14)	0% (12)	8% (13)	8% (13)
Heat-shock construct		90% (20)	83% (23)	76% (21)	23% (26)	5% (20)

*_mys_ eye clones were identified by the linked *cho* mutation

†*Heat shock/mys* constructs used in these experiments contained only *4A-mys* cDNA.

‡A *mys* eye clone was scored as rescued by the *heat shock/mys* construct when at least one *cho mys* ommatidium, completely surrounded by *cho mys* ommatidia had a wild-type phenotype. The total number of clones scored is given in brackets.

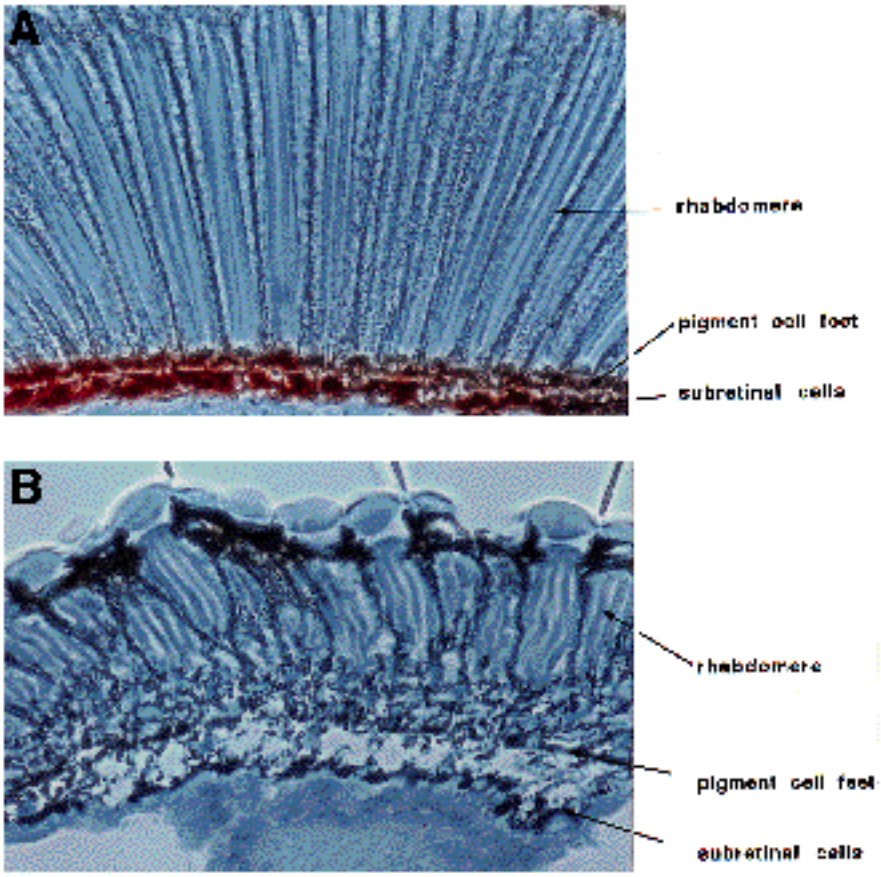

Fig. 4. Eye phenotype of mys^{nj42}/mys^{XR04} flies. (A) Longitudinal section of the basal surface of a wild-type retina. The layer of pigment cell feet forms the fenestrated membrane, which is separated by the basement membrane (white) from the underlying subretinal cells. (B) Longitudinal section of the basal surface of a mys^{nj42}/mys^{XR04} retina. Note the separation between the pigment cell feet comprising the fenestrated membrane and the layer of pigmented subretinal cells found below the basement membrane. Also note that photoreceptors form in these mutant flies but are disorganized.

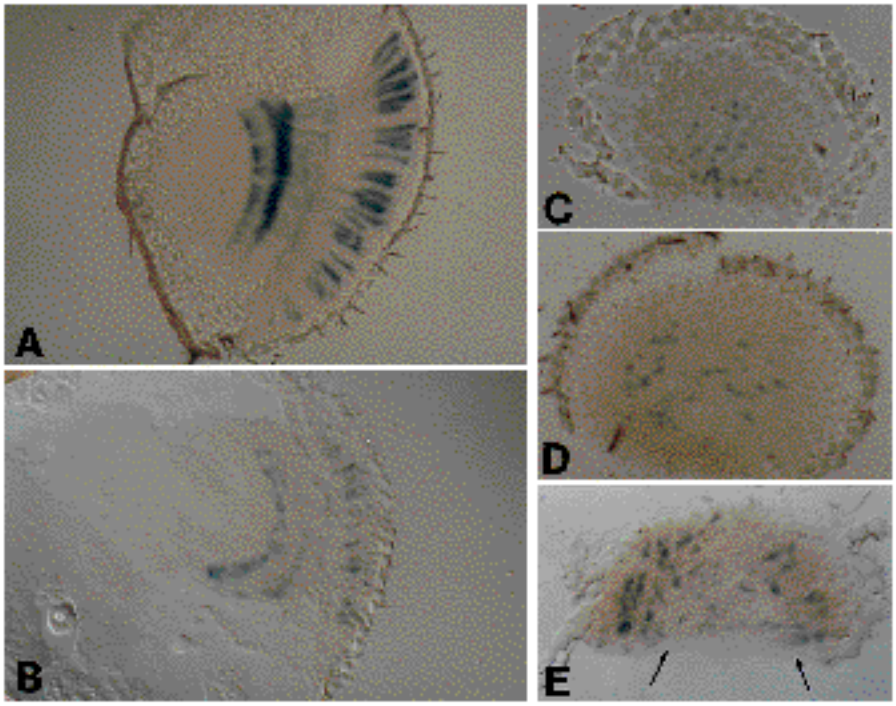

Fig. 5. Histochemical analysis of *Rh4-lacZ* expression. 12 μm cryostat sections of head tissues were prepared and stained as described in Materials and Methods. (A) Section through *P[Rh4.lacZ]* head tissues showing longitudinal profiles of R7 cell bodies and their synaptic terminals in the medulla. re, retina; me, medulla. (B) Similar section of mys^{nj42}/mys^{XR04} ; *P[Rh4. lacZ]* head tissue. Note that R7 cell bodies are present and that these cells extend axons into the medulla. (C,D) Tangential sections through the distal retina of wild-type (C) and mys^{nj42}/mys^{XR04}; *P[Rh4.lacZ]* flies (D). Note that both sections show R7 cells. Density measurements of 16 wild-type and 17 mys^{nj42}/mys^{XR04} ; P[*Rh4.lacZ*] sections gave values of 19.5±1.8 R7 cells/26.5 μm^2 and 16.3±2.4 R7 cells/26.5 μm^2, respectively. (E) Tangential section of a *w* $mys^{XG43}/+$ eye containing a homozygous *w* mys^{XG43} somatic clone (white area in center, arrows). Note that R7 cell bodies are observed even in the complete absence of *mys* expression. See Fortini and Rubin (1990) for a further description of *P[Rh4.lacZ]*.

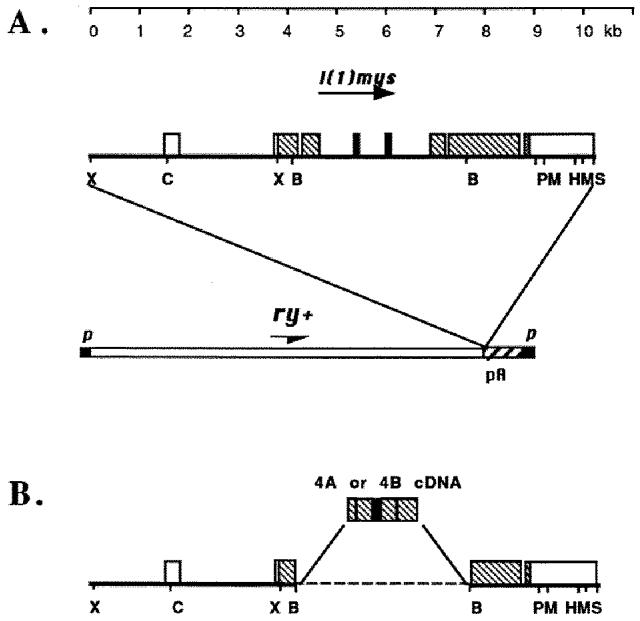

Fig. 6. The structures of *P[mys⁺], P[mys^4A] and P[mys^4B]*. (A) Structure of *P[mys⁺]*. Boxes represent exons; white areas are untranslated; black areas are the 4A- and 4B-alternative exons. The top line gives the scale in kilobases; the arrow shows the direction of transcription. The HZ50PL vector includes a copy of the *Drosophila rosy*⁺ gene (*ry*⁺) as a visible selection marker (open box), the 3′ untranslated region of the *Drosophila* hsp70 (cross hatched box) with its polyadenylation site (pA) and the P-element sequences required for transposition (black boxes). (B) Structure of *P[mys^4A]* and *P[mys^4B]*. In these constructs, 3.4 kb of genomic sequence from *P[mys⁺]* including five introns was replaced with 4A- or 4B-specific cDNA. H, *Hin*dIII; C, *Sac*II; S, *Sal*I; M, *Sma*I; P, *Spe*I; X, *Xho*I; B, *Bam*HI.

the loss of PS integrins during eye development, we examined the eyes of *mys^nj42/mys^XR04* flies grown at 18°C. Twelve eyes examined under antidromic illumination showed extremely disorganized rhabdomeres in the ommatidia. Ten eyes were embedded in plastic and sectioned. In sections perpendicular to the plane of the retina, disorganization of the photoreceptors was obvious (Fig. 4). In addition, at the basal surface of the retina, a gap was often observed between the pigment cell feet constituting the fenestrated membrane and the layer of pigmented subretinal cells found below the basement membrane (Fig. 4). Since the fenestrated membrane is first observed late in pupation (stage P8, 55 hours before eclosion at 20°C, see Cagan and Ready, 1989), an abnormality in its attachment to the basement membrane is consistent with the late requirement for *mys*⁺ expression (see above).

The cells of the retina develop by a cascade of inductive events (see Rubin, 1989; Lawrence and Tomlinson, 1991 for reviews). The photoreceptor cells differentiate first, in a sequence beginning with the formation of the R8 photoreceptor, followed by the induction and differentiation of R2 and R5, then R3 and R4, R1 and R6 and, finally, R7. The other cells of the ommatidium, the cone and pigment cells, are subsequently added to this initial cluster of photoreceptor precursors. Although *mys^XG43* somatic clones appear to have the normal number of photoreceptors per ommatidium, their disorganization makes accurate counts difficult (Zusman et al., 1990). In addition, it is unknown whether all cell types (R1-R8) are present. In order to address these issues, we examined *mys* mutant ommatidia for the presence of the R7 photoreceptor by introducing into *mys^nj42/mys^XR04* flies a chromosome carrying the transposon, *P[Rh4-lacZ]*, containing the bacterial reporter gene *lacZ* under the control of the R7-specific rhodopsin (Rh4) promoter (Fortini and Rubin, 1990). The presence of R7 photoreceptors in these flies with approximately normal density was confirmed in sections stained with X-gal (Fig. 5A-D). Longitudinal sections of these eyes showed that the R7 photoreceptor axons project into the medulla, the second optic ganglion of the brain (Fig. 5B) and thus are at least partially differentiated, despite their disorganization. The lower intensity of *lacZ* signal in the mutant eye was often seen and may reflect the disorganized arrangement of the mutant R7 cells which therefore do not lie entirely within the plane of section. It could also reflect a partial deficit in R7 differentiation. However, the presence of differentiated R7 photoreceptors indicates that the inductive signals required for their formation occurred and suggest that the early forming R1-R6, and R8 photoreceptors are also present.

Since the *mys^nj42* and *mys^XR04* alleles of the *myospheroid* locus are not null (Wilcox et al., 1989; Bunch et al., 1992), we also tested for the presence of R7 photoreceptors in homozygous clones of a null allele. The eyes of *y cho mys^XG43/+ ; P[Rh4-lacZ]* flies with homozygous *y cho mys^XG43* eye clones were also sectioned in cross section and stained. Fig. 5E shows that R7 photoreceptors are still present in ommatidia that have completely lost PSβ integrin expression. The lower intensity of *lacZ* signal probably reflects the fact that the distorted photoreceptors are sectioned at oblique angles. The presence of R7 cells in *mys* null clones suggests that the differentiation of these photoreceptors does not require the PS integrins.

P-element-mediated rescue of abnormalities caused by loss of PS integrins

A genomic fragment including the *mys* transcribed sequences and 1.5 kb of 5′ flanking DNA was inserted into the P-element transformation vector HZ50-PL (see Fig. 6, and Materials and Methods for further details). Two lines of transgenic flies containing this wild-type transposon on an autosome were used to test for rescue of four phenotypes, one embryonic (dorsal hole) and three postembryonic (Fig. 7), resulting from abnormalities in PS integrins (see Fig. 7 for description). These transformants showed similar and significant amounts of rescue (Table 2). Thus, one copy of the *P[mys⁺]* transposon produces rescue of the dorsal hole phenotype in embryos. A single copy of *P[mys⁺]* also completely eliminates the disorganization of photoreceptors (Fig. 7) and of the wing blisters in homozygous *mys^XG43* somatic clones (Table 2). In addition, *P[mys⁺]* rescues abnormalities observed in flies hemizygous for the viable allele, *mys^nj42*. The *mys^nj42* flies are incapable of flight and do not show the escape jump response. This is thought to be due to wasting of mesothoracic and metathoracic tergotrochanteral muscles, which attach in the thorax and in the leg of the fly (Costello and Thomas, 1981; de La Pompa et al., 1989). Presumably as a result of the thoracic muscle

Table 2. Mutant *mys* phenotypes: rescue by specific genomic *mys* constructs

	Phenotypes assayed				
	mys⁻ clones*		*mys*^{nj}/*Y*		*mys*⁻/*Y*
Transposon†	Wing blisters*	Mutant ommatidia*	Heldout wings	Blisters	Dorsal hole in embryos
NONE	15%(302)	100%(29)	98%(172)	9%(172)	100%(82)
WILD-TYPE *P[mys⁺]*	0%(345)	0%(17)	0%(116)	0%(116)	0%(157)
4A-SPECIFIC *P[mys^{4A}]*	0%(276)	0%(17)	0%(212)	0%(212)	100%(66)
4B-SPECIFIC *P[mys^{4B}]*	0%(268)	0%(20)	0%(214)	0%(214)	29%(180)‡

**mys*⁻ eye clones were identified by the linked *cho* mutation. *mys*⁻ wing clones were identified by the presence of wing blisters (See Materials and Methods).

†Two independent lines carrying each of P[*mys^{4A}*] and P[*mys^{4B}*] were tested; the results were equivalent and have been pooled. Data for a single P[*mys⁺*] transformant line are shown for comparison.

‡The larvae without a dorsal hole were flaccid.

defects, *mys^{nj42}* hemizygous flies hold their wings at an abnormal angle to their body axes. This heldout phenotype is enhanced when the flies are grown at 29°C (Fig. 7). The *P[mys⁺]* transposon prevents this heldout wing phenotype even when flies are grown at 29°C. In addition, this transposon prevents the wing blisters observed in *mys^{nj42}*/Y flies (Table 2), which are similar to those seen in homozygous *mys^{XG43}* clones.

P[mys⁺] expresses PSβ at levels up to 34% of the endogenous PSβ level (Fig. 8). This low level of expression may explain the observation that, although the transposon rescues the dorsal hole phenotype in *svb mys^{XG43}* ; *P[mys⁺]* animals, these embryos rarely hatch. In order to determine whether extra doses of *P[mys⁺]* allow rescue to later stages, *svb mys^{XG43}* embryos were produced containing two copies of P[*mys⁺*].As described in Materials and Methods, if effective rescue requires two copies of *P[mys⁺]*, one would expect a yield of 4% *svb* progeny. In fact, we observed that 7% of those embryos, which completed embryonic development, hatched and showed normal larval morphology, were *svb* (Table 3). Since *svb* results in larval lethality, we also tested for rescue to the adult stage by adding two copies of the transposon to flies containing a *y mys^{XG43}* chromosome. Since 0/369 expected *y mys^{XG43}*/Y adult flies were observed, we conclude that two copies of *P[mys⁺]* allow development to hatching but do not support viability to adult stages.

Table 3. Embryonic development in *mys-flies* containing two genomic *mys* transposons

Crosses[1]: *FM7/Y;P[mys*]* × *w svb mys^{XG43}/FM7; P[mys*] or + P[mys*]*[2] transposons	Hatched larvae from the crosses		
	svb larvae	*svb⁺* larvae	% *svb* larvae
NONE	0	119	0%
P[mys⁺] plus *P[mys⁺]*	8	110	7%**
P[mys^{4A}] plus *P[mys^{4B}]*	6	56	10%**
P[mys^{4A}] plus *P[mys^{4A}]*	0	248	0%
P[mys^{4B}] plus *P[mys^{4B}]*	12	382	3%†

[1]See Materials and Methods for the details of the crosses used to obtain two copies of *P[mys*]* in embryos. Since *svb mys* embryos carrying no or one copy of *P[mys*]* fail to hatch, 4% of *svb* embryos are expected, if two copies of *P[mys*]* rescue to hatching.

[2]At least two independent lines for each transposon were tested. The results were equivalent and have been pooled.

**Not significantly different from the expected value of 4% if two copies of *P[mys*]* rescue to hatching.

†Significantly different ($P \leq 0.05$) from rescue by two copies of *P[mys⁺]* or one each of *P[mys^{4A}]* and *P[mys^{4B}]*.

Either alternatively spliced form of PSβ can rescue the postembryonic phenotypes

We have shown that the *lethal(l)myospheroid* gene is alternatively spliced and has two mutually exclusive fourth exons (4A and 4B) which encode peptide segments that are 40% identical (Yee et al., unpublished data; see also Fig. 6) Since crosslinking studies and analyses of human mutations suggest that this portion of the β integrin subunit is near the ligand-binding site and near a segment required for αβ dimerization (see Hynes, 1992 for review), it seemed likely that the alternative splicing might provide functional specificity of PS integrins during development.

In order to address this possibility, we have undertaken a functional analysis of the two forms of PSβ, testing whether one or both of its 4A and 4B forms can rescue the three postembryonic *mys* phenotypes (wing blisters, disorganized photoreceptors and heldout wings). *P[mys^{4A}]* and *P[mys^{4B}]* derivatives of *P[mys⁺]* were generated by replacing 3.4 kb of genomic sequence with the corresponding segments of 4A- or 4B-specific cDNAs (see Materials and Methods and Fig. 6), so that only one form of PSβ would be produced from each construct. These mutant transposons were stably integrated into the *Drosophila* genome by P-element transformation. Two independent lines of each strain were examined for expression of PSβ and were used to test for rescue of the previously mentioned phenotypes resulting from abnormalities in PS integrins (Fig. 7, Table 2).

Fig. 8 shows a Western blot of lysates of hemizygous *y*

svb mys^{XG43} embryos with one copy of *P[mys^{+}], P[mys^{4A}]* or *P[mys^{4B}]* probed with antibodies against PSβ. *P[mys^{4A}]* and *P[mys^{4B}]* expressed PSβ at significant levels, similar to each other and comparable to that of *P[mys^{+}]*. Thus, the rescuing abilities of the 4A-and 4B-specific β subunits can be compared without complications caused by different protein levels derived from particular *P[mys*]* transgenes.

Table 2 shows that one copy of either *P[mys^{4A}]* or *P[mys^{4B}]* crossed into *w mys^{nj42}* flies, or in *y cho mys^{XG43}/+* flies with *y cho mys^{XG43}* homozygous clones, is sufficient to rescue any of the three *mys* postembryonic phenotypes scored. Therefore, the alternative splicing of the *lethal(l)myospheroid* gene is not required for the development of the *Drosophila* wing, for the organization of the photoreceptors or for development of tergotrochanteral muscles. In order to determine whether the two forms of PSβ show the same temporal requirements during wing development, we compared the ability of *P[hsp.mys^{4A}]* and *P[hsp.mys^{4B}]* to rescue the wing blister phenotype in homozygous *mys^{XG43}* clones. Temperature-shift experiments performed on third instar larvae and pupae and assays for wing blisters in *mys^{XG43}* clones were performed as described above. The results of these rescue experiments suggest that expression of either *mys^{4A}* or *mys^{4B}* beginning by early pupal stages of development (stages P1-P3) and continuing to stage P12 is sufficient to prevent wing blisters (Fig. 3). Thus, the temporal requirements for either the 4A or 4B form of PSβ appear to be similar during wing development.

Since either the 4A or 4B form of PSβ is sufficient to rescue the wing, muscle and retinal phenotypes, we examined the expression pattern of the two *mys* messages employing an RNase protection assay (Fig. 9). RNA from wing and eye imaginal discs was obtained from young pupae during stages P1-P3 (Bainbridge and Bownes, 1981; Cagan and Ready, 1989). Imaginal discs are easily accessible during these stages and, in the case of the wing discs, are just within the developmental period requiring *mys* expression (see above). Although both forms of *mys* message are found in the wing disc, the 4A mRNA represents 89%. In eye discs, 4A mRNA is the only form detected during these stages. RNA from thoraces contained approximately equal amounts of the 4A and 4B forms.

The 4B form of PSβ is necessary during embryonic development

In contrast to the results found for the postembryonic *mys* phenotypes, neither one copy of *P[mys^{4A}]* nor one copy of *P[mys^{4B}]* allowed complete rescue of the embryonic dorsal hole phenotype observed in the cuticle of *svb mys^{XG43}* (Table 2). All 66 *svb mys^{XG43}* embryos containing one copy of *P[mys^{4A}]* resembled the *svb mys^{XG43}* embryos with no transposon, ie. had a dorsal hole. In contrast, only 29% of the 180 *svb mys^{XG43}* embryos with one copy of *P[mys^{4B}]* had dorsal holes in their cuticles. The remainder of these embryos showed rescue of the dorsal hole phenotype, but appeared more flaccid than embryos with one copy of the genomic P[*mys*$^{+}$] construct. Few, if any, *svb mys^{XG43}* embryos with one copy of any of the transposons hatched.

To examine further the functional requirements of PSβ-4A and PSβ-4B during embryonic development, two copies of *P[mys^{+}], P[mys^{4A}]* or *P[mys^{4B}]* or one copy of *P[mys^{4A}]* plus one copy of *P[mys^{4B}]*, were crossed into *svb mys^{XG43}* embryos. Since two copies of *P[mys^{+}]* allow rescue of embryonic development (see above and Table 3), *svb mys^{XG43}/Y; P[mys*]/P[mys*]* animals were examined for their ability to hatch as larvae. Larvae containing one copy of *P[mys^{4A}]* plus one copy of *P[mys^{4B}]* hatched in numbers equivalent to those containing two copies of *P[mys^{+}]* (Table 3). Therefore, *P[mys^{4A}]* plus *P[mys^{4B}]* is equivalent to two copies of *P[mys^{+}]*, as might be expected. In contrast, although some (3%) of the embryos bearing two copies of *P[mys^{4B}]* were able to complete embryonic development and hatch, this was not observed with two copies of *P[mys^{4A}]*.

Collectively, the results of these experiments indicate that two copies of *P[mys^{+}]* are sufficient to rescue to hatching and that one copy each of *P[mys^{4A}]* plus *P[mys^{4B}]* is equivalent. *P[mys^{4A}]* alone is incapable of rescue of the dorsal hole phenotype or of hatching even when present in two copies, whereas *P[mys^{4B}]* was effective when present in two copies (Table 3) but less so when present in a single copy (Table 2). Thus, while the 4B form is sufficient by itself to support development to hatching, its functions can be enhanced by coexpression of the 4A form. Therefore, both forms of PSβ, 4A and 4B, can contribute to embryonic development but the 4B form is the more crucial. This is consistent with the observation that both forms of PSβ are abundant during embryogenesis (Fig. 9).

DISCUSSION

In this paper, we have examined the temporal and spatial requirements for PS integrins during embryonic, larval and pupal development and have initiated studies of the functional significance of alternative splicing of the common β subunit, PSβ, encoded by the *lethal(l)myospheroid* gene. The results indicate requirements for PS integrins during several developmental processes. Consideration of our data, together with others in the literature, suggests that the most stringent requirements for PS integrins arise in situations demanding strong adhesion of cells or cell sheets. In the absence of PS integrins, many developmental processes proceed approximately normally until a point where mechanical stresses produce rupture of the adhesions. This interpretation is illustrated in Fig. 10 and the various developmental processes will be considered separately below.

PS integrins in wing development

Prior to this work, it was known that PS integrins show restricted patterns of expression in wing imaginal discs (Brower et al., 1984; 1985) and that mutations in two integrin genes, *mys* encoding PSβ and *if* encoding PS2α, cause blistering of the wings (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990). Our experiments using heat-shock-inducible expression of *mys* cDNAs allow conclusions about the times at which the PS integrins are required during wing development. The development of the wing disc involves a series of morphogenetic events (Waddington, 1941; Milner and Muir, 1987; Fristrom et al., 1993). During most of pupal development, the basal surfaces

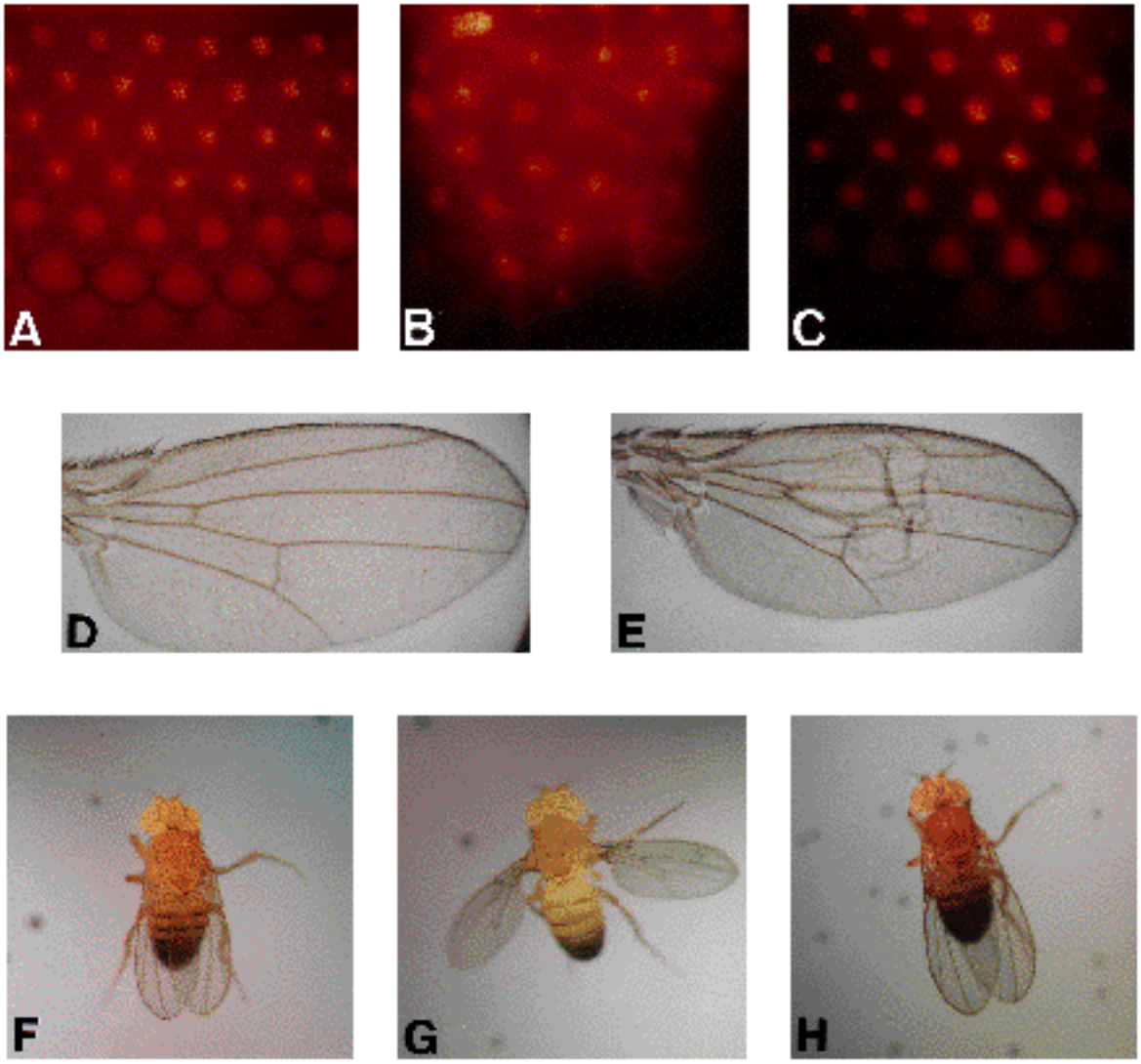

Fig. 7. Postembryonic *mys* phenotypes. (A-C) Eyes viewed under antidromic illumination Plane of focus is at the level of the rhabdomeres. (A) A wild-type eye showing the normal organization of rhabdomeres. (B) A *cho* mys^{XG43}/+ eye containing a *cho* mys^{XG43}/*cho* mys^{XG43} clone showing disorganized rhabdomeres. (C) A *cho* mys^{XG43}/+ eye containing a *cho* mys^{XG43}/*cho* mys^{XG43} ; *P[*mys^{+}*]* clone. Note wild-type rhabdomere organization demonstrating rescue by *P[*mys^{+}*]*. (D) A wing from an irradiated mys^{XG43}/+; *P[*mys^{+}*]* /+ fly. Note the wild-type appearance seen in irradiated flies of this genotype, even though irradiation would produce mys^{XG43}/ mys^{XG43} clones and blisters in 15-20% of the wings if *P[*mys^{+}*]* were not present (see Table 2). (E) A wing containing a mys^{XG43}/mys^{XG43} clone, which generates a blister in the middle of the wing. (F) A *w* mys^{nj42} *f/Y* fly raised at 25°C showing the normal wing posture. (G) A *w* mys^{nj42} *f/Y* fly raised at 29°C showing the 'heldout' wing phenotype in which the fly holds its wings at 90° to the body axis. (H) A *w* mys^{nj42} *f/Y; P[*mys^{+}*]* fly raised at 29°C showing the normal wing posture, demonstrating rescue by *P[*mys^{+}*]*.

of the dorsal and ventral epithelia of the wing are apposed. At two periods, one early in pupation and one late, the epithelia separate and then come back into apposition. Cellular processes arising from the basal surfaces of each of the epithelial sheets maintain connections between them. These cellular processes, known as transalar arrays, contain organized cytoskeleton and are joined by intercellular junctions at their tips (Morgensen and Tucker, 1987, 1988; Tucker et al., 1986; Fristrom et al., 1993). Blisters formed in the absence of PS integrins correspond to separations of the dorsal and ventral epithelia suggesting that the connections between the epithelia break in their absence and that a major role for PS integrins during wing morphogenesis is to maintain these connections (Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990). This suggestion is consistent with the evidence that expression of PSβ prior to pupation is not required to maintain apposition of the wing surfaces but becomes necessary early in pupation, at the time of the first wing expansion, (Fig. 3A) and remains necessary through most of pupal life including the period of late expansion (Fig. 3B). Because we do not know the half life of PS integrins in the wing disc, it is less clear when the integrin requirement ends. Brabant and Brower (1993) have observed similar results using a heat-shock *mys* transgene in $mys^{nj42}if^{3}$ flies. Although highly suggestive, the heat-shock experiments do not prove that PS integrins are required specifically for maintenance of contacts during wing expansion and certainly do not rule out other functions

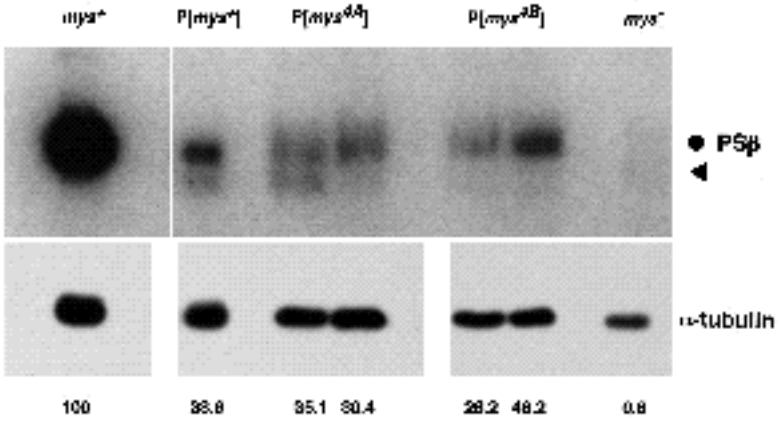

Fig. 8. Western blot analysis of flies bearing *P[mys⁺], P[mys^4A]* or *P[mys^4B]*. Lysates of *mys^XG43/Y* embryos containing one copy of transposon were analysed on two western blots. Two independent lines of 4A- and 4B-specific constructs were used. The same blots were probed with antisera against the cytoplasmic tail of PSβ (top panel) and α-tubulin (lower panel). The PSβ protein is marked with a dot and presumed PSβ precursor by an arrowhead. The level of PSβ expression was determined and compared among different lines by normalization against tubulin. Expression is indicated as a percentage of endogenous expression and is shown at the bottom.

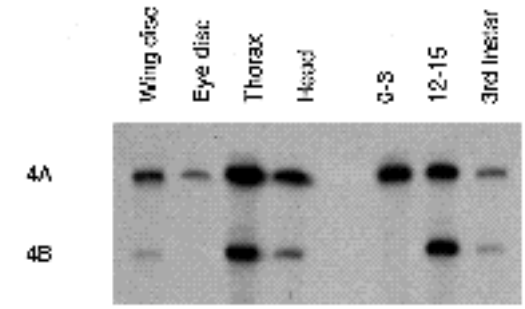

Fig. 9. Tissue-specific and developmental regulation of 4A/4B exon expression; RNAase protection analysis. A 706 nucleotide probe (nucleotides 827-1533, according to MacKrell et al., 1988) which includes the sequence of the 4A exon was used to analyze each RNA sample. The presence of 4A-containing transcripts is indicated by the upper, fully protected band, whereas 4B-containing transcripts are indicated by the lower band of 625 nucleotides.

during wing morphogenesis. However, Fristrom et al. (1993) have recently shown that PS integrins are concentrated at the junctions between the transalar processes. This location is highly concordant with a role in maintaining the interalar connections by mediating adhesion between the cellular processes and possibly also connections to the cytoskeleton (see below).

PS integrins in eye development

The late requirement for PS integrin expression is even more marked in the development of the eye disc. Because a switch in expression from PS1α in the undifferentiated disc epithelium to PS2α in the differentiating epithelium behind the morphogenetic furrow occurs during third larval instar (Brower et al., 1985), it has seemed likely that PS integrins play a role in the differentiation and/or pattern formation of the eye. However, our earlier experiments generating *mys⁻* clones in the eye discs suggested that much of eye development can proceed in the absence of PS integrins (Zusman et al., 1990). The heat-shock *mys* rescue experiments reported here (Table 1) confirm that the major requirement for PSβ in normal ommatidial development arises in the latter half of pupation. The development of photoreceptors, including R7, in *mys^nj42/mys^XR04* flies (Fig. 5E) also indicates that much of the differentiation of the eye can proceed in flies with mutant PSβ. The data presented here and previously (Zusman et al., 1990) suggest that bristles, cone cells and lenses and photoreceptors all develop in the typical hexagonal array. Furthermore, R7 photoreceptors form in normal numbers in *mys^nj42/mys^XR04* flies and project axons into the medulla (Fig. 5). The level of analysis does not preclude some abnormalities in the axonal projections of R1-R8 in PS integrin mutant flies but the distribution of R7 axons (Fig. 5B) and the apparently normal development of the lamina and medulla in flies with *mys⁻* clones (Zusman et al., 1990) both argue for normal or near normal projections of photoreceptor axons. This is so since development of lamina neurons requires retinal axon projections (Meyerowitz and Kankel, 1978; Fischbach and Technau, 1984; Selleck and Steller, 1991).

More subtle defects below the resolution of our analyses may occur; for example, we can only infer the differentiation of R1-R6 and R8 based on the induction of R7 and the disorganization of the photoreceptors in the adult mutant flies precludes analysis of their detailed spatial relationships. A descriptive analysis of the time course of eye development in, say, *mys^nj42/mys^XR04* flies and more extensive studies of retinal axonal projections might reveal further more subtle defects. Despite these caveats, the presently available data are consistent with normal, or near normal, development of the eye until relatively late in pupation up to and including formation of the fenestrated membrane. This occurs at 100-120 hours of pupal development at 20°C with eclosion at 160 hours (Cagan and Ready, 1989) and involves flattening of the feet of the secondary and tertiary pigment cells on the basement membrane to form the pigmented fenestrated membrane, to which the photoreceptors and cone cells are attached (Cagan and Ready, 1989; and see Fig. 4A). In the deformed retinas of *mys^nj42/mys^XR04* flies, this layer of pigment cell feet is visible but is detached from the underlying basement membrane (Fig. 4B). A plausible model for the mutant phenotype is that, at the time of retinal thickening and/or the morphogenetic movements that bring the retina to lie above the lamina, the mechanical stresses lead to the detachment of the pigment cell feet from the basement membrane along with the attached photoreceptors and cone cells. This presupposes that integrins are involved in attachment of the pigment cell feet, for which there is as yet no direct evidence. However, the elaborate arrays of actin filament bundles in the flattened pigment cell feet (Cagan and Ready, 1989) are highly reminiscent of the stress fibers

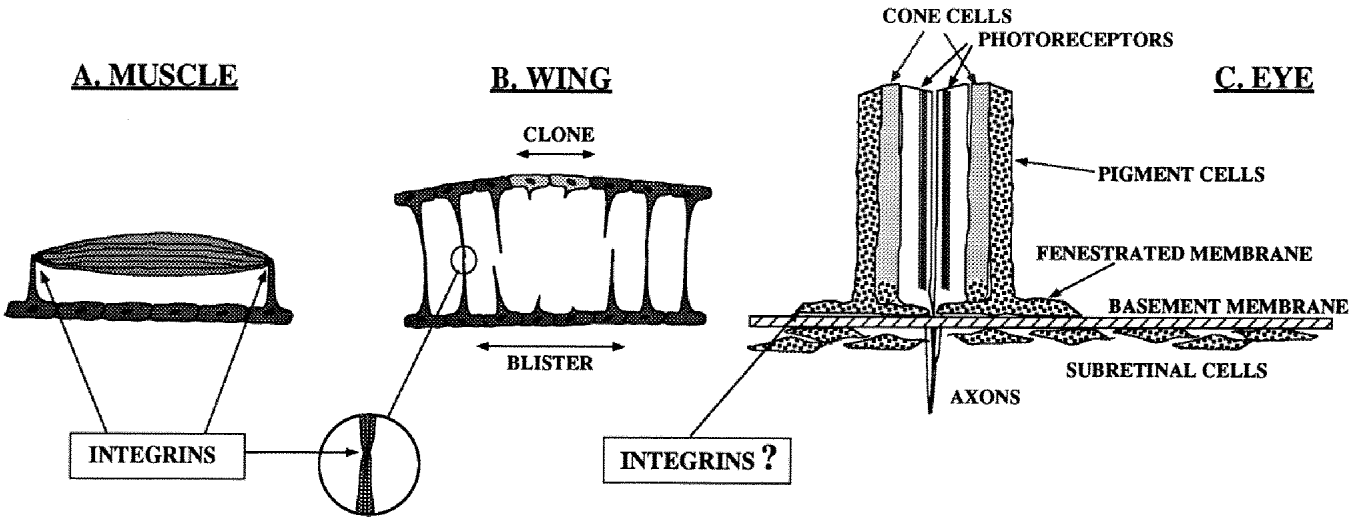

Fig. 10. Models for integrin function in *Drosophila* development. (A) Since the somatic musculature in *mys* embryos prematurely pulls away from hypodermal cells and since antibody localization studies place the PS integrins in embryonic muscle attachments (See text for references) a likely function for the PS integrins is to keep the somatic musculature of the embryo attached to the overlying body wall. (B) The blistering phenotype observed in the wings of mys^{XR04}/mys^{nj42} animals and in mys^{XG43} homozygous somatic clones demonstrates that loss of the PS integrins results in a separation of the dorsal and ventral epithelia of the wing blade. Our temperature-shift experiments show a requirement for PS integrins during much of pupation. An obvious model predicts that the PS integrins are found during pupal stages at the basal portion of the cytoplasmic processes connecting the two wing epithelial layers and that they function to prevent separation. Fristrom et al. (1993) have shown PS integrins to be concentrated there. (C) The separation of cell layers observed along the basal portion of mys^{XR04}/mys^{nj42} eyes and the disorganized photoreceptor phenotypes observed in mys^{XG43} somatic clones show a requirement for PS integrins in maintaining the organization of the *Drosophila* retina. Since our experiments also show a late requirement for PS integrins in the developing eye, we suggest that PS integrins may function along the basal surface of the retina where they may be responsible for holding the fenestrated membrane of the pigment cell feet to the basement membrane below. All three situations diagrammed show defects in the absence of PS integrins, which appear to arise only when mechanical stresses lead to rupture of the adhesion normally maintained by the PS integrins.

in well-spread vertebrate fibroblasts.Such stress fibers align over arrays of integrin molecules, which attach the cells to the substratum (Chen et al., 1985; Damsky et al., 1985).

This proposed connection of PS integrins with organized cytoskeleton in the retinal pigment cells is similar to their known associations with cytoskeleton in the transalar arrays in the wing disc (discussed above) and in muscle-tendon cell attachment sites (Bogaert et al., 1987; Leptin et al., 1989). The parallels among these three systems are illustrated in Fig. 10, which proposes that a major role for PS integrins in each case is to stabilize strong attachments involving submembrane cytoskeletal structures at adherens junctions. Many vertebrate integrins subserve similar functions. Thus, the mutant phenotypes observed (wing blisters, disorganized photoreceptors and muscle detachment) are all proposed to arise only at times of mechanical stress. In each of these systems, development of the relevant cell types (wing epithelia, photoreceptors and pigment cells, muscles) initially proceeds relatively normally in the absence of PS integrins. Any cell adhesion processes involved in the earlier development apparently do not require PS integrins, presumably because of the existence of other adhesion molecules.

PS integrins during embryogenesis

Earlier studies have described the various defects arising during embryogenesis of homozygous or hemizygous mys^{-} embryos (Wright, 1960; Newman and Wright, 1981; Wieschaus and Noell, 1986; Leptin et al., 1989; Zusman et al., 1990). The wild-type genomic transposon $P[mys^{+}]$ can rescue mys^{-} embryos to hatching but not to adulthood (Tables 2 and 3 and unpublished data). It is unclear whether this failure is because of a quantitative defect ($P[mys^{+}]$ transformants express at 16-34% of wild-type levels) or reflects the fact that the *mys* promoter used lacks elements necessary for appropriate expression at later stages. We have attempted to use longer segments of 5′ flanking sequence, but have been unable to obtain transformants because of the presence of apparent 'poison' sequences in the flanking DNA (unpublished data).

The exact nature of the mutant phenotype in embryos remains unclear, although it includes defects in germband retraction, visceral mesoderm, dorsal closure and somatic muscle attachment. Our experiments have directly tested for rescue of only some of these defects and for overall development to hatching. Elucidation of the potential involvement of PS integrins in the various processes will require further study. However, our studies of the functional significance of alternative splicing of PSβ do begin to dissect different roles for PS integrins during embryogenesis.

Functional consequences of alternative splicing of PSβ

The two alternative fourth exons of the *lethal(l)myospher - oid* gene encoding PSβ are mutually exclusive and homologous (Yee et al., unpublished data). Each exon encodes 29 amino acids apparently comprising a disulfide-bonded loop. These segments are homologous with one another and with

equivalent segments of vertebrate integrins and lie in a part of the β subunit close to the ligand-binding site and to regions involved in αβ dimerization (Hynes, 1992). The alternative exons might, therefore, alter α subunit selections and/or affinity or specificity of ligand binding. Our transgene rescue experiments test whether the two forms of PSβ differ in their functional capacities in vivo. It appears that either form will suffice in the postembryonic functions that we have tested, namely in wing and eye development and muscle attachment (Table 2). Both forms are present in the adult thorax (Fig. 9) and can be detected at the attachment sites of body wall muscles in embryos (Yee, G. unpublished data) but it appears that the 4A form is the only one present in early pupal eye discs and the predominant form in wing discs (Fig. 9). Nevertheless, in these tissues, the 4B form apparently provides sufficient function when produced from a transgene. Thus, in the postembryonic processes assayed here, there is as yet no clear evidence for a distinction in functions between the two splice forms of PSβ.

In contrast, when we tested the ability of the two forms to support dorsal closure and hatching of *mys*⁻ embryos, we found a clear distinction (Tables 2 and 3). The 4A form, which is the most widely expressed and represents the previously cloned form, appears incapable of supporting dorsal closure and even when present in two copies, does not support hatching. A single copy of the 4B form, on the other hand, supported some dorsal closure, although less than the wild-type genomic form (Table 2). Two copies of *P[mys^{4B}]* were able to rescue some *mys*⁻ embryos to hatching, although not quite as effectively as either two copies of *P[mys^{+}]* or one copy of *P[mys^{4B}]* plus one of *P[mys^{4A}]* (see Table 3). Since all three transgenes expressed PSβ at comparable levels (Fig. 8), these results suggest that each of the two forms, PSβ-4A and PSβ-4B, performs distinct functions in embryogenesis, with the 4B form being more crucial than the 4A form. The strong expression of the 4B form late in embryogenesis may suggest a role in some late events (see Fig. 8). Further analyses of the expression of the two splice isoforms of PSβ and of the defects in mutant embryos and in those expressing the 4A and 4B PSβ transgenes should provide further insights into the roles of PS integrins in various morphogenetic processes during embryogenesis.

We thank Mark Fortini, Walter Costello, Eric Wieschaus and Michael Wilcox for *Drosophila* stocks. We also wish to thank Helena Kashevsky, Sue Bergin, Blanca Klumpar and Larry Zusman for technical and photographic assistance and Colleen Leslie for preparation of the manuscript. This work was supported by the Howard Hughes Medical Institute (to R. O. H.) and grants from ACS and NSF (to F. C. K.).

REFERENCES

Albelda, S. M. and Buck, C. A. (1990). Integrins and other adhesion molecules. *FASEB J.* **4**, 2868-2880.

Bainbridge, S. P. and Bownes, M. (1981). Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. Exp. Morph.* **66**, 57-80.

Bogaert, T., Brown, N. H. and Wilcox, M. (1987). The *Drosophila* PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell* **51**, 929-940.

Brabant, M. C. and Brower, D. L. (1993). PS2 integrin requirements in *Drosophila* embryo and wing morphogenesis. (in press).

Brower, D. L., Piovant, M. and Reger, L. A. (1984). Developmental analysis of *Drosophila* position-specific antigens. *Dev. Biol.* **108**, 120-130.

Brower, D. L. and Jaffe, S. M. (1989). Requirement for integrins during *Drosophila* wing development. *Nature* **342**, 285-287.

Brower, D. L., Wilcox, M., Smith, R. J. and Reger, L. A. (1984). Related cell-surface antigens expressed with positional specificity in *Drosophila* imaginal disks. *Proc. Natl. Acad. Sci. USA* **81**, 7485-7489.

Brown, N. H. and Kafatos, F. C. (1988). Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425-437.

Brown, N. H., King, D. L., Wilcox, M. and Kafatos, F. C. (1989). Developmentally regulated alternative splicing of *Drosophila* PS2α transcripts. *Cell* **59**, 185-195.

Bunch, T. A., Salatino, R., Engelsjerd, M. C., Mukai, L., West, R. F. and Brower, D. L. (1993). Characterization of mutant alleles of *mysopheroid*, the gene encoding the β subunit of the Drosophila PS integrins. *Genetics* (in press).

Burridge, K., Fath, K., Kelly, T., Nuckolls, G., and Turner, C. (1988). Focal Adhesions: Transmembrane junctions between the extracellular matrix and the cytoskeleton. *Ann. Rev. Cell Biol.* **4**, 487-525.

Cagan, R. L. and Ready, D. F. (1989). The emergence of order in the *Drosophila* pupal retina. *Dev. Biol.* **136**, 346-362.

Chen W. T., Hasegawa, T., Hasegawa, C., Weinstock, C., and Yamada, K. M. (1985) Development of cell surface linkage complexes in cultivated fibroblasts. *J. Cell Biol.* **100**, 1103-1114.

Chomczynsli, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt. Biochem.* **162**, 156-159.

Costello, W. J., and Thomas, J. B. (1981). Development of thoracic muscles in muscle-specific mutant and normal *Drosophila melanogaster*. *Soc. Neurosci. Abs.* **7**, 543.

Damsky, C. M., Knudsen, K. A., Bradley, D., Buck, C. A. and Horwicz, A. F. (1985). Distribution of the CSAT cell-matrix antigen on myogenic and fibroblastic cells in culture. *J. Cell Biol.* **100**, 1528-1539.

de la Pompa, J. L., Garcia, J. R. and Ferrus, A. (1989). Genetic analysis of muscle development in *Drosophila melanogaster*. *Dev. Biol.* **131**, 439-454.

Faure, G. (1910). Liquido conservatore per framenti di organi e per piccoli 7 organisismi inerti. *Ann. Botanici* **8**, 63-64.

Fawcett, J., Holness, C. L., Needham, L. A., Turley, H., Gatter, K. C., Mason, D. and Simmons, D. L. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. *Nature* **360**, 477-480.

Fischbach, K. R. and Technau, G. (1984). Cell degeneration in the developing optic lobes of the *sin oculis* and *small optic-lobes* mutants of *Drosophila melanogaster*. *Dev. Biol.* **104**, 219-239.

Fortini, M. E. and Rubin, G. M. (1990). Analysis of *cis*-acting requirements of the RH3 and RH4 genes reveals a bipartite organization to rhodopsin promoters in *Drosophila melanogaster*. *Genes Dev.* **4**, 444-463.

Francescini, N. (1975). Sampling of the visual environment by the compound eye of the fly: Fundamentals and applications. In: *Photoreceptor Optics* (eds. Synder, A. W. and Menzel, R.) pp. 98-125.

Fristrom, D,, Wilcox, M. and Fristrom, J. (1993) The distribution of PS integrins, laminin A and F-actin during key stages in *Drosophila* wing. *Development* **117**, 509-526.

Gergen, J. P. and Wieschaus, E. F. (1985). The localized requirements for a gene affecting segmentation of *Drosophila*: Analysis of larvae mosaic for *runt. Dev. Biol.* **109**, 321-335.

Hemler, M. (1990). VLA proteins in the integrin family: structure, functions, and their role in leukocytes. *Annu. Rev. Immunol.* **8**, 365-400.

Hiromi, Y. and Gehring, W. J. (1987). Regulation and function of the *Drosophila* segmentation gene *fushi tarazu. Cell* **50**, 963-974.

Horwitz, A., Duggan, K., Beckerle, M. C. and Burridge, K. (1986). Interaction of plasma membrane fibronectin receptor with talin, a transmembrane linkage. *Nature* **320**, 531-533.

Hynes, R. O. (1987). Integrins: a family of cell surface receptors. *Cell* **48**, 549-554.

Hynes, R. O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**, 11-27.

Hynes, R. O. and Lander, A. D. (1992). Contact and adhesive specificities in the associations, migrations and targeting of cells and exons. *Cell* **68**, 303-322.

Ingolia, T. D., Craig, E. A. and McCarthy, B. J. (1980). Sequence of three

copies of the gene for the major *Drosophila* heat shock induced protein and their flanking regions. *Cell* **21**, 669-679.

Johnson, D. A., Gautsch, J. W., Sportsman, J. R. and Elder, J. H. (1984). Improved technique utilizing non-fat milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal.Tech.* **1**, 3-8.

Lawrence, P. A. and Tomlinson, A. (1991). A marriage is consummated. *Nature* **352**, 193.

Leptin, M., Aebersold, R. and Wilcox, M. (1987). *Drosophila* position-specific antigens resemble the vertebrate fibronectin-receptor family. *EMBO J.* **6**, 1037-1043.

Leptin, M., Bogaert, T., Lehmann, R. and Wilcox, M. (1989). The function of PS integrins during *Drosophila* embryogenesis. *Cell* **56**, 401-408.

Lindsley, D. L. and Zimm, G. (1985). The genome of *Drosophila melanogaster*. Part I: Genes A-K. *Drosophila Inform. Serv.* **62**.

Lindsley, D. L. and Zimm, G. (1990). The genome of *Drosophila melanogaster*. Part IV: Genes L-Z, balancers, transposable elements. *Drosophila Inform. Serv.* **68**.

Lis, J. T., Simon, J. A. and Sutton, C. A. (1983). New heat shock puffs and β-galactosidase activity resulting from transformation of *Drosophila* with an *hsp70-lacZ* hybrid gene. *Cell* **35**, 403-410.

MacKrell, A. J., Blumberg, B., Haynes, S. R. and Fessler, J. H. (1988). The *lethal myospheroid* gene of *Drosophila* encodes a membrane protein homologous to vertebrate integrin β subunits. *Proc. Natl. Acad. Sci. USA* **85**, 2633-2637.

Mahowald, A P., Caulton, J. H. and Gehring, W. J. (1979). Ultrastructural studies of oocytes and embryos derived from female flies carrying the *grandchildless* mutation in *Drosophila subobscura*. *Dev. Biol.* **69**, 118-132.

Meyerowitz, E. M. and Kankel, D. R. (1978). A genetic analysis of visual system development in *Drosophila melanogaster*. *Dev. Biol.* **62**, 112-142.

Milner, M. J. and Muir, J. (1987). The cell biology of *Drosophila* wing metamorphosis in vitro. *Roux's Arch. Dev. Biol.* **195**, 63-73.

Mogensen, M. M. and Tucker, J. B. (1987). Evidence for microtubule nucleation at plasma membrane associated sites in *Drosophila*. *J. Cell Sci.* **88**, 95-107.

Mogensen, M. M. and Tucker, J. B. (1988). Intermicrotubular actin filaments in the transalar cytoskeletal arrays of *Drosophila*. *J. Cell Sci.* **91**, 431-438.

Mismer, D. and Rubin, G. M. (1987). Analysis of the promoter of the *ninaE* opsin gene in *Drosophila melanogaster*. *Genetics* **116**, 565-578.

Newman, S. M. and Wright, T. R. (1981). Histological and ultrastructural analysis of developmental defects produced by the mutation *lethal(1)myospheroid* in *Drosophila melanogaster*. *Dev. Biol.* **86**, 393-402.

Norton, P. A. and Hynes, R. O. (1990). *In vitro* splicing of fibronectin pre-mRNAs. *Nucleic Acids Res.* **18**, 4089-4097.

Otey, C. A., Pavaiko, F. M. and Burridge, K. (1990). An interaction between alpha-actinin and β_1 integrin subunit in vitro. *J. Cell Biol.* **111**, 721-729.

Pelham, H. R. B. (1982). A regulatory upstream promoter element in the *Drosophila* Hsp 70 heat shock gene. *Cell* **33**, 517-528.

Ready, D. F. Hanson, T. E. and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **55**, 217-240.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Rubin, G. M. (1989). Development of the *Drosophila* retina: Inductive events studied at single cell resolution. *Cell* **57**, 519-520.

Selleck, S. B., and Steller, H. (1991). The influence of retinal innervation on neurogenesis in the first optic ganglion of *Drosophila*. *Neuron* **6**, 83-99.

Shattil, S. J. and Brugge, J. S. (1991). Protein tyrosine phosphorylation and the adhesive functions of platelets. *Curr. Opin. Cell Biol.* **3**, 869-879.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341-348.

Tomlinson, A. (1988). Cellular interactions in the developing *Drosophila* eye. *Development* **104**, 183-193.

Tucker, J. B., Milner, J. J., Currie, D. A., Muir, J. W., Forrest, D. A. and Spencer, M. (1986). Centrosomal microtubule-organizing centres and a switch in the control of protofilament number for cell-associated microtubules during *Drosophila* wing morphogenesis. *Eur. J. Cell Biol.* **41**, 279-289.

van der Meer, J. M. (1977). Optical clean and permanent whole mount preparation for phase contrast microscopy of cuticular structures of insect larvae. *DrosophilaInf. Service* **52**, 160.

Waddington, C. H. (1941). The genetic control of wing development in *Drosophila*. *J. Genet.* **41**, 75-139.

Waddington, C. H. (1962). *New patterns in genetics and development.* New York: Columbia University Press.

Wieschaus, E., Nusslein-Volhard, C. and Jurgens, G. (1984). Mutations affecting the pattern of the larval cuticle in *D. melanogaster*. III. Zygotic loci on the X-chromosome. *Roux's Arch. Dev. Biol.* **193**, 296-307.

Wieschaus, E. and Noell, E. (1986). Specificity of embryonic lethal mutations in *Drosophila* analyzed in germline clones. *Roux's Arch. Dev. Biol.* **195**, 63-73.

Wilcox, M., DiAntonia, A. and Leptin, M. (1989). The function of PS integrins in *Drosophila* wing morphogenesis. *Development* **107**, 891-898.

Wright, T. R. F. (1960). The phenogenetics of the embryonic mutant, *lethal myospheroid*, in *Drosophila melanogaster*. *J. Exp. Zool.* **143**, 77-79.

Zusman, S., Patel-King, R. S., ffrench-Constant, C. and Hynes, R. O. (1990). Requirements for integrins during *Drosophila* development. *Development* **108**, 391-402.

(Accepted 5 April 1993)