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Null mutations in the α_{PS2} and β_{PS} integrin subunit genes have distinct phenotypes

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SUMMARY

The two *Drosophila* position-specific (PS) integrins are expressed on complementary sides of sites where different cell layers adhere to each other, such as the attachments of the embryonic muscles to the epidermis. While there is suggestive evidence that the PS integrin-mediated adhesion is via the extracellular matrix, it is also possible that it occurs through the direct interaction of the two integrins, $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$. To help distinguish between these possibilities a comparison between the phenotypes caused by the absence of the β_{PS} subunit and the absence of one of the PS α subunits, α_{PS2}, has been made. Two pieces of evidence are provided that prove that the α_{PS2} subunit is encoded by the locus *inflated* (*if*). Firstly, three new *if* alleles have been isolated, each of which is associated with a molecular lesion in the α_{PS2} gene, and each of which results in the complete loss of *if* activity. Secondly, a 39 kb fragment of genomic DNA that encompasses the α_{PS2} gene completely rescues *if* mutations when introduced into the germline by P-element-mediated transformation. A comparison of the null *inflated* phenotype with that of the locus that encodes the β_{PS} subunit, *myospheroid* (*mys*), reveals that while the β_{PS} subunit is required for the adhesion of the epidermis along the dorsal midline, the α_{PS2} subunit is not. In *if* mutant embryos, the muscles remain attached to the other cell layers significantly longer than in a *mys* mutant embryo. This shows that the $\alpha_{PS2}\beta_{PS}$ integrin only contributes part of the adhesive activity at the sites of PS integrin adhesion, and rules out a model where PS integrin function occurs solely by the direct interaction of the two PS integrins.

Key words: integrins, morphogenesis, cell-cell adhesion, extracellular matrix, integrin genes, embryogenesis, *Drosophila*

INTRODUCTION

The assembly of an organism from the cells within the developing embryo requires a variety of molecular interactions between cells. Cells within an epithelial cell layer are connected to the lateral cell surfaces of their neighbours by multiple cell surface proteins, including cell adhesion molecules and the components of specialised junctions. To form the organism, the different cell layers generated at gastrulation must also stick to each other. This type of adhesion is defective in *Drosophila* embryos mutant for the *myospheroid* (*mys*) locus (Wright, 1960; see Brown, 1993 for review) which encodes an integrin subunit (MacKrell et al., 1988). Integrins are a family of heterodimeric cell surface receptors (see Hynes, 1992 for review). Each integrin is composed of an α and a β subunit, which are unrelated in sequence. To date 14 different α subunits have been identified in vertebrates and 8 different β subunits, and 20 different α/β combinations have been observed. Most commonly an individual β subunit is found to form heterodimers with a variety of different α subunits to generate different receptors, but individual α subunits are also found in association with different β subunits, revealing that each subunit contributes to the integrin's specificity for ligands. The majority of integrins are receptors for the major components of the extracellular matrix, such as fibronectin, laminin and collagen, but integrins also bind to other types of cell surface protein.

In *Drosophila* four integrins subunits have been identified to date. The β_{v} subunit was cloned by its sequence similarity to other β subunits and is expressed in a single tissue, the midgut (Yee and Hynes 1993). The other three subunits, α_{PS1}, α_{PS2} and β_{PS}, compose the Position-specific (PS) integrins, which are widely expressed. They were initially identified through screens for monoclonal antibodies that recognise spatially restricted cell surface antigens on imaginal discs, the sacs of cells in the larva that give rise to the adult epidermis (Wilcox et al., 1981; Brower et al., 1984). Their name refers to the fact that the expression of the α subunits is determined by the position of a cell within the imaginal discs rather than the type of cell. The two PS integrin α subunits have complementary patterns of expression at the sites where PS integrins mediate adhesion. At the developing larval muscle attachment sites, $\alpha_{PS1}\beta_{PS}$ is expressed in the epidermal cell membrane, while $\alpha_{PS2}\beta_{PS}$ is expressed in the muscle cell membrane (Bogaert et al., 1987; Leptin et al., 1989). As the muscle attachments form, the two membranes interdigitate and large adherens junctions are formed (Lai-Fook, 1967; Tepass and Hartenstein, 1994). In the developing adult wing,

$\alpha_{PS1}\beta_{PS}$ is expressed in the cells that will give rise to the dorsal surface of the wing blade, while $\alpha_{PS2}\beta_{PS}$ is expressed in the presumptive ventral cells (Wilcox et al., 1981; Brower et al., 1984). During metamorphosis, the basal surface of the dorsal cell layer becomes attached to the basal surface of the ventral cell layer and the two surfaces are connected by large adherens junctions similar to those seen at the muscle attachments (Mogensen and Tucker, 1987; Fristrom et al., 1993; see Brown, 1993 for comparison). In both cases the PS integrins are concentrated at the site of these basal adherens junctions (Bogaert et al., 1987; Fristrom et al., 1993), although it is not yet clear whether the integrins are actual junction components. In *mys* mutant embryos the muscles detach and round up (Wright, 1960), and in clones of *mys* mutant cells within the adult wing (induced by mitotic recombination) the two surfaces of the wing blade separate (Brower and Jaffe, 1989; Zusman et al., 1990). Thus in both cases the two PS integrins are found on opposite sides of the interacting cell layers and they are required for the interaction of the cell layers.

The PS integrins could mediate the adhesion of the different cell layers by binding directly to each other, or by binding to other molecules. It has been found recently that the vertebrate integrin $\alpha_2\beta_1$ can bind directly to $\alpha_3\beta_1$ (Symington et al., 1993) setting a precedent for a model whereby $\alpha_{PS1}\beta_{PS}$ binds directly to $\alpha_{PS2}\beta_{PS}$ to stick the different cell layers together. This would be consistent with the localisation of the PS integrins at sites of cell-cell contact in culture (Peel and Milner, 1992). An alternative model is that the PS integrins mediate the adhesion of the cell layers by binding to other cell surface proteins or to components of the extracellular matrix, as the majority of the vertebrate integrins do. The latter hypothesis is supported by the finding that cells expressing $\alpha_{PS2}\beta_{PS}$ adhere to mammalian fibronectin and vitronectin (Hirano et al., 1991; Bunch and Brower, 1992).

One way to distinguish between the two models is to compare the phenotypes resulting from mutations in the genes encoding each of the PS integrin subunits. If the PS integrins function solely (i.e. there are not additional α subunits involved) by binding to each other directly, then loss of one integrin would be equivalent to losing both since the remaining integrin would have nothing to bind to. Therefore the phenotype resulting from the loss of one α subunit should be identical to the phenotype resulting from the loss of the β_{PS} subunit. Conversely if the two PS integrins bind to independent ligands then the *mys* (β_{PS}^-) phenotype will be more severe than an α mutant phenotype.

While the evidence demonstrating that the *myospheroid* (*mys*) locus encodes the β_{PS} subunit is now good (Digan et al., 1986; MacKrell et al., 1988; Leptin et al., 1989; Bunch et al., 1992; Zusman et al., 1993), prior to the work described here, it was less certain that a mutation in the α_{PS2} subunit had been identified. There were three lines of evidence to suggest that the α_{PS2} integrin subunit is encoded by the *inflated* (*if*) locus. Both the *if* locus and the α_{PS2} gene map near to *rudimentary* (Weinstein, 1918; Falk et al., 1984; Rawls et al., 1986; Bogaert et al., 1987), α_{PS2} protein expression is reduced in *if* alleles, and there are similarities between the *if* phenotypes and those of *mys* (Brower and Jaffe, 1989; Wilcox et al., 1989; Brabant and Brower, 1993). These features of the *if* locus are also consistent with an alternative hypothesis where *if* encodes a product that regulates the synthesis of the closely linked α_{PS2} subunit gene (Brower and Jaffe, 1989). The phenotypes of the embryonic lethal alleles of *if* that have been examined are distinctly weaker than *mys* (Wilcox et al., 1989; Brabant and Brower, 1993). There are three possible explanations for this difference: (1) the α_{PS2} subunit is not involved in all the functions that the β_{PS} performs, (2) there is residual α_{PS2} activity in the lethal alleles examined to date, and (3) the *if* locus does not encode α_{PS2}.

In this paper I prove that the *if* locus does encode the α_{PS2} subunit, generate new *if* alleles which are null mutations, and compare the *if* null phenotype with that of *mys*.

MATERIALS AND METHODS

Genetics

Genetic loci and the mutant alleles used are described in Lindsley and Zimm (1992), with the exception of three mutant alleles which were isolated in the course of this work. In this paper the deficiency *Df(1)80f3c* within the *Dp(1:4)*r^+f^+, is referred to as *Dp f3c*. For the experiments shown here the *myospheroid* allele mys^{XG43} was used, which is a null allele (Leptin et al., 1989; Bunch et al., 1992).

The *if* alleles if^{B4} and $T(1{:}2)if^{B6}$ were isolated from the following screen: Isogenic *y f* males were mutagenised by exposure to a ^{137}Cs γ-ray source to a dose of 4000 r, and crossed to virgin females homozygous for the if^3 allele. Approximately 30,000 F_1 females, heterozygous for the mutagenised *y f* chromosome over the if^3 chromosome, were screened for an inflated wing phenotype. From 47 females that were initially selected, 4 yielded new mutations that are allelic to if^3 and if^{k27e}. The alleles $if^{B4}$and $T(1{:}2)if^{B6}$are described in more detail in the results. One of the other new alleles, $if^{B2.1}$, is associated with a complex rearrangement, and the other is a partly viable allele, $T(1{:}4)if^{V2}$, which will be described in more detail elsewhere. The deficiency, *Df(1)rif*, was isolated from a separate F_1 screen for P-element induced *if* alleles: the P-element in the nearby gene *rudimentary* (*r*), from the allele r^{hd1} (Tsubota et al., 1985), was mobilised using the Δ2,3 source of P-transposase (Robertson et al., 1988) and crossed to if^3*; cn ; ry*^{506} flies. The heterozygous females, *cv v* r^{hd1} *f/* if^3 *; cn /+; ry*^{506} *or P[ry+, Δ2,3] ry*^{506}*/ ry*^{506} were screened for inflated wings. One *if* allele was isolated from a screen of approximately 44,000 F_1 females, and was found to be a deletion of 130 kb which removes both *r* and *if*, hence the name *Df(1)rif*. The deficiency is mutant for at least one other gene judging from its embryonic phenotype, but does not extend much more distally than *r* since it is complemented by a duplication *Dp f3c* (a terminal deficiency, *Df(1)80f3c* within the *Dp(1:4)*r^+f^+) that has had its distal endpoint mapped just distal to *r* (Falk and Halladay, 1986; see Fig. 1). The proximal endpoint of *Df(1)rif* was mapped to within 1 kb upstream of the α_{PS2} gene start of transcription (data not shown), so it removes approximately 130 kb of the X chromosome including the entire α_{PS2} transcription unit. This deficiency does not remove either of the Minute loci that flank *if*, *M(1)14C* and *M(1)15D*, which are deleted in the other deficiencies that remove the *if* locus, *Df(1)*r^{D1} and *Tp(1:2)*r^{+75c}.

The most useful allele is if^{B4} because it can be rescued completely by the pRR1PS2 transposon. The translocation $T(1{:}2)if^{B6}$ is complemented by pRR1PS2 and *Dp f3c* to produce viable males which are sterile, presumably an effect of the X to autosome translocation. The embryonic lethal phenotypes of if^{B4} and $T(1{:}2)if^{B6}$ are indistinguishable.

Polytene chromosomes of the if alleles were examined as described in protocol 18 of Ashburner (1989). The *if* alleles were mapped by

genomic Southern analysis using standard procedures. Once the if^{B4} deletion was localised within the α_{PS2} gene, a small fragment of the mutant chromosome was amplified by PCR using primers flanking the deletion and sequenced using Sequenase (US Biochemicals).

Germline clones of if^{B4} were generated as described in Chou and Perrimon (1992) although a different insertion of the FRT site was chosen. A recombinant chromosome was made that contained the if^{B4} allele and an insertion of an FRT site, $FRT^{9\text{-}2}$ at position 18E (T.-B. Chou and N. Perrimon, personal communication). A recombinant chromosome containing the dominant female sterile mutation ovo^{D2} and $FRT^{9\text{-}2}$ was kindly provided by E. Wieschaus. Larvae of the genotype *y w* if^{B4} *f* $FRT^{9\text{-}2}$/ ovo^{D2} $FRT^{9\text{-}2}$; FLP^{F38}/+ were heat shocked at 37°C for 2 hours to generate germline clones.

P-element transformation of the α_{PS2} gene

The P-element construct containing the entire α_{PS2} gene was made in a new P-element vector, pRosyRhino 1 (to be described elsewhere). This vector is based on the origin of replication of pSC101, which is stable at 5-8 copies per copy of the *E. coli* chromosome (Hasunuma, 1977). A low copy number vector was tried because the construct containing the entire α_{PS2} gene in a high copy vector was found to be unstable, and large fragments of *Drosophila* DNA have previously been successfully propagated in low copy number vectors (O'Connor et al., 1989). The α_{PS2} gene was assembled into this vector, to create the plasmid pRR1PS2, by inserting four fragments derived from lambda clones of genomic DNA (going 5′ to 3′: a 3.3 kb *Spe*I to *Not*I, a 24.3 kb *Not*I to *Bss*HII, a 4.5 kb *Bss*HII to *Sac*II, and a 6.8 kb *Sac*II to *Xho*I), into pRosyRhino in a 3 step procedure (details are available on request). The large plasmid pRR1PS2 (48 kb) was grown in high salt medium (2% tryptone, 0.5% yeast extract, 0.3 M NaCl, 2.5 mM KCl, 30 μg/ml kanamycin sulfate) at 37°C until early mid-log stage of growth and then was shifted to 24°C to grow to late log; these conditions increase the supercoiling of the DNA, and so decrease the viscosity of the DNA and aid injection (M. O'Connor, personal communication). The DNA was injected with phsπ helper into *cn* ; ry^{506} embryos as described by Spradling (1986). From a total of 2,760 embryos injected, 1,925 hatched and approximately 1,500 were crossed to *cn* ; ry^{506} mates. Approximately 10% of the G_0 adults showed a phenocopy ry^+ eye color, and the single G_0 male that was found to have transformed offspring was one of these. Two independent transpositions occurred in the germline of this male: one on the second chromosome and one on the third chromosome.

Histology

To examine the embryonic phenotypes of the integrin mutations, the mutant stocks were outcrossed to remove modifiers that might have built up in the stock. It should also be noted that since each of the lethal *if* alleles can be rescued by the

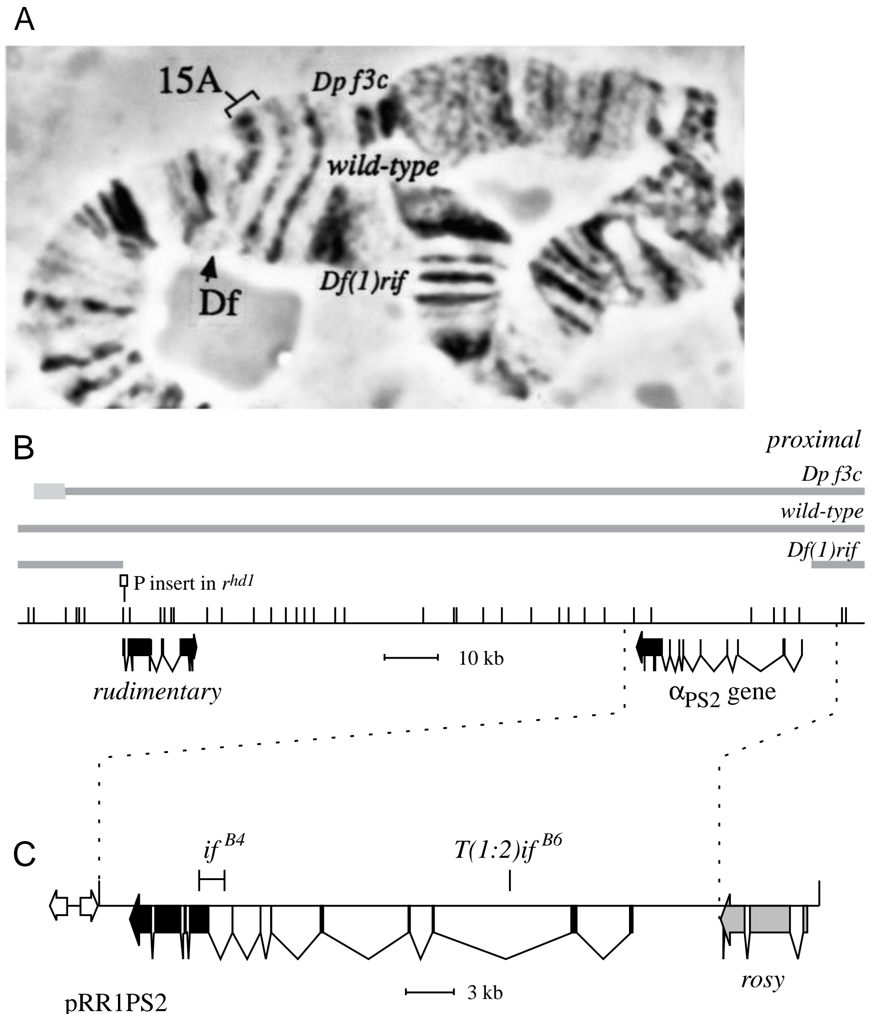

Fig. 1. The α_{PS2} gene is the *inflated* locus. (A) The extent of the small P-induced deficiency *Df(1)rif* is shown. Four chromosomes are present in this salivary gland chromosome preparation. At the top is the duplication *Dp f3c* (a terminal deficiency, *Df(1)80f3c* within the *Dp(1:4)r⁺f⁺)* which has a portion of the X-chromosome, 14F-16A, attached to the distal tip of the 4th chomosome (no. 1). At the left, it is apposed to the wild-type chromosome (no. 2), which is paired to the deficiency containing chromosome (no. 3) at the bottom. The proximal part of the duplication, 15C to 16A, is separated from the wild-type X chromosome and the rest of the duplication chromosome, consisting of most of the 4th chromosome, is paired to the wild-type 4th chromosome (no. 4). The small bands 15A1-2, 3-4, 5 are removed in *Df(1)rif* leaving a single thin band which could be 15A1 or 15A5 or a combination of the two. (B) The relationship between *Df(1)rif* and the molecular map of the region is diagrammed. The thick lines indicate the three chromosomes shown in (A). The thicker shaded rectangle shows the distal end of *Dp f3c* (Falk and Halladay, 1986). The proximal end of *Df(1)rif* was mapped by genomic Southern data (not shown) and the distal end was inferred from the position of the P-element in the starting chromosome (Tsubota et al., 1985), as drawn below the schematic *Df(1)rif* chromosome. The *Eco*RI sites of the genomic DNA of the region are indicated on the thin line, and the *rudimentary* and α_{PS2} transcription units are drawn below that. (C) An enlarged view of the α_{PS2} transciption unit is shown within the plasmid used for P-element-mediated transformation of the α_{PS2} gene. The positions of the two γ-ray induced *if* alleles are shown on this diagram: the extent of the if^{B4} deletion is indicated as is the breakpoint of the $T(1{:}2)if^{B6}$ translocation. The entire 48.5 kb plasmid pRR1PS2 is shown, the P-element ends that flank the segment that transposes into the germline are indicated at the 3′ end of the α_{PS2} gene and the 5′ end of the *rosy* gene (the two vertical lines above the central horizontal line). The two white arrows at the left indicate the origin of replication and kanamycin resistance gene of the pRosyRhino plasmid. When integrated into the genome this construct fully rescues *inflated* alleles.

pRR1PS2 transposon, the *if* allele bearing chromosomes are free of other lethal mutations. Embryos were fixed and stained with antibodies using standard procedures. Antibodies used were: the CF6G11 mouse monoclonal antibody against β_{PS} (Brower et al., 1984), the DA1B6 mouse monoclonal antibody against fasciclin III (Brower et al., 1980; Patel et al., 1987) and a rabbit antiserum against muscle myosin heavy chain (Kiehart and Feghali, 1986). The secondary antibodies were from BioRad. To stain embryos containing a P-element construct containing the myosin heavy chain promotor fused to *lacZ* (Hess et al., 1989) with X-gal, the embryos were fixed and devitelinised as for antibody staining, except that the length of time the embryos were in methanol was kept as short as possible. The embryos were stained overnight at 37°C in the solution described in Glaser et al. (1986). Cuticle preparations were made in Hoyers:lactic acid as described in Wieschaus and Nüsslein-Volhard (1986). Photography was performed on either a Zeiss Axiophot or an Axioplan microscope with Kodak Tech-Pan, Ilford 100 delta or ASA 100 Gold II colour print film.

RESULTS

New *inflated* alleles have lesions in the gene encoding α_{PS2}

At the time this work was initiated two *inflated* (*if*) alleles were available, the viable allele if^{3} and the lethal allele if^{k27e}. The if^{3} allele is obviously not a null, while if^{k27e} might be a null but is hard to work with, because it was induced on a chromosome containing a strong *rudimentary* allele, which is too close to *if* to be easily removed by recombination (see below). Therefore in order to isolate new *if* alleles two screens were performed, and they resulted in the isolation of three new *inflated* (*if*) mutations.

The aim of the first screen was to isolate *if* mutations caused by insertion of the P transposable element. In order to increase the probability of a P-element insertion into *if* (Tower et al., 1993), a P allele of the nearby gene *rudimentary* was used as the source of the P-elements (Tsubota et al., 1985). The positioning of the restriction maps of the α_{PS2} and *rudimentary* (*r*) genes onto the large chromosomal walk of the region (Surdej et al., 1990) has revealed that these two genes are reasonably close to each other (see Fig. 1), with the 5′ ends separated by 130 kb and the 3′ ends by 80 kb. A screen of 44,000 F_1 females did not yield any P-element insertion alleles of *if*; however a small deficiency for the *if* locus was recovered, *Df(1)rif* (*r* to *if*), which removes most of the three small bands at 15A on the X-chromosome (and therefore has endpoints of 15A1-2; A4-5; see Fig 1). The characteristics of this mutation suggest that it arose through a two step process where the jumping of the P-

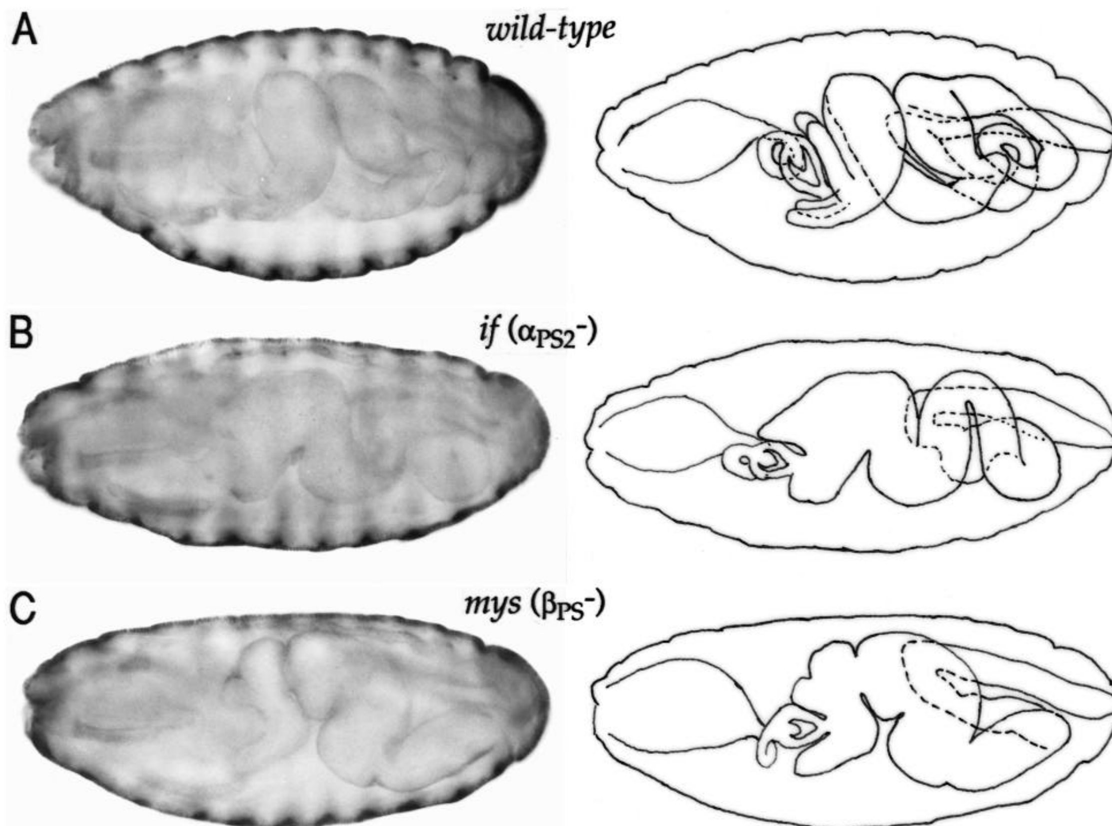

Fig. 2. The midgut defect of *inflated* is distinct from *myospheroid*. Stage 16 embryos stained for fasciclin III, which stains the epidermis, the visceral mesoderm and some cells within the nerve cord (Patel et al., 1987) and corresponding drawings outlining the gut; anterior is left, dorsal up. The elongation of the midgut and gastric caeca seen in the wild-type embryo (A) is not found in the *if* (B) or *mys* (C) embryos. However the posterior midgut becomes somewhat elongated in the *if* embryo, compared with the *mys* embryo, which may result in the relative enlargement of the anterior midgut. In the *if* mutant embryo one can see the clustering of the visceral mesoderm in the ventral side of the midgut constriction.

element to the 5′ end of the α_{PS2} gene was followed by a deletion, leaving a single P-element at the site of deletion.

Two new embryonic lethal *if* alleles were recovered from a screen of mutations induced by γ-rays (see Materials and Methods for details). The if^{B4} allele was found to be cytologically normal and have a small deletion within the α_{PS2} gene (Fig. 1C). The nucleotide sequence of the area surrounding the deletion was obtained following amplification and revealed that 1,036 bp had been deleted from intron 8 and 577 bp from exon 9. The first 8 exons encode only the first 220 amino acids of the 1364 amino acid mature α_{PS2} subunit. Although an alternative acceptor could be sought by the splicing machinery to overcome the loss of the exon 9 splice acceptor, most of the potential cryptic splice acceptors would result in a frameshift and premature termination. This allele appears to be a null allele by genetic tests. Thus by both genetic and molecular criteria, the if^{B4} allele is a null mutation.

The second γ-ray allele was found to be a translocation that breaks within the 2nd intron of the α_{PS2} gene (see Fig. 1), and was therefore renamed $T(1{:}2)if^{B6}$ (15A; 41D). The result of this mutation is that the signal peptide and first 38 amino acids of the α_{PS2} protein are on a different chromosome from the rest of the α_{PS2} protein. This mutation should completely inactivate the α_{PS2} gene, and as expected behaves as a null allele when tested genetically. Thus all three new *if* mutations are associated with molecular lesions in the α_{PS2} gene, and each of these mutations result in the complete loss of *if* gene activity.

39 kb of genomic DNA encompassing the α_{PS2} transcription unit rescues *if* alleles

In order to confirm the assignment of *if* as the locus encoding the α_{PS2} subunit, the ability of the α_{PS2} gene to rescue *inflated* mutations was tested. Because the gene is alternatively spliced (Brown et al., 1989) and because there appear to be essential regulatory regions in the introns (A. Dokadis, M. Leptin, N. H. B, and F.C. Kafatos, unpublished observations), the entire 30 kb transcription unit and some flanking DNA was cloned into a P-element vector to make the plasmid pRR1PS2 (see Fig. 1). It was necessary to construct a new P-element vector in order to grow a construct containing the 39 kb of DNA encompassing the α_{PS2} gene in *E. coli* without deletions (see Materials and Methods for details). Only one of the over 2700 embryos injected gave rise to transformed progeny, indicating that the large size of the pRR1PS2 plasmid significantly reduced the rate of integration. Two different lines were recovered from the single G_0 male, each of which contained a single insertion of the pRR1PS2 construct, as revealed both by genomic Southern and in situ hybridisation (data not shown).

Both pRR1PS2 insertions were tested to see whether they complement *if* alleles. They complemented the blistered wing phenotype of the if^3 mutation and the embryonic lethality of the if^{B4} mutation to give fully fertile adult flies. The pRR1PS2 transgenes fully complemented if^{k27e}, if^{B4}, and $T(1{:}2)if^{B6}$ in all cases tested. The P-element copies of α_{PS2} gene did not rescue the lethality of *Df(1)rif*, demonstrating that this deficiency also removes other essential genes. Thus a 39 kb fragment of genomic DNA (containing the entire 31.5 kb primary transcription unit of α_{PS2}, 5.7 kb upstream of the start of transcription of the α_{PS2} gene and 2 kb downstream of the polyadenylation site) fully complements *if* alleles, demonstrating that the *if* locus maps within this segment of genomic DNA. This result is consistent with the *if* locus encoding α_{PS2}, but does not eliminate the possibility that *if* consists of a transcription unit within one of the

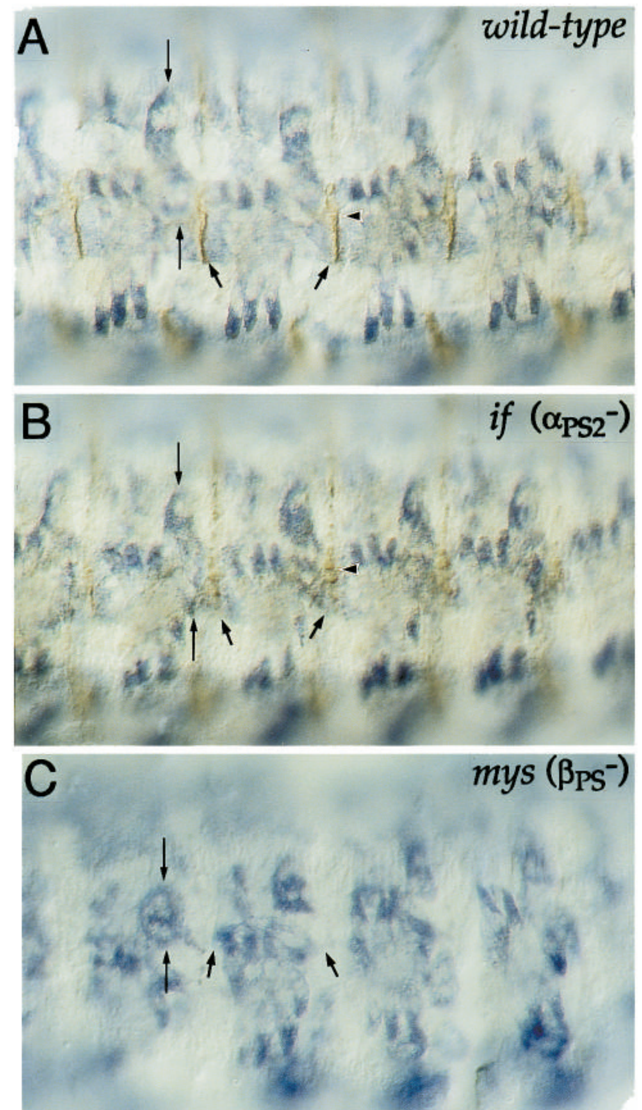

Fig. 3. Detachment of somatic muscles is not detected in mid stage 16 *inflated* mutant embryos. Stage 16 embryos were stained with antibodies against β_{PS} (brown) and muscle myosin (blue). Embryos were dissected to remove internal tissues, and mounted in Araldite; anterior is left, dorsal up. (A) In wild-type embryos the β_{PS} is strongly expressed in both the muscles and the epidermis, at the attachment sites of the longitudinal and oblique muscles, which attach at the segment boundary (arrowhead). The ventral corners of a lateral longitudinal muscle (no. 4) are indicated in one of the segments by the short arrows, and the ends of a dorsal transverse muscle (No. 18) are indicated by the long arrows. Staining of β_{PS} at the termini of the transverse muscles is weak at this stage. (B) In *if* mutant (*y v* if^{B4} *f/Y*) embryos β_{PS} is only found in the epidermis and it is not localised to the basal surface, giving a ‘cobblestone’ appearance (arrowhead). In this embryo, none of the muscles have detached yet. (C) In *mys* mutant embryos (*y* mys^{XG43}*/Y*) there is no staining of the β_{PS} subunit and many of the muscles have detached and are rounded up, e.g muscles 18 (long arrows) and 4 (short arrows).

introns of the α_{PS2} gene or flanking it. However, the fact that two of the new *if* mutations map to sites within this segment that are separated by 18 kb, yet both inactivate the α_{PS2} gene, rules out the possibility that the *if* locus is a different, closely linked gene. Therefore the ability of the pRR1PS2 construct to complement *if* alleles and the demonstration that *if* mutations are associated with molecular lesions in the α_{PS2} gene, prove conclusively that the *if* locus encodes the integrin α_{PS2} subunit.

The null *inflated* phenotype and its similarity to the *myospheroid* phenotype

Now that I have proved that the *inflated* (*if*) locus encodes the α_{PS2} subunit and have isolated null *if* alleles, it is possible to determine the phenotype that results from the absence of the α_{PS2} subunit. I have examined the embryonic phenotype of the strong *if* alleles. As the α_{PS2} subunit forms a heterodimer with the β_{PS} subunit, encoded by *myospheroid* (*mys*), I have compared the phenotypes of *if* and *mys* mutations to resolve different models of integrin function. The defects caused by mutations in the genes encoding the PS integrin subunits are first apparent during late stages of embryogenesis. The phenotype of *mys* has been characterised in some detail by Wright (1960; Newman Jr. and Wright, 1981), and the description of the *mys* phenotype below is derived from this description and my own observations. The earliest that zygotic integrin mutant phenotypes can be identified is during stage 16 (13-16 hours after egg deposition; *Drosophila* embryogenesis last for 21-22 hours at 25°C, and has been divided into 17 stages by Campos-Ortega and Hartenstein, 1985). The *mys* phenotype can be divided into four features: (1) a defect in midgut morphogenesis, (2) detachment of the somatic muscles, (3) a failure in nerve cord condensation, and (4) a hole in the dorsal epidermis. I will compare normal development, the *mys* phenotype and the *if* phenotype for each of these features in turn.

Midgut morphogenesis

The midgut is formed by the migration of the anterior and posterior midgut primordia toward each other along lateral bands of visceral mesoderm, which are required for the migration (Azpiazu and Frasch, 1993; Bodmer, 1993). The midgut spreads to enclose the yolk sac, resulting in a heart shaped midgut which is then subdivided by the formation of three constrictions (Campos-Ortega and Hartenstein, 1985; Reuter and Scott, 1990). Four evaginations occur at the anterior end of the midgut to form the gastic caeca, and at the junction between the foregut and the midgut the proventriculus develops. The midgut elongates to become coiled within the embryo by mid stage 16 (14 hours; Fig 2A). The first departure from normal development in *mys* mutant embryos can be observed at early stage 16 (13 hours) because the constrictions do not fully form. At this point the visceral mesodermal cells accumulate at the base of the constrictions and the tips of only two broad gastric caeca (not shown). The midgut and gastric caeca then fail to elongate (Fig. 2C) and by the end of stage 16 (16 hours) the proventriculus is also observed to be abnormal (not shown). In *if* mutant embryos the constrictions appear to form normally and defects are not observed until the gut elongates, when the visceral muscle can also be seen to detach and clump (Fig. 2B). As with *mys*, only two broad gastric caeca are formed in *if* mutant embryos, but in contrast the proventriculus appears to develop normally. The failure in the elongation of the midgut is reproducibly different between *if* and *mys* mutants. In *if* mutant embryos the anterior portion of the midgut remains particularly large, resulting in a more spherical yolk-filled sac at the anterior of the midgut, while the posterior end does narrow in diameter (Fig. 2B). In *mys* the failure of the midgut to narrow occurs throughout the length of the midgut (Fig. 2C). This difference suggests that the failure in the adhesion of the visceral mesoderm cells to the endoderm and to each other is more pronounced in *mys* than in *if*, resulting in the loss of adhesion at an earlier point, and the uniform failure of midgut elongation. In the *if* mutant embryos the remaining β_{PS} activity results in the partial constriction of the posterior midgut which pushes the yolk into the anterior midgut, enlarging it.

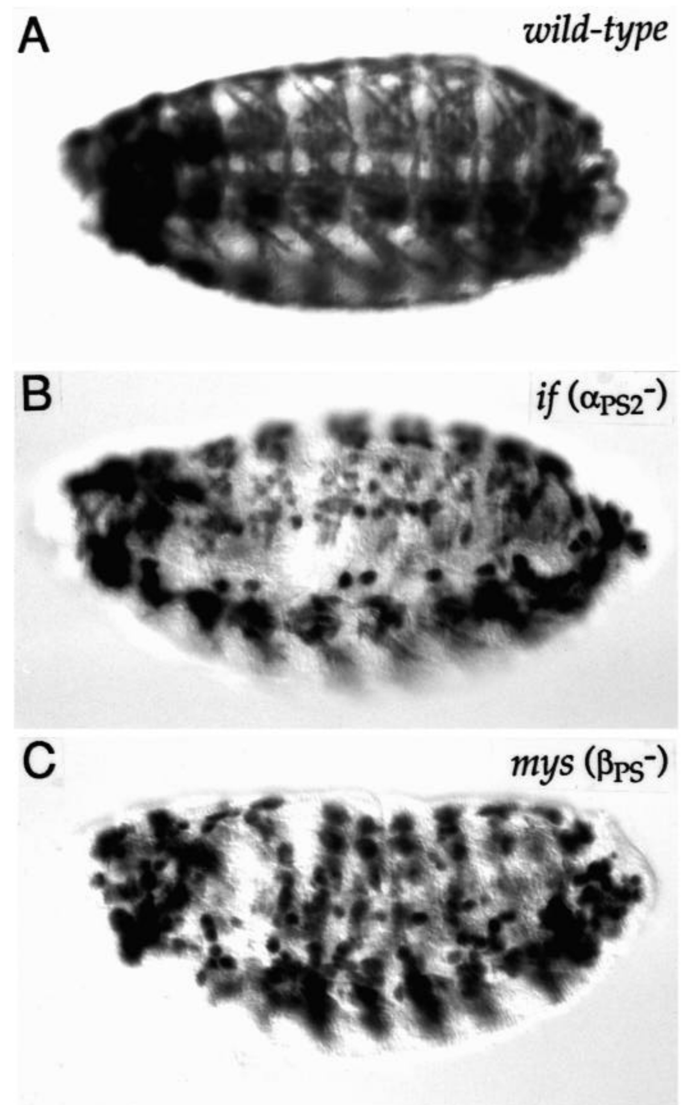

Fig. 4. Both *inflated* and *myospheroid* have a similar ‘detached’ muscle phenotype by stage 17. Stage 17 embryos are stained with X-gal to reveal muscle-specific β-galactosidase activity produced by the muscle myosin *lac*Z fusion gene. The genotypes of the different embryos are (A) *wild-type*, (B) *y v if^{B4} f/Y*, (C) *y mys^{XG43}/Y*.

Somatic muscle detachment

The pattern of somatic muscles is essentially complete by the start of stage 16 (13 hours; Bate, 1990) and the muscles begin to contract shortly afterwards (13.5 hours). In Fig. 3 mid stage 16 (15 hours) embryos are shown stained with antibodies against the β_{PS} subunit (brown) and muscle myosin (blue). The wild-type embryo (Fig. 3A) shows strong β_{PS} staining at the segment borders where the lateral longitudinal (muscle 4, numbering as in Bate, 1990; the ventral corners of this muscle are indicated by the short arrows in Fig. 3) and oblique (muscles 5, 19, 20) muscles attach. The attachment sites of the transverse muscles (for example the dorsal transverse muscle 18; the ends of this muscle are indicated by the long arrows in Fig. 3) have weak expression of β_{PS} at this stage. In *mys* mutant embryos (Fig. 3C) little β_{PS} staining is detected and many of the muscles have detached and rounded up by the middle of stage 16. In contrast, *if* mutant embryos (Fig. 3B) have a normal pattern of muscles at this stage. In *if* mutant embryos the β_{PS} subunit is only detected in the epidermal cells, giving a cobblestone appearance rather than the sharp line of expression in both the muscles and epidermal cells seen in wild-type embryos (compare the staining at the arrowheads in Fig. 3A,B). By the end of stage 16 (16 hours) a few muscles detach in if^{B4} mutant embryos, generally the lateral longitudinal muscles 4 and 12 and the transverse muscle 8, as was found for if^{k27e} in a previous study (Drysdale et al., 1993). Staining of whole-mount embryos with antibodies is hindered after stage 16 due to the synthesis of the cuticle which blocks the penetration of the antibody, but the histological reagent for the enzyme β-galactosidase is still able to penetrate the cuticle even during stage 17. Therefore a transgene consisting of the promoter of the muscle myosin heavy chain gene linked to *lac*Z (Hess et al., 1989) was used to visualise the muscle pattern during stage 17. By this stage, *if* and *mys* mutant embryos look very similar (Fig. 4), demonstrating that the extent of muscle detachment is the same although the timing of detachment differs between *if* and *mys*.

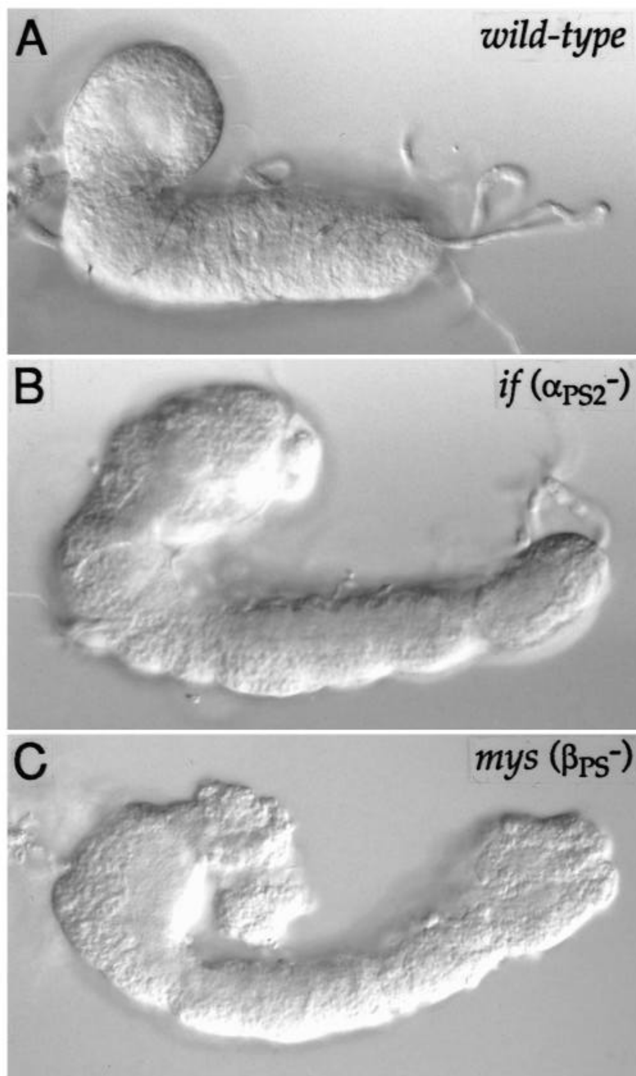

Fig. 5. *inflated* and *myospheroid* share a failure in the condensation of the ventral nerve cord. Ventral nerve cords were dissected from 20- to 24-hour embryos and viewed with Nomarski optics, anterior is left, dorsal up. The genotypes of the embryos from which the nerve cords were derived are (A) *wild-type*, (B) *y v* if^{B4} *f/Df(1)rif, v*, (C) *y* mys^{XG43}*/Y*.

Nerve cord condensation

In a wild-type embryo the ventral nerve cord condenses from a structure occupying most of the length of the embryo at stage

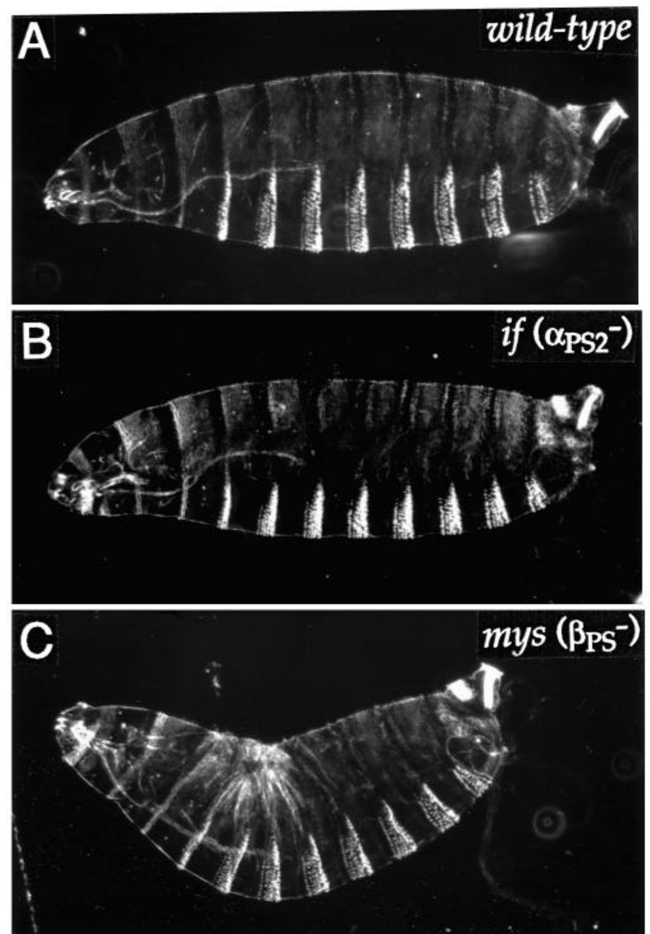

Fig. 6. *inflated* does not have the *myospheroid* dorsal hole phenotype. Cuticle preparations are shown of three embryos, anterior is left, dorsal up. (A) *wild-type*, (B) *y v* if^{B4} *f/Y*, (C) *y* mys^{XG43}*/Y*.

13 to one occupying appoximately 60% of the length by hatching (Fig. 5A). In both *mys* and *if* mutant embryos, the ventral nerve cord only partially contracts (Fig. 5B,C). The supraoesophageal ganglia and presumptive optic lobes become distorted in *mys* embryos because they are pushed though the dorsal hole (see below), otherwise the phenotype is the same in the two genotypes. Since the α_{PS2} subunit is not strongly expressed in the nerve cord (Bogaert et al., 1987), this phenotype may be a secondary effect of the defect in the musculature.

Dorsal hole

During the first half of embryogenesis the presumptive dorsal epidermis is bisected along the midline by amnioserosa cells. After germline shortening, dorsal closure occurs and the two edges of the dorsal epidermis meet along the dorsal midline to create a continous epidermis, which subsequently secretes the cuticle (Fig. 6A). In *mys* mutant embryos, dorsal closure occurs normally, but shortly afterwards the edges of the epidermis partially separate, resulting in a hole in the epidermis and the resulting cuticle (Fig. 6C). Later morphogenetic movements promote a 'secondary dorsal closure' which results in the grooved appearance of the epidermis surrounding the hole, and the constriction of internal tissues, such as the brain and midgut, which have been extruded through the hole.

In contrast to *mys*, in *if* mutant embryos there is no rupture along the dorsal midline and the epidermis is normal (Fig. 6B), even in embryos deficient for the *if* locus (not shown). One possible explanation for this difference between *if* and *mys*, which is also relevant to the differences discussed above, is that there is a large store of maternal *if* product, α_{PS2}, which is able to partially complement the loss of zygotic gene activity. However, *mys* itself has a maternal contribution, since embryos that lack both maternal and zygotic *mys* have a more severe phenotype, where germband retraction does not occur (Wieschaus and Noell, 1986; Leptin et al., 1989). The maternally provided β_{PS} is not essential, since a single paternal wild-type *mys* allele supports normal development. There would have to be a much more substantial store of maternal *if* product for the phenotype resulting from the lack of maternal and zygotic *if* to be the same as for *mys*. To test this, germline clones of if^{B4} were generated using the FRT-FLP system in conjunction with ovo^D (Chou and Perrimon, 1992). The embryos mutant for *if* both maternally and zygotically were found to undergo germ band retraction normally and form a normal epidermis (not shown). This demonstrates that $\alpha_{PS2}\beta_{PS}$ is not required to hold the dorsal edges of the epidermis together. This is consistent with the observation that the expression of α_{PS2} in the embryo is primarily restricted to the mesoderm (Bogaert et al., 1987). As with *mys*, embryos from *if* mutant germlines are fully rescued by a zygotic allele of if^+ and the lack of maternal *if* activity does enhance the zygotic phenotype, although not as dramatically as for *mys*. Some of the embryos lacking both maternal and zygotic *if* activity have many of the somatic muscles detached during stage 16, approaching the zygotic *mys* phenotype (not shown).

DISCUSSION

Two lines of evidence presented in this paper prove that the *inflated* (*if*) locus encodes the α_{PS2} subunit. A 39 kb fragment of genomic DNA encompassing the α_{PS2} gene fully rescues *if* alleles and 3 new *if* alleles have been isolated, each of which has a molecular lesion in the α_{PS2} gene. Furthermore, the demonstration that the new *if* alleles are complete loss of function (null) alleles has allowed a comparison to be made between the null phenotype of *if* and that of *myospheroid* (*mys*), which encodes the β_{PS} subunit. This has shown that mutations in the two genes have similar but distinct defects in the attachment of the somatic and visceral muscles to the epidermis and midgut epithelium respectively, an identical defect in nerve cord condensation, but that only *mys* has a defect in the dorsal epidermis.

The attachment of the embryonic muscles to the other cell layers is accompanied by strong complementary expression of the two integrins, $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$, at the sites of attachment (Bogaert et al., 1987; Leptin et al., 1989). Both *mys* and *if* mutations cause defects in the maintenence of the attachment of the somatic muscles to the epidermis and the attachment of the visceral muscles to the midgut epithelium. However, in both cases the defects caused by loss of the α_{PS2} subunit (*if*) are significantly weaker and delayed compared with the loss of the β_{PS} subunit (*mys*). This shows that the $\alpha_{PS2}\beta_{PS}$ integrin only contributes to part of the β_{PS}-mediated cell-cell adhesion and suggests that $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$ have independent roles at the sites of PS integrin function. Therefore a model where PS integrin function is only mediated by a direct interaction between the two PS integrins, $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$, can now be ruled out, since a prediction of this model is that embryos missing one of the integrins (*if*) should have an equivalent phenotype to embryos missing both integrins (*mys*), because the remaining $\alpha_{PS1}\beta_{PS}$ would have nothing to bind to (Fig. 7A). In the absence of an α subunit, the β_{PS} subunit is unlikely to be able to reach the cell surface to provide any adhesive activity, since surface expression of integrin β subunits requires an α subunit (Cheresh and Spiro, 1987).

There are at least three ways to account for the residual β_{PS}-dependent adhesive activity present in an *if* mutant embryo. This activity could be provided by another α subunit that is expressed in the muscle and able to partly complement the absence of α_{PS2} (Fig. 7B). Alternatively, non-integrin cell surface proteins could be the ligands for the PS integrins (Fig. 7C). Finally the PS integrins could bind to components of the extracellular matrix, provided there are some other cell surface molecules that have some affinity for the extracellular matrix (Fig. 7D). This last model is supported by additional experimental evidence. *Drosophila* cells expressing $\alpha_{PS2}\beta_{PS}$ attach to surfaces coated with the vertebrate proteins, fibronectin and vitronectin (Hirano et al., 1991; Bunch and Brower, 1992). In *mys* mutant embryos extracellular matrix assembly is delayed (Fig. 7D; Wright, 1960; Newman Jr. and Wright, 1981) suggesting that the PS integrins do bind to extracellular matrix proteins which 'seed' the assembly of the matrix. The *mys* phenotype is analogous to the block in the formation of fibronectin fibrils observed in amphibian embryos injected with antibodies against the β_1 integrin subunit (Darribere et al., 1990).

The retraction of the visceral mesoderm and clumping of the cells within the midgut constrictions in *mys* (Wright, 1960; Newman Jr. and Wright, 1981) and *if* mutant embryos suggests that the visceral muscle attachments, which are from muscle to muscle (Tepass and Hartenstein, 1994), are also PS integrin

dependent. This could be a secondary effect, if the attachment of the visceral muscles to each other is too weak to maintain the integrity of this layer in the absence of PS integrin-mediated attachment of these muscles to the midgut epithelium. Alternatively somatic and visceral muscle could both use the PS integrins to form functionally equivalent muscle attachments, even though somatic muscles attach to the epidermis while the visceral muscles attach to each other. Since α_{PS1} does not appear to be expressed in the visceral mesoderm (Leptin et al., 1989; Werhli et al., 1993), attachments between the visceral muscles would be mediated solely by $\alpha_{PS2}\beta_{PS}$. If this is true, one would predict that an embryo mutant for α_{PS1} would retain a visceral muscle monolayer. Conversely, if clumping is a secondary effect caused by defective adhesion of the visceral muscles to the midgut epithelium, then an α_{PS1} mutant embryo would also have this defect. The resolution of this question awaits the isolation of mutations in the α_{PS1} subunit.

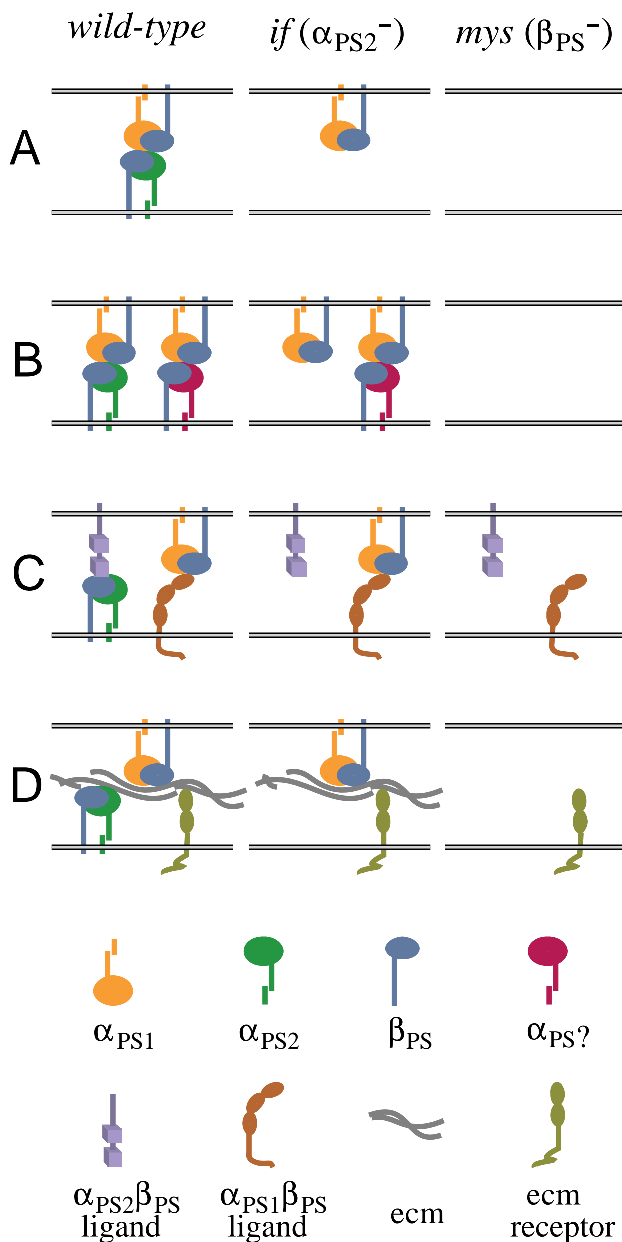

Fig. 7. The effect of integrin subunit mutations on different models of integrin-mediated cell-cell attachment. In each case, the appearance of an integrin on the cell surface is assumed to require both subunits. (A) If the two integrins interact directly the *if* mutant would have the same effect as the *mys* mutant, and since this is not the case this model is ruled out. The other models are attempts to explain why the integrin-mediated adhesion remains intact longer in an *if* mutant than a *mys* mutant. The residual adhesive activity in an *if* mutant must be β_{PS}-dependent, and could be (B) additional α subunit(s), (C) non PS integrin cell surface ligands or (D) components of the extracellular matrix (ecm) combined with additional ecm receptors. The delay in the deposition of the ecm in *mys* mutant embryos is indicated by the absence of the ecm.

By discarding the direct interaction model it is difficult to understand the significance of the complementary expression of the two PS integrins. It is possible that the α_{PS2} subunit cytoplasmic tail is required to interact with muscle-specific cytoskeletal proteins, but this does not explain the complementary expression of the two PS integrins in the developing adult wing. Alternatively, the two integrins could bind to different sites on the same extracellular matrix molecule, or they could set up a matrix that has different sides, which are important for the localisation of other molecules.

During embryogenesis both the epidermis and the gut become associated with a layer of muscles. The muscles appear to have little or no effect on the development of the epidermis, since some mutations that severely disrupt the muscles have no effect on the epidermis (Drysdale et al., 1993). In contrast, the development of the midgut is completely dependent on the visceral muscles. The migration of the two midgut primordia to form the midgut has been shown recently to require the visceral mesoderm, since it does not occur in embryos mutant for the homeobox containing gene *tinman*, which have not specified the visceral mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993). Furthermore in embryos mutant for the homeobox containing gene *bagpipe*, which is downstream from *tinman*, the visceral mesoderm appears to be specified in a patchy manner and although the midgut primordia fuse to form the sac shaped midgut, no constrictions are formed (Azpiazu and Frasch, 1993). The formation of the central midgut constriction and the evagination of the gastric caeca require the product of the *decapentaplegic* (*dpp*) gene, a member of the TGF-β family of secreted factors (Immergluck et al., 1990; Panganiban et al., 1990). Dpp is expressed in the visceral mesoderm and induces changes in the expression of at least one gene in the midgut epithelium, *labial*. The normal formation of the central midgut constriction in *if* mutant embryos indicates that the Dpp signalling at this position occurs normally. The observation that *if* mutant embryos are unable to complete the elongation of the midgut indicates that the reception of the Dpp signal by the endoderm is not sufficient to specify the full extent of morphogenetic changes of the midgut. Thus late events in midgut morphogenesis also require interaction between the visceral mesoderm and the midgut epithelium.

One of the most striking features of the *mys* phenotype, the hole in the dorsal epidermis, is not found in *if* mutant embryos. Two trivial explanations for this result, that the α_{PS2} mutations are not null mutations or that α_{PS2} has a large maternally provided component, have been ruled out. Therefore the main-

tenence of dorsal closure by the PS integrins does not require the function of the α_{PS2} subunit and must be performed by β_{PS} in association with α_{PS1} and/or additional α subunits that have yet to be identified. As with the defect in nerve cord condensation, this defect in the adhesion of the dorsal epidermis occurs in a region of the embryo that does not have high levels of PS integrin expression. It is thus currently unclear how the β_{PS} subunit contributes to the adhesion of the epidermis along the dorsal midline.

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