

Functions of the cytoplasmic domain of the β_{PS} integrin subunit during *Drosophila* development

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SUMMARY

Integrins constitute a family of membrane-spanning, heterodimeric proteins that mediate adhesive interactions between cells and surrounding extracellular matrices (or other cells) and participate in signal transduction. We are interested in assessing integrin functions in the context of developing *Drosophila melanogaster*. This report, using mutants of the β_{PS} subunit encoded by the *myspheroid* (*mys*) locus, analyzes the relationships between integrin protein structure and developmental functions in an intact organism.

As a first step in this analysis, we demonstrated the ability of a fragment of wild-type *mys* genomic DNA, introduced into the germ line in a *P*-element vector *P[mys⁺]*, to rescue phenotypes attributed to lack of (or defects in) the endogenous β_{PS} during several discrete morphogenetic events. We then produced in vitro a series of modifications of the wild-type *P[mys⁺]* transposon, which encode β_{PS} derivatives with mutations within the small and highly conserved cytoplasmic domain. In vivo analysis of these mutant transposons led to the following conclusions.

(1) The cytoplasmic tail of β_{PS} is essential for all developmental functions of the protein that were assayed.

(2) An intron at a conserved position in the DNA sequence encoding the cytoplasmic tail is thought to participate in important alternative splicing events in vertebrate β integrin subunit genes, but is not required for the developmental functions of the *mys* gene assayed here.

(3) Phosphorylation on two conserved tyrosines found in the C terminus of the β_{PS} cytoplasmic tail is not necessary for the tested developmental functions.

(4) Four highly conserved amino acid residues found in the N-terminal portion of the cytoplasmic tail are important but not critical for the developmental functions of β_{PS} ; furthermore, the efficiencies with which these mutant proteins function during different morphogenetic processes vary greatly, strongly suggesting that the cytoplasmic interactions involving PS integrins are developmentally modulated.

Key words: *Drosophila*, integrin, cytoplasmic domain, cell adhesion, *myspheroid*, PS integrin

INTRODUCTION

Multicellular organisms rely on a sophisticated set of adhesive interactions to maintain their integrity. During development, these interactions must be both strong and plastic enough to allow extensive cellular rearrangements to take place. Several adhesive components (reviewed in Hynes and Lander, 1992) are shared between vertebrates and invertebrates, among them the large family of integrin proteins (Albelda and Buck, 1990; Hemler, 1990; Hynes, 1992). These integral membrane proteins are found on most cell types as α - β heterodimers with large extracellular and small cytoplasmic domains. Extracellular ligands of integrins include matrix components, such as

fibronectins, laminins and collagens, and members of the immunoglobulin superfamily of cell surface proteins such as VCAM-1, ICAM-1 and ICAM-2 (Hynes, 1992). The known intracellular ligands include the cytoskeletal proteins talin (Horwitz et al., 1986) and α -actinin (Otey et al., 1990).

Integrins have been shown to be coupled to a variety of signal transduction pathways (Shattil and Brugge, 1991; Hynes, 1992; Juliano and Haskill, 1993). Integrins concentrate at sites of cell-extracellular matrix adhesion known as focal contacts, along with molecules with which they are known to interact, including extracellular ligands, cytoskeletal proteins and protein kinases (Burrige et al., 1988). Focal contacts are thus both points of contact and sites for interactions between

the external and internal environments of the cell, in which integrins play a central linking role.

In an effort to understand the structure-function relationships of integrins, several groups have initiated analyses using cell culture assays to test physiological effects of specific mutations. Particular attention has been given to the small and highly conserved cytoplasmic domain of the integrin β subunits (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992). However, integrins are thought to play crucial roles not only in cellular events such as oncogenic transformation, hemostasis, inflammation and immune response (see reviews above), but also in numerous morphogenetic processes in development, including gastrulation, neural crest migration and neuronal pathfinding. Therefore, studies of integrin function in the context of the whole organism are desirable, and are best pursued in genetically favorable species.

In *Drosophila melanogaster*, previous work by MacKrell et al. (1988) and Leptin et al. (1989) has identified the X-linked *myospheroid* (*mys*) locus as a gene encoding a β integrin subunit. This gene is expressed in many cell types throughout development (Leptin et al., 1989; Zusman et al., 1990). In the developing embryo, its protein product, the β_{PS} integrin subunit, forms heterodimers with two α subunits: α_{PS1} , found in the epidermis, fat body and gut (Leptin et al., 1989), and α_{PS2} , expressed primarily in cells of mesodermal origin (Bogaert et al., 1987). β_{PS} is also expressed in later life, and indeed was first identified in imaginal discs (Brower et al., 1985). Examination of the effects of mutants, including the embryonic recessive lethal allele *mys^{XG43}* and the adult viable allele *mys^{nj42}*, revealed a requirement for β_{PS} during several embryonic and postembryonic morphogenetic events (Wright, 1960; Leptin et al., 1989; Brower and Jaffe, 1989; Wilcox et al., 1989; Zusman et al., 1990, 1993; Brabant and Brower, 1993).

We have begun to explore the structure-function relationships of *Drosophila* integrins by focusing on the *in vivo* functions of the β_{PS} cytoplasmic domain during development. This experimental system offers the attractive prospect of addressing the role of the protein and its cytoskeletal associations, and their regulation and possible involvement in signal transduction in the context of an intact developing organism. We first defined the boundaries of a functional β_{PS} gene by *P*-element-mediated transformation and documented the phenotypes that this DNA fragment can rescue. We then used these phenotypic assays to analyse a set of mutants dissecting the functions of the cytoplasmic domain of β_{PS} in developing *Drosophila*. Our results strongly suggest that *Drosophila* PS integrins engage in cytoplasmic interactions very similar to those of the vertebrate β_1 integrins and, furthermore, that some aspects of these interactions are modulated during development.

MATERIALS AND METHODS

Fly strains

Two mutant alleles of *myospheroid* were used: *mys^{XG43}* (Wieschaus et al., 1984) and *mys^{nj42}* (Costello and Thomas, 1981). Chromosomes bearing *mys^{XG43}*, together with the marker mutations *white* and *shavenbaby* (Wieschaus et al., 1984) (*w svb^{YP17} mys^{XG43}*), or with the marker mutations *yellow* and *chocolate* (*y cho mys^{XG43}*) were constructed and kept balanced over the *FM7* chromosome. Balancer chromosomes *CyO* and *TM3, Sb ry* were used to map and maintain trans-

formant lines. See Lindsley and Zimm (1985, 1990) for detailed marker descriptions.

Isolation and analysis of cDNA and genomic clones

cDNA libraries were derived from a *dp cn bw* strain of *Drosophila melanogaster* (Brown and Kafatos, 1988), and screened with a ³²P-labeled *EcoRI* fragment of the genomic clone B1 containing part of the *mys* locus (Digan et al., 1986). 23 embryonic and 13 disk-positive cDNA clones were analyzed by Southern hybridization or sequencing. 9 embryonic and 6 disk cDNAs were found to contain the complete open reading frame reported by MacKrell et al. (1988); they were subjected to an *in vitro* translation assay (Brown and Kafatos, 1988) to determine the size of the encoded protein. All 15 isolates yielded a $110 \times 10^3 M_r$ glycosylated product precipitable with monoclonal antibodies against β_{PS} (data not shown).

A genomic library (gift from Ron Blackman, University of Illinois, Urbana, Illinois) was constructed in the EMBL3 vector, using DNA of the same *Drosophila* strain used to prepare cDNA libraries. Five overlapping genomic clones (*gA3*, *gA5*, *gA6*, *gA10* and *gA11mys*) were isolated by screening this library with the probe described above. The 5' and 3' termini of the full-length cDNA inserts were located within the genomic DNA by restriction mapping and sequencing. The intron-exon structure of the gene was determined by sequencing of complete cDNA and genomic clones and will be reported in detail elsewhere (Yee et al., unpublished data). The structure of the gene is shown diagrammatically in Fig. 3A.

Construction of *P[mys⁺]*

The genomic clone *gA10mys* contains a novel *SalI* site introduced during library construction, located 200 bp 3' to the second *SmaI* site in the 3' untranslated portion of *mys*. A 10.5 kb fragment of *gA10mys* contained between an *XhoI* site 1.5 kb upstream of the putative transcription start site and the *SalI* site was subcloned between the unique *KpnI* and *SalI* sites of plasmid HZ50PL (Hiromi and Gehring 1987), producing the plasmid *P[mys⁺]* (see Fig. 3A).

Construction of mutant derivatives of *P[mys⁺]*

The *BglII-SpeI* fragment of *P[mys⁺]*, which encodes the putative cytoplasmic domain of β_{PS} , was subcloned into the M13mp19 vector. Mutants were generated in the resulting plasmid using the Muta-Gene *in vitro* mutagenesis protocol (Bio-Rad). Plasmids containing the desired mutations were identified by sequencing. The mutant *BglII-SpeI* fragments were used to replace the equivalent fragment in *P[mys⁺]*, yielding *P[mys^{t1}]*, *P[mys^{t2}]*, *P[mys^{rYYF}]*, *P[mys^{rYYA}]*, *P[mys^{rDEA}]* and *P[mys^{rFFA}]* (see Figs 3B, 6A). *P[mys^{dim}]*, which lacks the 75 nt intron found in the cytoplasmic tail-encoding portion of *mys*, was constructed by replacing the *BglII-SpeI* fragment of *P[mys⁺]* with the equivalent fragment derived from a cDNA clone (Figs 3B, 6B).

Germ-line transformation

Stable integration of *P[mys⁺]* and its derivatives into chromosomal DNA of the *cn; ry⁵⁰⁶* strain of *Drosophila* was achieved according to published procedures (Spradling and Rubin 1982; Rubin and Spradling 1982).

Genetic crosses employed in assays for embryonic functions of β_{PS}

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

$$\begin{array}{c} \frac{w \text{ svb } \textit{mys}^{XG43}}{FM7} \times \frac{+}{Y} ; \frac{P[\textit{mys}^*]}{P[\textit{mys}^*]} \\ \downarrow \\ \frac{w \text{ svb } \textit{mys}^{XG43}}{Y} ; \frac{P[\textit{mys}^*]}{+} \end{array}$$

where $P[mys^*]$ is either $P[mys^+]$, $P[mys^{I1}]$, $P[mys^{I2}]$, $P[mys^{YYF}]$, $P[mys^{rDEA}]$, $P[mys^{rFFA}]$ or $P[mys^{din}]$ carried on the second or on the third chromosome.

Cuticular preparations of embryos aged 40 to 60 hours were made and analyzed according to published protocols (Wieschaus and Nüsslein-Volhard, 1986).

Genetic crosses employed in assays for postembryonic functions of β_{PS}

Adult mys^{nj42} flies used in assays for held-out wing posture were produced in the cross below.

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

$$\downarrow$$

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

Eye and wing clones were generated as described (Zusman et al., 1990) in flies derived in the following way:

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

$$\downarrow$$

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

Genetic crosses used to produce embryos containing two copies of $P[mys^*]$

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

$$\downarrow$$

$$F1: \frac{w\ svb\ mys^{XG43}}{+}; \frac{P[mys^*]}{+} \times \frac{FM7}{Y}, \frac{P[mys^*]}{+}$$

The $w\ svb\ mys^{XG43}/FM7$ F₂ progeny were crossed to their $FM7/Y; P[mys^*]/+$ fathers in single pair matings. The F₃ progeny of several of these pair matings were tested for the presence of svb and mys^{XG43} to ensure that the alleles had not been lost through recombination. They were then combined to establish a stock producing $w\ svb\ mys^{XG43}/FM7$ embryos with zero, one or two copies of $P[mys^*]$. Approximately 25% of all *shavenbaby* embryos produced in this scheme are expected to carry two copies of the transposon.

Embryonic protein preparation and western blot analysis

Embryos of the appropriate genotypes, as determined by the cuticular marker *shavenbaby*, were individually selected under a dissecting microscope. Total lysates were prepared as previously described (Leptin et al., 1989), usually from batches of 15 embryos. SDS denaturing gel electrophoresis, electroblotting and immunological detection were performed according to standard protocols (Johnson et al., 1984), using anti- β_{PS} monoclonal antibodies 9A5 and 12A5 (a gift of the late Michael Wilcox, MRC, Cambridge, UK) and ¹²⁵I-labelled anti-rat Ig secondary antibody (Amersham Life Sciences). To control for equal loading of samples, blots were reprobbed with an anti- α -tubulin monoclonal antibody 4A1 (a gift of Margaret T. Fuller, Stanford University, USA) followed by ¹²⁵I-labelled anti-mouse Ig secondary antibody (Amersham Life Sciences). Quantitation was performed using a PhosphorImager Apparatus and its associated ImageQuant computer package (Molecular Dynamics).

RESULTS

The β_{PS} integrin subunit is required for several developmental functions

As a prelude to testing for rescue of function through transformation, four distinct mutant phenotypes attributable to lack of, or defects in, β_{PS} during development were documented. One phenotype was embryonic (Fig. 1), the other three were scored in the adult (Fig. 2 and Zusman et al., 1993).

Embryos homozygous or hemizygous for null alleles of *mys* are unable to complete or maintain dorsal closure normally or to form stable somatic muscle attachments (Wright, 1960); they die before hatching. One of these alleles is the recessive lethal, mys^{XG43} , which lacks detectable levels of β_{PS} (Leptin et al., 1989). To allow convenient and unambiguous identification of hemizygous *mys* embryos, we constructed a strain with an X-chromosome carrying mys^{XG43} linked to *shavenbaby*

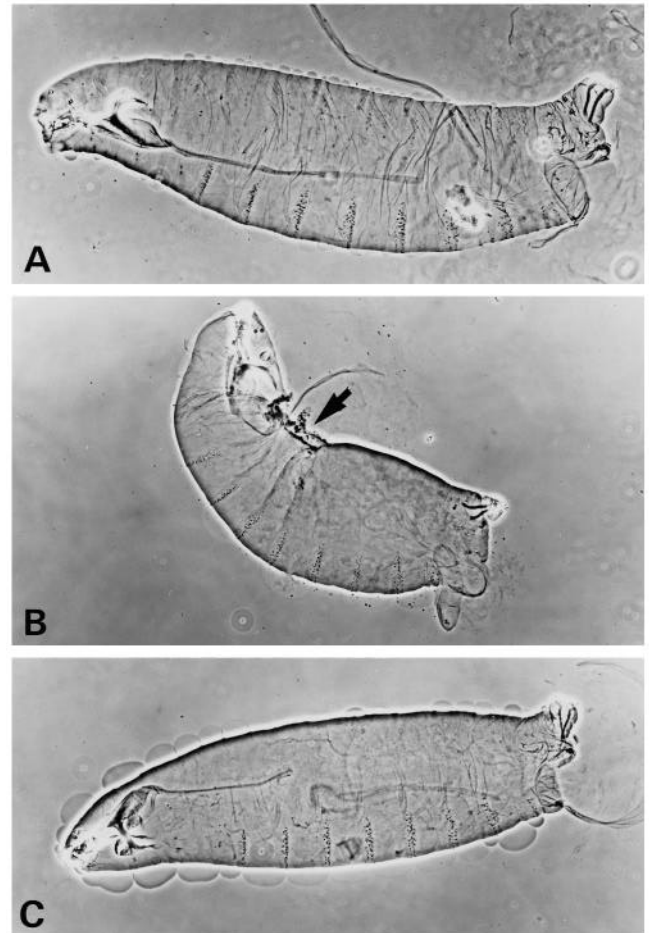


Fig. 1. Embryonic requirement for the β_{PS} integrin subunit. In all panels, the embryos are oriented with their anterior end on the left and ventral side at the bottom. (A) A cuticular preparation of a control $w\ svb^{yp17}/Y$ embryo showing the shortened ventral denticles characteristic of *shavenbaby* mutations. The cuticle is otherwise wild type. (B) A cuticular preparation of a $w\ svb^{yp17}\ mys^{XG43}/Y$ embryo. Note the large hole, marked by the arrow, in the dorsal portion of the cuticle. (C) A cuticular preparation of a $w\ svb^{yp17}\ mys^{XG43}/Y; P[mys^+].1/+$ embryo. Note the wild-type appearance of the dorsal cuticle demonstrating rescue by the transgene.

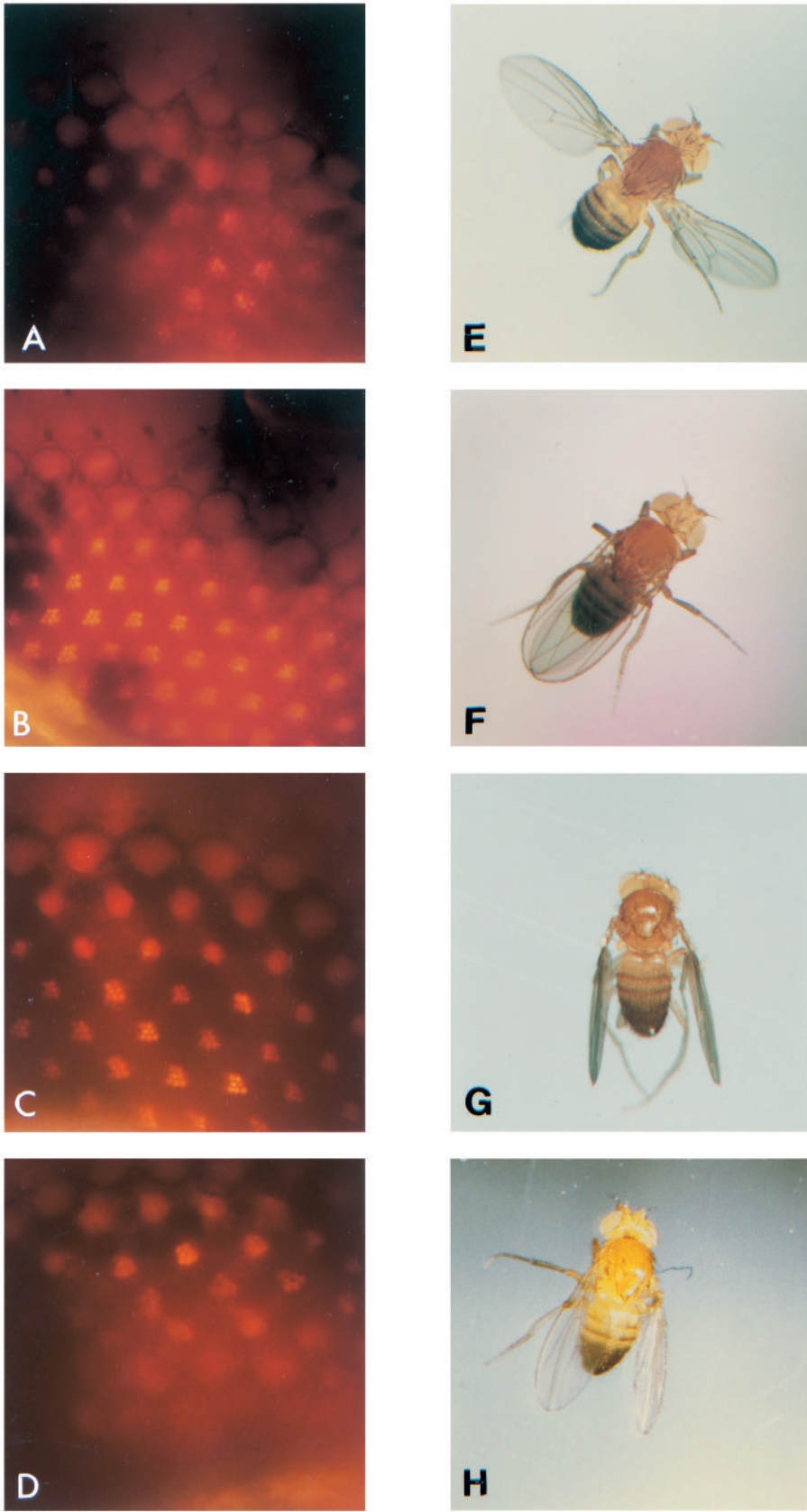


Fig. 2. Postembryonic requirement for the β_5 integrin subunit. (A-D) Eyes viewed under antidiromic illumination; plane of focus is at the level of rhabdomeres, which are evident as regularly spaced yellow dots in the wild type. Mitotic clones homozygous for *cho* are marked by red brick color. (A) An eye containing a *cho mys^{XG43}/cho mys^{XG43}* clone. Note that the rhabdomeres are not organized in the regular pattern. (B) An eye containing a *cho mys^{XG43}/cho mys^{XG43}; P[mys⁺]/+* clone. Note the rescue of ommatidia to wild-type rhabdomere organization. (C) An eye with a *cho mys^{XG43}/cho mys^{XG43}; P[mys^{rDEA}]/+* clone. Note its wild-type appearance. (D) An eye with a *cho mys^{XG43}/cho mys^{XG43}; P[mys^{rFFA}]/+* clone. Note disorganized rhabdomeres, similar to those seen in the absence of transposon. (E-H) Flies raised at 29°C and examined for wing posture. (E) A *w mys^{nj42f}/Y* fly holds his wings at 90° to the body axis. (F) A *w mys^{nj42f}/Y; P[mys⁺]/+* fly is wild type with respect to wing posture, demonstrating rescue by the transgene. (G) A *w mys^{nj42f}/Y; P[mys^{rDEA}]/+* fly holds his wings at a slight angle to the body axis, demonstrating partial rescue by the transgene. (H) A *w mys^{nj42f}/Y; P[mys^{rFFA}]/+* fly with ‘droopy’ wings; note similarity to the fly rescued with *P[mys^{rDEA}]*.

Table 1. Mutant *mys* phenotypes rescued by derivatives of *P[mys⁺]*

Transposon	Protein level ^a	Phenotype assayed			
		Dorsal hole in cuticle (<i>mys^{XG43}</i>)	Disorganized ommatidia (<i>mys^{XG43}</i>)	Wing blisters (<i>mys^{XG43}</i>) ^b	Heldout wing (<i>mys^{uj42}</i>)
<i>no transposon</i>	0%	100% (253)	100% (29)	15% (128)	97% (251)
<i>P[mys⁺]</i> lines #1	7.2-22%	2% (52)	22% (9)	2% (142)	1% (288)
2	7.9%	0% (157)	0% (17)	0% (345)	0% (153)
<i>P[mys^{t1}]</i> lines #2	21-23%	100% (101/2)*	100% (7)	9% (74)	100% (24)
3	12%	100% (67/2)*	100% (4)	14% (80)	100% (51)
4	2.6%	ND	ND	ND	94% (67)
<i>P[mys^{t2}]</i> lines #3	1.1%	100% (63)	100% (7)	12% (66)	100% (9)
4	1.6%	100% (30)	100% (2)	14% (14)	100% (13)
5	2.9%**	100% (11)	ND	ND	97% (30)
<i>P[mys^{dln}]</i> line #2	ND	0% (18)	0% (13)	0% (143)	3% (110)
<i>P[mys^{YYF}]</i> lines #1	6%	7% (30)	0% (8)	0% (148)	1% (129)
2	6.4-21%	0% (35)	0% (6)	0% (105)	20% (258)
5	12-22%	0% (32)	ND	ND	2% (106)
<i>P[mys^{rDEA}]</i> lines #1	16%	6% (18)	0% (10)	3% (175)	25% (201) ^c
2	9%	0% (46/2)*	0% (5)	0.4% (222)	33% (182) ^c
<i>P[mys^{rFFA}]</i> lines #1	11%	80% (44)	100% (10)	1.5% (266)	18% (96) ^c
3	6%	57% (63)	100% (9)	1% (418)	63% (210) ^c

Percentage of individuals or structures that are mutant for the given phenotype is shown in boldface; the total number of such individuals or structures analyzed is given in brackets. *mys^{XG43}* is a null allele assayed in hemizygous embryos for a dorsal cuticular defect and in homozygous cell clones for eye and wing defects. *mys^{uj42}* is a viable allele assayed for wing posture in hemizygous males at 29°C.

^aProtein expression levels for each transformant line are shown as % of the endogenous (see Fig. 4 for details).

^bIn this assay, all wings are scored for the presence of a blister. The low percentage of mutant structures in the absence of a transposon (15%) reflects the frequency of clone generation rather than incomplete penetrance of the mutant phenotype.

^cAmong flies scored as wild type for wing posture some exhibited an unusual 'droopy wing' phenotype (see Fig. 2G,H), which was not observed in flies rescued with the other mutant transposons.

*Due to homozygous lethality of these transformant lines, only half of the scored embryos contain a copy of a mutant transposon, as indicated by the /2 after the total number of scored embryos shown in brackets. For *P[mys^{t1}]*, all embryos exhibited the dorsal hole, and thus the incidence of mutant phenotype for the transposon-bearing embryos is inferred to be 100%. For *P[mys^{rDEA}]*, only 44% of the embryos had a dorsal hole; thus, the transposon-bearing half of the embryos must be phenotypically normal.

**Embryos that express twice this amount of protein also fail to complete dorsal closure (see text).

ND Not determined.

(*svb*), a recessive larval lethal mutation that causes drastic reduction in the size of ventral denticles without otherwise affecting the visible embryonic phenotype (Wieschaus et al., 1984; Fig. 1A). The effectiveness of this indirect marking system was confirmed: all *svb* (and therefore *mys^{XG43}*) embryos die with a characteristic hole in the dorsal cuticle (Fig. 1B; Table 1), whereas none of their *svb⁺* (and therefore *mys⁺*) siblings do (data not shown). In control crosses, *svb mys⁺* embryos die without a dorsal hole (Wieschaus et al., 1984).

During postembryonic development of imaginal disks, loss of β_{PS} in mitotic clones homozygous for *mys^{XG43}* does not result in cell lethality but causes morphological defects consistent with lack of proper attachment between cell layers (Brower and Jaffe, 1989; Zusman et al., 1990, 1993; Brabant and Brower, 1993). Generation of *mys^{XG43}* homozygous clones in the developing wing blade results in blisters, with the dorsal and ventral wing surfaces failing to adhere to each other. In this study, we did not use a linked marker to identify precisely the mutant clones in the wing; however, the presence of such a marker in the experiments of Brower and Jaffe (1989) and of Zusman et al. (1990), permits the conclusion that the low frequency of wings with blisters (15%; Table 1) reflects the frequency with which clones are generated, rather than incomplete penetrance of the wing phenotype. In the eye, the ommatidial arrays are disorganized within the homozygous *mys^{XG43}*

clones, which are identified by a linked mutation in the eye color gene, *chocolate* (Fig. 2A and Table 1).

The fourth phenotype scored was based on the adult viable allele, *mys^{uj42}*. Flies that are hemizygous or homozygous for this allele express an aberrant form of β_{PS} at levels comparable to wild-type expression (Wilcox et al., 1989). These flies are incapable of flight, probably as a consequence of defects in the major thoracic indirect flight muscles (de la Pompa et al., 1989). The severity of the mutant phenotype is exacerbated in flies reared at 29°C, which hold their wings at an abnormal angle to the body (Fig. 2E); this 'held-out' phenotype is almost fully penetrant (Table 1).

P-element transformation defines the boundaries of a functional *mysospheroid* gene

We extended the initial molecular characterization of the locus (Digan et al., 1986) by isolating full-length *mys* cDNA clones (see Materials and Methods for details) and mapping their 5' and 3' termini on a genomic DNA fragment, thus determining the minimum extent of the transcription unit to be 9 kb (Fig. 3A).

Germ-line transformation experiments were performed using the Carnegie 20-based *P*-element vector HZ50PL (Hiromi and Gehring, 1987) according to established methods. In preliminary experiments, we failed repeatedly to obtain

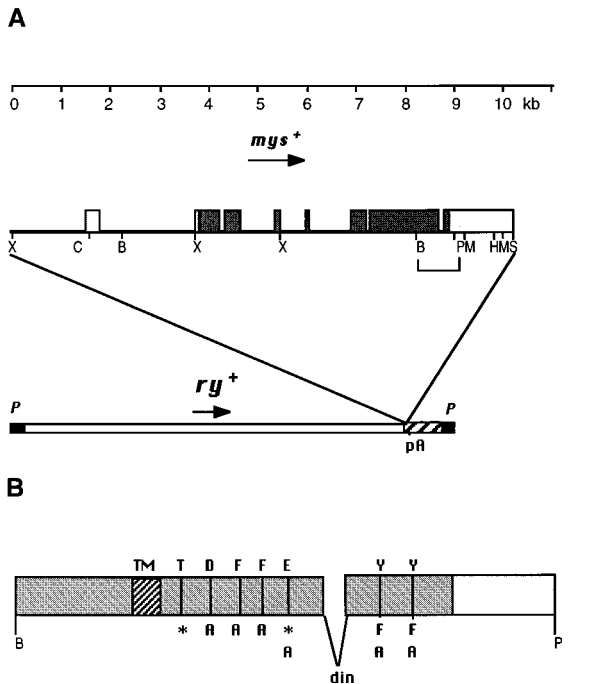


Fig. 3. The structure of *P[mys⁺]*, a *P*-element transposon containing a 10.5 kb genomic DNA fragment encompassing the *mys* gene. (A) The top line represents a scale in kilobases. The middle diagram shows the structure of the *mys* transcription unit (Yee et al., unpublished data), where boxes represent exons (light gray - untranslated, dark gray - coding sequences) and thin lines are introns or 1.5 kb of 5' flanking DNA. The following restriction sites are shown underneath: *Bgl*III (B), *Hind*III(H), *Sac*II(C), *Sall*(S), *Sma*I(M), *Spe*I(P) and *Xho*I(X). A bracket highlights the *Bgl*III-*Spe*I fragment (see part B below). The arrow shows the direction of transcription. This *mys* DNA was inserted in the transformation vector HZ50PL, replacing the *Kpn*I-*Sall* fragment that contains the *lacZ* gene. Only the portions of this construct that integrate into the chromosome are shown at the bottom. This vector includes a functional copy of the *Drosophila rosy* gene (*ry*⁺) as a visible selection marker (open box), the 3' untranslated region of the *Drosophila hsp70* gene (cross-hatched box) with its polyadenylation site (pA), and the *P*-element sequences required for transposition (black boxes). (B) A closer view of the *Bgl*III-*Spe*I restriction fragment (bracket in part A above), which was used as a shuttle for in vitro-generated mutants in the cytoplasmic tail. The region encoding the putative transmembrane domain (TM) is indicated by a cross-hatched box. Approximate positions at which mutations were introduced are shown by vertical lines. For amino acid replacement mutants, the wild-type residue is shown on top and its replacement on the bottom. The termination codons are marked with *. In the mutant *din*, a 75 nt fragment of DNA corresponding to an intron is deleted (see Materials and Methods and Fig. 6 for more detail).

transformants using a 16 kb *Drosophila* genomic fragment that encompassed the *mys* gene, suggesting the existence of a 'poison' sequence in the region (data not shown). However, we did obtain transformants with a smaller, 10.5 kb genomic fragment, which included all of the transcribed sequences and 1.5 kb of the 5' flanking DNA. In designing this construct, which we named *P[mys⁺]*, we deliberately minimized the flanking sequences and, as a result, the 3' end of the genomic fragment used falls just short of the natural polyadenylation site of the gene. The polyadenylation signal is provided by the

hsp70 sequences contained in the vector (see Materials and Methods and Fig. 3).

Adult flies carrying one or more copies of *P[mys⁺]* were identified by the eye color marker gene, *rosy*⁺(*ry*⁺), included in the transposon. Two independent, single insert germ-line integrants, *P[mys⁺].1* and *P[mys⁺].2*, were obtained with this wild-type construct, and their insertion sites were mapped to the third and second chromosomes, respectively (data not shown). Each of these inserts of *P[mys⁺]* was transferred into mutant *svb mys^{XG43}* embryos by genetic crosses in a way that excluded the possibility of recombination between these two latter loci (see Materials and Methods for details of the cross). Each insert was found capable of alleviating the mutant embryonic defects described in the previous section. In the vast majority of such embryos, a single copy of *P[mys⁺]* leads to complete rescue of the dorsal hole phenotype (Table 1; Fig. 1C) and of somatic muscle detachment as judged from observations of muscle contraction in living embryos (data not shown). The ability of the transposon to rescue in these assays is dependent upon its insertion site: *P[mys⁺].2* rescued all 157 tested embryos, while *P[mys⁺].1* failed to do so in 1 out of 52 examined embryos.

Similarly, *P[mys⁺]* was shown to provide the function of the wild-type gene during imaginal disk development (see Materials and Methods for crosses used in these studies, and Zusman et al., 1990, 1993 for details of the assays). In eye clones homozygous for *mys^{XG43}*, addition of a copy of *P[mys⁺].2* leads to complete rescue of the mutant phenotype while a copy of *P[mys⁺].1* rescues in 78% of the cases (Table 1, Fig. 2B). In the wing blade, introduction of a single copy of *P[mys⁺].2* eliminates the blisters, while addition of a copy of *P[mys⁺].1* reduces their occurrence significantly but not completely (Table 1). Introduction of a single copy of *P[mys⁺]* also alleviates the defects associated with *mys^{uj42}* at the non-permissive temperature of 29°C, namely, the abnormal wing posture and wing blistering (Fig. 2F; see Materials and Methods for description of the cross). As in all the previous assays, *P[mys⁺].2* rescues the mutant phenotypes more efficiently than does *P[mys⁺].1* (Table 1).

Thus *P[mys⁺]*, a transposon carrying 10.5 kb of genomic *mys* DNA, is capable of functionally replacing the endogenous *mys* gene during several stages of *Drosophila* development. However, at least in the available integrants, it is not as effective as the endogenous gene, as attested by the observation that transformants that lack endogenous β PS and carry one copy of *P[mys⁺]* die either as late embryos or as early first instar larvae (data not shown). These embryos appear to have normal cuticle and are capable of muscle contraction.

To examine why transformants fail to complete development, we analyzed by western blotting the β PS protein produced in both *P[mys⁺]* lines. Since this protein and its endogenous counterpart are expected to have the same electrophoretic mobility, only hemizygous *mys^{XG43}* embryos, which lack the endogenous protein, were used in this analysis. Total lysates prepared from such embryos by the squashing method described by Leptin et al. (1989) were subjected to western blotting with monoclonal antibodies against β PS. As an internal standard, the same blots were also probed with a tubulin antibody and the β PS signals were normalized against tubulin. Multiple analyses of samples from the same transformant line gave values within a three-fold range. This moderate

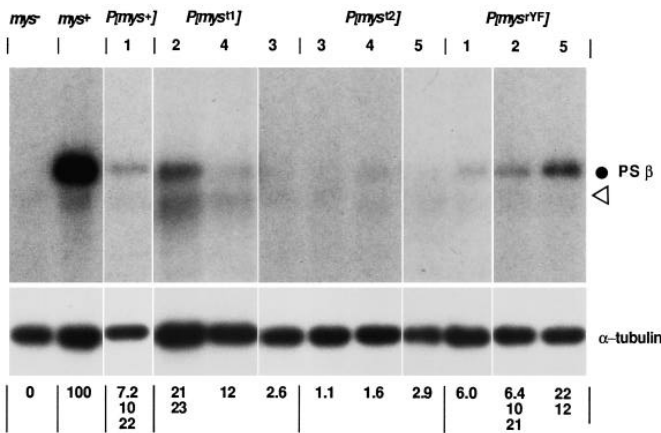


Fig. 4. Western blot analysis of β_{PS} protein in wild-type, mutant and transformant embryos. Protein extracts from hemizygous mys^{XG43} embryos carrying the indicated transposon were analyzed on two western blots, together with hemizygous positive (wild type, mys^+) and negative (non-transformed mys^{XG43} or mys^-) controls. The blots were probed with an antibody against β_{PS} (top panel) and then with an antibody against α -tubulin (lower panel). The position of β_{PS} is marked by a dot on the right, and that of the presumed β_{PS} precursor by an arrowhead. Relative levels of β_{PS} expressed by the different transposons, quantitated by a PhosphorImager and corrected for variations in loading by normalization against tubulin, are shown at the bottom as percentages of the endogenous expression level in the wild-type control. For each line, additional numbers represent estimates from independent experiments (not shown). The line $P[mys^+]$ shown is $P[mys^+].1$; a similar experiment, not shown here, indicated a value of 7.9% for β_{PS} expression in the $P[mys^+].2$ transformants. The following estimates were obtained for $P[mys^{DEA}]$ and $P[mys^{FFA}]$ transformants (data not shown): $P[mys^{DEA}].1$, 16%; $P[mys^{DEA}].2$, 9%; $P[mys^{FFA}].1$, 11%; $P[mys^{FFA}].3$, 6%.

variability might be due to differences in the embryonic stages analyzed, or to variable release of membrane versus cytoplasmic proteins in different preparations. These experiments, the results of which are shown in Fig. 4, revealed that both integrant lines of $P[mys^+]$ produce an appropriately sized

protein but at levels that are significantly reduced compared to wild type. We estimate the amount of protein produced by these lines to be 7-22% of the endogenous β_{PS} level.

The cytoplasmic domain of β_{PS} is necessary for proper function during development

Identification of a fragment of DNA containing a functional mys gene set the stage for analysis of the contributions of specific structural features of the protein to different aspects of its function. The putative cytoplasmic domain of the protein was chosen as the first target for analysis because the presumed cytoskeletal associations and the high degree of sequence conservation found within this region (Fig. 5) suggest an important role, and its small size (47 amino acids) makes it amenable to detailed dissection.

We began by asking whether this region of the protein was necessary for function. Two mutant derivatives of the original transposon, $P[mys^{t1}]$ and $P[mys^{t2}]$, were constructed in vitro (see Materials and Methods for details), each of which differs from the original transposon at one nucleotide position, resulting in a termination codon (Fig. 6). Consequently, the mutants encode truncated β_{PS} , which lacks virtually the entire putative cytoplasmic domain ($P[mys^{t1}]$), or the 30 most C-terminal amino acid residues of it ($P[mys^{t2}]$). Germ-line transformants containing mutant transposons were tested in rescue assays as described in the preceding section. The results of these tests, shown in Table 1, demonstrate that both truncations completely abolish phenotypic rescue in our assays.

To ascertain that the truncations exert their effect at the level of protein function rather than protein stability, we performed western blot analysis on three independent transformant lines of $P[mys^{t1}]$ and three lines of $P[mys^{t2}]$. Appropriately sized bands were detected for all of the lines tested (Fig. 4 and data not shown). Quantitative evaluation, summarized in Fig. 4 and Table 1, revealed that at least one of the $P[mys^{t1}]$ integrants, $P[mys^{t1}].2$, expresses β_{PS} at levels comparable to those of $P[mys^+].1$, thus ruling out the possibility that a simple reduction in quantity is responsible for the functional failure of this mutant protein. This conclusion could not be made for a single copy of $P[mys^{t2}]$, since all three lines tested were found to express at levels significantly lower than those of $P[mys^+].1$ (Fig. 4).

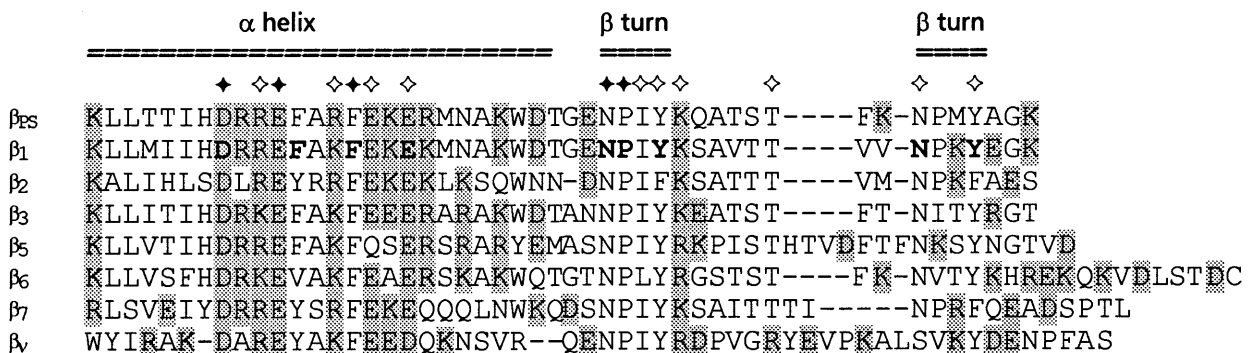


Fig. 5. Amino acid sequence alignment of the cytoplasmic domains of the known β integrin subunits. Charged residues are shaded to emphasize their clustering in the N-terminal half of the domain. Positions of absolutely conserved residues are marked with \blacklozenge . Positions containing only conservative substitutions, or those where identical residues are found in all but one of the β subunits are marked with \diamond . Amino acids that are important for the function of β_1 in fibroblasts are shown in bold (Reszka et al., 1992). The extent of the predicted secondary structure elements is shown at the top of the alignment.

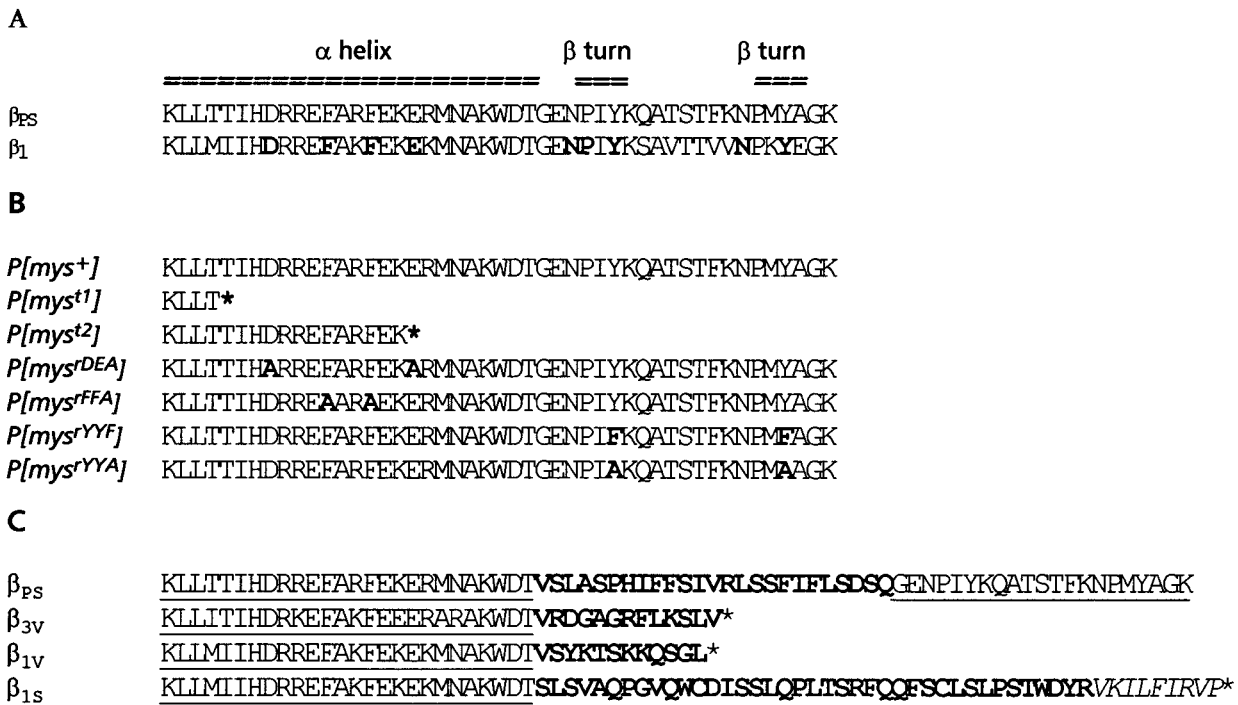


Fig. 6 (A) Cytoplasmic domain structure. The aligned sequences of β_{PS} and β₁ are shown, with the β₁ residues that are important for function in fibroblasts shown in bold (Reszka et al., 1992). (B) Mutations introduced in the cytoplasmic domain of β_{PS}. Shown here are the amino acid sequences of the cytoplasmic tail that are encoded by the wild-type transposon *P*[*mys*⁺] and its mutant derivatives *P*[*mys*^{t1}], *P*[*mys*^{t2}], *P*[*mys*^{YYF}], *P*[*mys*^{YYA}], *P*[*mys*^{DEA}] and *P*[*mys*^{FFA}] (see Materials and Methods for detailed description of the constructs). Bold letters correspond to point substitutions in each mutant derivative and asterisk indicates a premature termination codon. The transposons differ only within the area shown. (C) The potential splicing isoform of the cytoplasmic tail of β_{PS}. Shown here is the variant cytoplasmic tail of β_{PS}, which could be produced if the intron found in the corresponding region of the *mys* gene were retained in the mRNA. The underlined sequences are found in the canonical form; the intron-encoded variant sequence, inserted in frame, is shown in bold. The mutant transposon *P*[*mys*^{din}] lacks the cytoplasmic intron and, therefore, is unable to produce the variant cytoplasmic isoform. The alternative splicing isoforms of the vertebrate β integrins are shown below for comparison. In β_{3V} and β_{1V}, the retained intron sequence encodes a short novel peptide (bold) and a premature termination codon (*). In β_{1S} the novel C-terminal peptide is encoded partially by the retained intron sequence (bold) and partially by the exon that encodes the C-terminal peptide of the canonical isoform, but is translated in a different reading frame (italics).

However, by doubling the number of genomic copies of *P*[*mys*^{t2}].5 in *mys*^{XG43} hemizygous embryos, we were able to produce embryos that expressed mutant β_{PS} at levels comparable to transformants bearing other rescuing transposons. Embryos with two copies of the *P*[*mys*^{t2}] transposon, not readily distinguishable from those carrying zero or one copies, constitute a quarter of all *mys*^{XG43} hemizygous embryos produced in a cross that is outlined in the Materials and Methods. 215 *mys*^{XG43} hemizygous progeny of this cross were examined, and all 215 were found to contain the characteristic lesion in the dorsal cuticle. Since approximately 54 of these embryos were expected to express the product of *P*[*mys*^{t2}] at levels comparable to those of *P*[*mys*⁺] (and *P*[*mys*^{YYF}], see below), the functional failure of the truncated β_{PS} encoded by *P*[*mys*^{t2}] is not due simply to insufficient amounts of the protein.

A conserved intron found in the portion of the *mys* gene encoding the cytoplasmic tail is dispensable for the functions of *mys* during development

In the genes encoding the vertebrate integrins β₁ and β₃, the cytoplasmic tail is interrupted by an intron (Altruda et al., 1990; van Kuppevelt et al., 1989). These introns appear to undergo alternative splicing which can lead to their partial or complete

preservation in the mature transcript. The proteins encoded by these alternative transcripts differ from the canonical forms shown in Fig. 5 in that their C-terminal portions are replaced with peptides of unrelated sequences. Although two of the three predicted alternative splicing isoforms have been detected at the protein level (Languino and Ruoslahti, 1992; Balzac et al., 1993), they are found at very low frequencies and their biological significance has not been determined.

Interestingly, an intron is found at exactly the same position in the cytoplasmic domain-encoding portion of *mys* (Y. G., R. Patel-King, R. O. H, unpublished observation). This 75 bp-long intron contains an open reading frame which, if the intron were not excised, would lead to an in-frame insertion of 25 amino acids in the middle of the cytoplasmic domain. This peptide would bear no similarity to the proposed alternative cytoplasmic peptides of β₁ and β₃ (Fig. 6). It would be important to distinguish between the intriguing and biologically significant possibility that any potential cytoplasmic domain isoforms result from regulated splicing, and the less exciting possibility that they are due to faulty RNA processing. With this goal in mind, we constructed a mutant derivative of *P*[*mys*⁺], named *P*[*mys*^{din}], which encodes wild-type β_{PS} protein but lacks this intron, and thus lacks the ability to

produce the alternative cytoplasmic tail isoform shown in Fig. 6. This construct is indistinguishable from $P[mys^+]$ in its ability to function during embryogenesis and in support of adult thoracic muscle development and wing blade morphogenesis (Table 1). Therefore, the isoform potentially generated by the unspliced mRNA is not required for the functions tested.

Tyrosine phosphorylation is not essential for the functions of β_{PS} during development

Two tyrosines in the cytoplasmic tail of the vertebrate β_1 integrin are conserved in β_{PS} and in many other integrin β subunits. This conservation, together with the observation that β_1 integrin is phosphorylated on tyrosine in certain transformed cell lines (Hirst et al., 1986; Tapley et al., 1989), suggests that cytoplasmic tyrosine phosphorylation may be a mechanism for regulating integrin function. This model, which has attracted considerable attention, was tested by replacing the codons for both of the potentially phosphorylatable cytoplasmic tyrosines in $P[mys^+]$ with phenylalanine codons (Fig. 6; see Materials and Methods for details of construction). These replacements are expected to prevent phosphorylation while preserving the overall physical structure of the protein. Three independent lines stably transformed with this construct, referred to as $P[mys^{YYF}]$, were obtained. Their protein product was appropriately sized and expressed at levels comparable to those of the $P[mys^+]$ product (Fig. 4). When these lines were tested in our standard rescue assays, they were found to be phenotypically indistinguishable from the wild-type $P[mys^+]$ lines (Table 1). Therefore, phosphorylation of these conserved tyrosine residues does not appear to be necessary for correct function of β_{PS} in the processes assayed here.

Another potential role for these tyrosine-containing sequences is suggested by their homology to the vertebrate lysosomal targeting motifs, which have been proposed to be involved in controlling rates of turnover of cell surface proteins (NPXY; Chen et al., 1990; Peters et al., 1990). Tyrosine and phenylalanine are often functionally interchangeable in these motifs, while substitution with alanine abolishes the lysosomal targeting function (Bansal and Gierasch, 1991). We constructed a mutant derivative of $P[mys^+]$, $P[mys^{YYA}]$, which encodes a protein with both tyrosines in the cytoplasmic tail replaced with alanines. Surprisingly, attempts to obtain germline transformants of this construct in a series of injections with two independent isolates of the plasmid were unsuccessful. In comparison, between five and ten independent integrants were generated upon injecting a similar number of embryos with any of the other $P[mys^*]$ constructs. Although the injected embryos did not show appreciable increase in lethality, upon reaching adulthood they failed to express at the normal strong level the marker ry^+ gene that is contained in $P[mys^{YYA}]$ and they did not yield any ry^+ progeny. This result might be explained by postulating dominant toxicity of the mutant protein encoded by $P[mys^{YYA}]$ which acts to prevent cells that express it from contributing to the germ line (see Discussion).

Mutations of conserved amino acids in the highly charged portion of the β_{PS} cytoplasmic domain reveal developmental modulation of PS integrin functions

The N-terminal half of the cytoplasmic tail of β integrins is especially highly conserved; it is highly charged and is

predicted to form an α -helix. Reszka et al. (1992) reported that mutations at four residues in this portion of the β_1 integrin partially impaired the ability of the protein to associate with the cytoskeleton in cultured fibroblasts, while mutations at the other positions had no effect. We assayed the contribution of these four residues to the developmental functions of β_{PS} by constructing two mutagenized derivatives of $P[mys^+]$: $P[mys^{rDEA}]$, in which codons for asparagine and glutamine were replaced with alanine codons, and $P[mys^{rFFA}]$, in which the codons for two phenylalanines were changed to encode alanines (Fig. 6A). The mutagenized constructs were introduced into the germ line of *cn;ry* flies, and two independent autosomal insertion lines were established for each. Levels of protein expressed by each of these lines were quantitatively evaluated, and found to be comparable to the levels produced by $P[mys^+]$ (Table 1 and legend to Fig. 4), with levels ranging between 6 and 16% of endogenous *mys* expression.

The transformants were introduced into *mys*⁻ embryos by genetic crosses in order to test their ability to replace the function of the endogenous *mys* gene. As shown in Table 1, these two transposons are distinct from the previously tested mutant constructs in that they rescue the various tested mutant phenotypes with different efficiencies. In the embryo, both alleviate the dorsal closure defect associated with lack of β_{PS} ; however, while the degree of rescue effected by $P[mys^{rDEA}]$ is equal to that of $P[mys^+]$, $P[mys^{rFFA}]$ is only capable of partial rescue. This functional difference cannot be attributed to a quantitative advantage of $P[mys^{rDEA}]$ over $P[mys^{rFFA}]$, since $P[mys^{rDEA}].2$ is expressed at a similar, or slightly lower, level than $P[mys^{rFFA}].1$ (9% and 11% of wild type, respectively; Table 1). Both mutant transposons appear also to rescue the *myospheroid* muscle-attachment phenotype, since embryos rescued with either $P[mys^{rDEA}]$ or $P[mys^{rFFA}]$ can occasionally be seen moving within their vitelline membranes (data not shown); the relative efficiencies of the mutants in this respect were not evaluated.

In the developing eye disk, $P[mys^{rDEA}]$ is, again, fully functional (Fig. 2C) while $P[mys^{rFFA}]$ is non-functional (Fig. 2D) (Table 1); this is in contrast with the partial rescue observed with the latter in the embryonic assay. In the developing wing disk, however, both $P[mys^{rDEA}]$ and $P[mys^{rFFA}]$ are indistinguishable from the wild-type construct in their ability to replace the function of the endogenous β_{PS} and prevent blister formation (Table 1). With respect to thoracic muscle development, assayed as wing posture, $P[mys^{rDEA}]$ and $P[mys^{rFFA}]$ were roughly equivalent, and both effected significant but incomplete rescue of the held-out wing phenotype. This incomplete rescue was manifested in two ways. First, a significant proportion of the flies carrying either $P[mys^{rDEA}]$ or $P[mys^{rFFA}]$ exhibited the classical held-out phenotype observed in the absence of rescue (Table 1); second, some of the flies classified as wild type for wing posture in Table 1 frequently displayed an abnormal 'droopy' wing phenotype shown in Fig. 2G,H.

Thus, the mutant versions of β_{PS} encoded by $P[mys^{rDEA}]$ and $P[mys^{rFFA}]$ are clearly distinct from the other mutant β_{PS} derivatives in that their functional impairment is manifested in some, but not all, of the morphogenetic processes assayed here. Furthermore, the two mutant proteins are functionally distinct from each other, since their rescue efficiencies for two of the

four assayed mutant phenotypes (dorsal hole and disorganized ommatidia) differ significantly.

DISCUSSION

Functional characterization of *myspheroid*, the gene encoding β_{PS} integrin subunit

We have shown that a 10.5 kb fragment of genomic DNA from the *myspheroid* locus of *Drosophila*, when introduced at various sites in the fly genome via *P*-element-mediated germline transformation, contains sufficient sequence information to permit functional protein to be expressed during several postembryonic developmental stages. This is demonstrated by the ability of the transposon *P[mys⁺]* to rescue several mutant phenotypes, which are evidently caused by the absence of, or a defect in, the endogenous protein during imaginal disk morphogenesis.

The *P[mys⁺]* transposon is also able to rescue visible embryonic defects attributable to lack of the endogenous protein; however, its failure to support continued development of these apparently normal embryos indicates that one or more important regulatory elements of the gene reside outside the bounds of the genomic DNA included in *P[mys⁺]*. We cannot exclude the possibility that the element in question is responsible for β_{PS} production in specific cells, where it is essential for completion of embryogenesis. An alternative hypothesis is that this element is involved in the quantitative regulation of *mys* expression, since *P[mys⁺]*-encoded β_{PS} is found to be expressed at considerably lower levels than is the endogenous protein. So far we have been unable to construct a more efficiently expressed *mys⁺* transgene because of the presence of non-transformable DNA sequences just outside the insert shown in Fig. 3A. Doubling the copy number of the *P[mys⁺].2* insert (i.e. testing homozygotes rather than heterozygotes) did not result in survival (Zusman et al., 1993).

Structure-function analysis

The work presented here represents the first mutational analysis of integrin structure/function relationships in an intact, developing organism. Our results are summarized in Fig. 7, where the contrast between transformants bearing a wild-type *mys⁺* transposon or none at all provides a clear margin of scoring β_{PS} mutants in four distinct assays. These results establish that the cytoplasmic domain of the *Drosophila* β_{PS} integrin subunit is necessary for many developmental functions of the protein, but that phosphorylation of the highly conserved cytoplasmic tyrosines, as well as the presence of a potentially translatable intron, are dispensable for these functions. We also find that

several conserved residues in the N-terminal portion of the cytoplasmic domain are functionally important, although not essential; and, moreover, that these residues make different contributions to the overall function of the protein at different stages of morphogenesis.

Thus far, mutagenesis studies in cultured fibroblasts (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992) produced a wealth of information about structure/function relationships in the cytoplasmic domain of β_1 integrin. However, these studies assayed only one aspect of integrin function, namely, the ability of the cytoplasmic domain to interact with its cytoplasmic ligand in an already existing, preassembled focal contact structure, in the presence of the wild-type, endogenous integrin. This situation is likely to impose requirements that are different from, and considerably less stringent than, those found in cells of a developing organism. In the latter, the protein in question might be the sole mediator of adhesion; in addition, such factors as the rate of turnover of the surface protein and its phosphorylated state could make a critical difference that might go unnoticed in the fibroblast assay.

In vertebrate fibroblast cultures, complete deletion of the β_1 cytoplasmic domain prevents its localization to focal contacts, presumably reflecting failure of cytoskeletal associations. A similar truncation of β_{PS} in *P[mys^{t1}]* ablates function during development (Fig. 7), even though at least one transformant shows levels of expression equivalent to *P[mys⁺]*. Such extreme truncations in β_1 also partially interfere with $\alpha\beta$

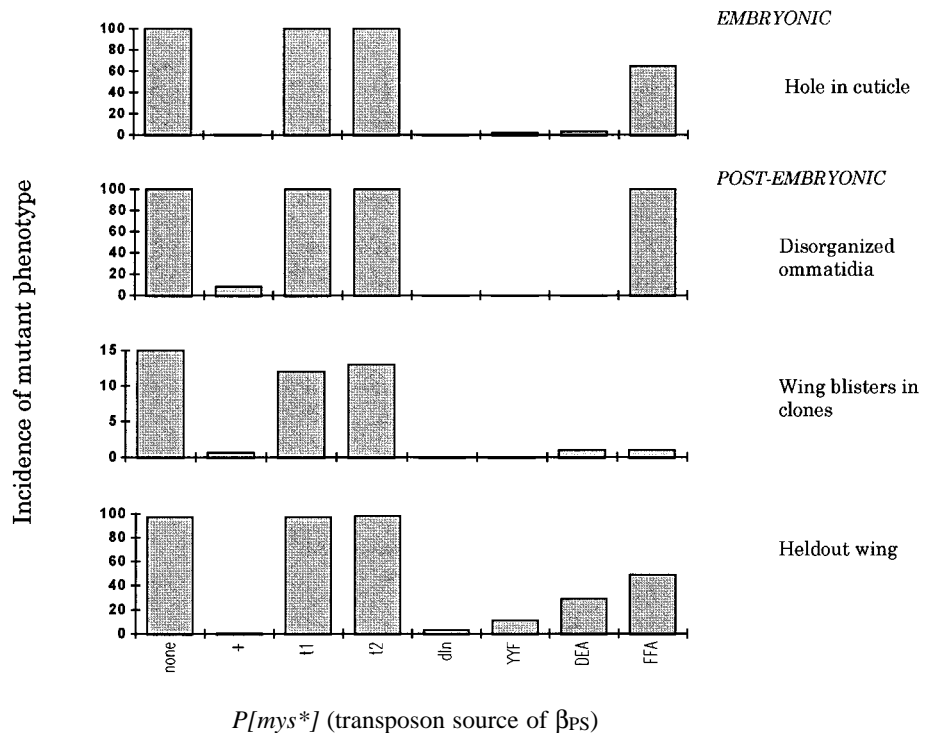


Fig. 7. Incidence of mutant phenotypes and their rescue by *P[mys⁺]* and its derivatives. The data for the phenotypic assays presented in Table 1 are summarized here in bar chart form to aid in comparing functional efficiencies of the mutant derivatives of *P[mys⁺]*. For each transposon, column height corresponds to the weighted average proportion of mutant individuals among all the independent transformant lines.

dimerization and potentially with export to the cell surface. Interestingly, the β_{PS} expressed from $P[mys^{l1}]$ shows an increased level of a lower molecular weight band on western blots (Fig. 4), which may represent accumulation of intracellular precursor as seen in tissue culture of vertebrate cells. Alternatively, this band may represent a degradation product. In the case of β_1 , less extreme truncations interfere less, or not at all, with dimerization but the truncated proteins still fail to localize properly at focal contacts (Hayashi et al., 1990; Marcantonio et al., 1990). In our experiments, the less extensive deletion encoded by $P[mys^{l2}]$ also eliminates function of β_{PS} (Fig. 7), even when the quantity of the mutant product is sufficient for rescue by wild-type or some replacement mutant proteins.

A mechanism by which integrin-mediated cytoskeletal associations may be regulated in vertebrates is suggested by reports of rare alternative splicing events in the cytoplasmic domain-encoding portions of the vertebrate β_1 and β_3 integrin genes. These splicing events lead to the retention of the entire intron or of a portion of it; as a result, the C-terminal half of the encoded cytoplasmic tails are replaced with peptides of unrelated sequences (van Kuppevelt et al., 1989; Altruda et al., 1990; Languino and Ruoslahti, 1992; Balzac et al., 1993). One such splicing isoform of β_1 appears to have the same dimerization and ligand-binding properties as the major form, but lacks the ability to form the proper associations with the cytoskeleton (Balzac et al., 1993). The biological roles of these splicing isoforms have yet to be determined. Significantly, an intron is also found at the equivalent position in the *mys* gene; its retention (undetected thus far) would lead to an in-frame insertion of 25 amino acids in the middle of the cytoplasmic domain (Fig. 6B). However, the observation that deletion of this intron does not diminish the ability of the $P[mys^{din}]$ transgene to replace the function of the endogenous *mys* gene (Fig. 7), together with a lack of sequence conservation within the alternative peptides (Fig. 6B), argues against a significant biological role for this alternative splicing event in the fly.

The C-terminal portion of β_{PS} contains two tyrosine residues within sequences reminiscent of target sites for tyrosine kinases, such as those found in the EGF receptor (Tamkun et al., 1986). The high degree of conservation of these sequences, in conjunction with the observations of increased tyrosine phosphorylation in vertebrate β_1 integrin in certain transformed cells (Hirst et al., 1986; Tapley et al., 1989), suggested phosphorylation as a mechanism of regulating β_{PS} function. We have tested this hypothesis by replacing both tyrosine residues in question with phenylalanines, which lack the hydroxyl group required for phosphorylation. Since this manipulation did not alter the phenotypic rescue (Fig. 7), phosphorylation on tyrosine, whether or not it takes place during normal development in *Drosophila*, does not make a significant contribution to the protein functions that are tested here.

A comprehensive mutagenesis screen conducted by Reszka et al. (1992) tested the effect of replacing single residues in the cytoplasmic tail of the avian β_1 on the integrin's ability to concentrate at focal adhesion sites, which is thought to be indicative of its ability to associate with the actin cytoskeleton. Surprisingly, mutations of 14 out of 18 residues constituting the highly conserved and highly charged N-terminal portion of the cytoplasmic domain left this function unaffected. Replacements made at the other four positions partially impaired the

function of β_1 integrin to localize to focal contact sites. Two of these are strongly conserved, negatively charged aspartic acid and glutamic acid residues, while the other two are phenylalanines, whose aromatic nature is also strongly conserved (Fig. 5). All four residues are located on the same face of the α -helix predicted for this portion of the protein, and are likely to come in direct contact with the cytoplasmic ligand, or ligands, of β_1 . Among the likely candidates for these ligands are α -actinin and talin which have been shown to bind directly to the cytoplasmic domain of β integrin subunits in vitro (Horwitz et al., 1986; Otey et al., 1990).

The present study assayed the effects of equivalent mutations in β_{PS} on the developmental functions of the protein. For efficiency, these point mutations were tested in pairwise combinations: aspartic and glutamic acids were replaced jointly by alanines in $P[mys^{rDEA}]$, and the two phenylalanines were similarly replaced to generate $P[mys^{rFFA}]$. The finding that both mutant proteins are partially compromised in their ability to function during development is consistent with the conclusions derived from the vertebrate study cited above. This consistency supports the notion that the cytoplasmic domains of β_{PS} and β_1 associate with very similar cytoplasmic ligands, and that the nature of these associations is highly conserved.

Even more significant is the observation that the mutant proteins encoded by $P[mys^{rDEA}]$ and $P[mys^{rFFA}]$ are distinct in their abilities to function during the different morphogenetic processes assayed here (Fig. 7). The product of $P[mys^{rDEA}]$ functions as well as the wild-type protein during embryogenesis and imaginal disk development, and seems partially compromised only during thoracic muscle morphogenesis. The product of $P[mys^{rFFA}]$ is non-functional during eye imaginal disk development, partially functional during embryogenesis and adult thoracic muscle development, and seems fully functional only during wing disk morphogenesis. These observations can be interpreted most readily as an indication that, in the course of development, β_{PS} associates with different cytoplasmic ligands, or that different morphogenetic processes impose different requirements on some aspects of integrin-mediated adhesion, such as its mechanical strength or plasticity.

The overall agreement between the results obtained thus far in the developing insect and in vertebrate cell culture suggests that a high degree of similarity exists between the components and the nature of the interactions in which integrins participate in these two very different situations. Furthermore, results reported here argue for significant modulation of these interactions during *Drosophila* development, the precise nature of which is yet to be discovered. In the future, the experimental approach used in this study may be used to assay the functions of the highly conserved residues in the cytoplasmic domain of β_{PS} which do not contribute to cytoskeletal binding in vitro. In the potentially more discriminating developmental assays, these residues may prove to be important for regulating integrin function. Cytoplasmic domains of α integrin subunits also make important, and as yet poorly understood, contributions to vertebrate integrin function (O'Toole et al., 1991; Chan et al., 1992). Analysis of the roles played by the cytoplasmic tails of α_{PS1} and α_{PS2} integrin subunits in the overall developmental function of PS integrins is another important future direction for mutant analysis that can be pursued efficiently in transgenic *Drosophila*.

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REFERENCES

- Albelda, S. M. and Buck, C. A. (1990). Integrins and other adhesion molecules. *FASEB J.* **4**, 2868-2880.
- Altruda, F., Cervella, P., Tarone, G., Botta, C., Balzac, F., Stefanuto, G., and Silengo, L. (1990) A human integrin β_1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. *Gene* **95**, 261-266.
- Balzac, F., Belkin, A. M., Koteliensky, V. E., Balabanov, Y. V., Altruda, F., Silengo, L., and Tarone, G. (1993). Expression and functional analysis of a cytoplasmic domain variant of the β_1 integrin subunit. *J. Cell Biol.* **121**, 171-178.
- Bansal, A. and Gierasch, L. M. (1991). The NPXY internalization signal of the LDL receptor adopts a reverse-turn conformation. *Cell* **67**, 1195-1201.
- Brabant, M. C. and Brower, D. L. (1993) PS2 integrin requirements in *Drosophila* embryo and wing morphogenesis. *Dev. Biol.* **157**, 49-59.
- Brower, D. L., Piovant, M. and Reger, L. A. (1985) 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.
- Brown, N. H. and Kafatos, F. C. (1988). Functional cDNA libraries from *Drosophila* embryos. *J. Mol. Biol.* **203**, 425-437.
- 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.
- Chan, B. M. C., Kassner, P. D., Schiro, J. A., Byers, H. R., Kupper, T. S., and Hemler, M. E. (1992) Distinct cellular functions mediated by different VLA integrin α subunit cytoplasmic domains. *Cell* **68**, 1051-1060.
- Chen, W. J., Goldstein, J. L., and Brown, M. S. (1990). NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of the low density lipoprotein receptor. *J. Biol. Chem.* **265**, 3116-3123.
- 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.
- 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.
- Digan, M. E., Haynes, S. R., Mozer, B. A., David, I. B., Forquignon, F. and Gans, M. (1986). Genetic and molecular analysis of *fs(1)h*, a maternal effect homeotic gene in *Drosophila*. *Dev. Biol.* **114**, 161-169.
- Hayashi, Y., Haimovish, B., Reszka, A., Boettiger, D. and Horwitz, A. (1990). Expression and function of chicken integrin β_1 subunit and its cytoplasmic domain mutants in mouse NIH 3T3 cells. *J. Cell Biol.* **110**, 175-184.
- Hemler, M. (1990). VLA proteins in the integrin family: structure, functions, and their role in leukocytes. *Annu. Rev. Immunol.* **8**, 365-400.
- Hiroimi, Y. and Gehring, W. J. (1987). Regulation and function of the *Drosophila* segmentation gene - *fushi tarazu*. *Cell* **50**, 963-974.
- Hirst, R., Horwitz, A., Buck, C., and Rohrschneider, L. (1986) Phosphorylation of the fibronectin receptor complex in cells transformed by oncogenes that encode tyrosine kinases. *Proc. Natn. Acad. Sci. USA* **83**, 6470-6475.
- 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. and Lander, A. D. (1992). Contact and adhesive specificities in the associations, migrations, and targeting of cells and exons. *Cell* **68**, 303-322.
- Hynes, R. O. (1992). Integrins: versatility, modulation and signaling in cell adhesion. *Cell* **69**, 11-25.
- 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.
- 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.
- Juliano, R. L. and Haskill, S. (1993) Signal transduction from the extracellular matrix. *J. Cell Biol.* **120**, 577-585.
- Lakonishok, M., Muschler, J. and Horwitz, A. F. (1992). The $\alpha_5\beta_1$ integrin associates with a dystrophin-containing lattice during muscle development. *Dev. Biol.* **152**, 209-220.
- Languino, L. R. and Ruoslahti, E. (1992) An alternative form of the integrin β_1 subunit with a variant cytoplasmic domain. *J. Biol. Chem.* **267**, 7116-7120.
- Leptin, M., Boegart, T., Lehman, R. and Wilcox, M. (1989). The function of PS integrins during *Drosophila* embryogenesis. *Cell* **56**, 401-408.
- Lindsley, D. L. and Zimm, G. (1990). The genome of *Drosophila melanogaster*. Part IV: Genes L-Z, balancers, transposable elements. *Drosophila Inform. Serv.* **68**.
- Lindsley, D. L. and Zimm, G. (1985). The genome of *Drosophila melanogaster*. Part I: Genes A-K. *Drosophila Inform. Serv.* **62**.
- 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. Natn. Acad. Sci. Acad.* **85**, 2633-2637.
- Marcantonio, E. E. and Hynes, R. O. (1988). Antibodies to the conserved cytoplasmic domain of the integrin β_1 subunit react with proteins in vertebrates, invertebrates and fungi. *J. Cell Biol.* **106**, 1765-1772.
- O'Toole, T. E., Mandelman, D., Forsyth, J., Shattil, S. J., Plow, E. F. and Ginsberg, M. (1991) Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of α_{IIb} . *Science* **254**, 845-847.
- Otey, C. A., Pavaiko, F. M. and Burridge, K. (1990). An interaction between alpha-actinin and the β_1 integrin subunit in vitro. *J. Cell Biol.* **111**, 721-729.
- Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A. and von Figura, K. (1990). Targeting of a lysosomal membrane protein: a tyrosine-containing endocytosis signal in the cytoplasmic tail of lysosomal acid phosphatase is necessary and sufficient for targeting to lysosomes. *EMBO J.* **9**, 3497-3506.
- Reszka, A. A., Hayashi, Y., and Horwitz, A. F. (1992) Identification of amino acid sequences in the integrin β_1 cytoplasmic domain implicated in cytoskeletal association. *J Cell Biol.* **117**, 1321-1330.
- Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.
- Shattil, S. J. and Brugge, J. S. (1991). Protein tyrosine phosphorylation and the adhesive functions of platelets. *Curr. Opin. Cell Biol.* **3**, 869-879.
- Solowska, J., Guan, J.-L., Marcantonio, E. E., Trevithick, J. E., Buck, J. E. and Hynes, R. O. (1989). Expression of normal and mutant avian integrin subunits in rodent cells. *J. Cell Biol.* **109**, 853-861.
- Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germ line chromosomes. *Science* **218**, 341-348.
- Tamkun, J. W., DeSimone, D. W., Fonda, D., Patel, R. S., Buck, C., Horwitz, A. F. and Hynes, R. O. (1986). Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell* **46**, 271-282.
- Tapley, P., Horwitz, A. F., Buck, C. A. and Burridge, K. (1989). Integrins isolated from Rous sarcoma virus-transformed chicken embryo fibroblasts. *Oncogene* **4**, 325-333.
- van Kuppevelt, T., Languino, L. R., Gailit, J. O., Suzuki, S. and Ruoslahti, E. (1989) An alternative cytoplasmic domain for the integrin β_3 subunit. *Proc. Natl. Acad. Sci. USA* **86**, 5415-5418.
- Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at embryos. In *Drosophila: A Practical Approach*. (ed. D. B. Roberts), pp. 199-226. IRL Press: Oxford.
- Wieschaus, E., Nüsslein-Volhard, C. and Jurgens, G. (1984). Mutations affecting the pattern of the larval cuticle in *D. melanogaster*. III. Zygotic loci on the X-chromosome. *Wilhelm Roux's Arch. Dev. Biol.* **193**, 296-307.
- Wilcox, M., DiAntonio, 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**, 77-99.
- Yee, G. H. and Hynes, R. O. (1993) A novel, tissue-specific integrin subunit, β_v , expressed in the midgut of *Drosophila melanogaster*. *Development* **118**, 845-858.
- Zusman, S., Grinblat, Y., Yee, G., Kafatos, F. C., and Hynes, R. O. (1993) Analyses of PS integrin functions during *Drosophila* development. *Development* **118**, 737-750.
- Zusman, S., Patel-King, R. S., French-Constant, C. and Hynes, R. O. (1990). Requirements for integrins during *Drosophila* development. *Development* **108**, 391-402.