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Disruption of C-Terminal Cytoplasmic Domain of βPS Integrin Subunit Has Dominant Negative Properties in Developing *Drosophila*

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We have analyzed a set of new and existing strong mutations in the *myospheroid* gene, which encodes the βPS integrin subunit of *Drosophila*. In addition to missense and other null mutations, three mutants behave as antimorphic alleles, indicative of dominant negative properties. Unlike null alleles, the three antimorphic mutants are synthetically lethal in double heterozygotes with an *inflated* (αPS2) null allele, and they fail to complement very weak, otherwise viable alleles of *myospheroid*. Two of the antimorphs result from identical splice site lesions, which create a frameshift in the C-terminal half of the cytoplasmic domain of βPS. The third antimorphic mutation is caused by a stop codon just before the cytoplasmic splice site. These mutant βPS proteins can support cell spreading in culture, especially under conditions that appear to promote integrin activation. Analyses of developing animals indicate that the dominant negative properties are not a result of inefficient surface expression, or simple competition between functional and nonfunctional proteins. These data indicate that mutations disrupting the C-terminal cytoplasmic domain of integrin β subunits can have dominant negative effects in situ, at normal levels of expression, and that this property does not necessarily depend on a specific new protein sequence or structure. The results are discussed with respect to similar vertebrate β subunit cytoplasmic mutations.

INTRODUCTION

The integrin family of cell surface receptors is important for adhesion between the extracellular matrix and the cytoskeleton (Yamada and Miyamoto, 1995; Dedhar and Hannigan, 1996). Binding of extracellular ligands and/or the formation of integrin complexes can trigger integrin association with a number of cellular components, including both cytoplasmic proteins and other membrane proteins. This association makes strong connections between the matrix and cytoskeleton, and also transmits signals that can regulate cellular functions such as proliferation, differentiation, and migration, often in cooperation with information from other cell surface receptors (Dedhar and Hannigan, 1996; Howe *et al.*, 1998). Signals also can be transmitted in the opposite direction, as it is clear that events inside the cell can regulate the extracellular binding activities of the integrin αβ heterodimers (Fernandez *et al.*, 1998; Hughes and Pfaff, 1998).

The PS integrins of *Drosophila* are important for a variety of embryonic and postembryonic morphogenetic events (Stark *et al.*, 1997; Brown *et al.*, 2000). Like most vertebrate integrins, the PS1 and PS2 integrins are receptors for extracellular matrix components (Gotwals *et al.*, 1994). To date, five different αPS subunit genes have been identified: *mew*, encoding αPS1; *inflated*, encoding αPS2; *scab*, encoding αPS3; and two other as yet poorly characterized αPS3-like genes (Hynes and Zhao, 2000). At least the first three of these encode polypeptides that combine with βPS subunits, encoded by the *myospheroid* (*mys*) gene, to generate PS1 (αPS1βPS), PS2 (αPS2βPS), or PS3 (αPS3βPS) integrins.

The primary sequences of integrins indicate a high degree of structural conservation. The PS integrins are no exception, and their sequences are as similar to vertebrate integrins as these are to one another (Gotwals *et al.*, 1994). One structural constraint probably is associated with the interactions of α and β subunits that must be important in propagating conformational changes between the short cytoplasmic tails of the protein and the extracellular ligand binding domains

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(Humphries, 1996). In any case, integrin structure and function appear to be strongly conserved in evolution, and therefore basic information gleaned from studies of one integrin is likely to be applicable to others.

Much of what we know about integrin structure-function derives from studies involving site-directed mutagenesis followed by transfection into cultured cells for functional assays. Although very successful, this approach has limitations. For example, expression levels in transfected cells are often artificially high or unbalanced, and only phenotypes manifested by cultured cells can easily be examined. Also, it is impractical to sample more than a relatively small number of mutagenic changes. These limitations are ameliorated by the standard "forward" genetic approach of random mutagenesis followed by selection of mutants in situ, in developing animals. Mutations affecting required functions that are specific to certain cell types can be identified, and the animal tells the experimenter which mutations alter function, without regard to preconceived notions as to the functions of specific residues. Of course, "blind" genetic screens also have drawbacks; the important point is that this complementary approach has the potential to provide insights that would not readily be forthcoming from directed mutagenesis studies.

Genetic screens of this sort are not easily feasible with vertebrates, although β subunit mutations are revealed in human clinical syndromes, such as leukocyte adhesion deficiency (β2) and Glanzmann thrombasthenia (β3). Random mutagenesis screens can more easily be accomplished in cell culture (Baker *et al.*, 1997), or with invertebrates such as *Drosophila melanogaster* and *Caenorhabditis elegans*. *Drosophila* provides a particularly good system for pursuing screens for integrin mutations, because the PS integrin genes are well characterized genetically and molecularly, and many different integrin-dependent functions have been defined during fly development (Brown *et al.*, 2000).

Previously, a number of mutant alleles of *myospheroid* (βPS) were generated and partially characterized (Wright, 1960, 1968; Costello and Thomas, 1981; Newman and Wright, 1981; Wieschaus *et al.*, 1984; Leptin *et al.*, 1989; Bunch *et al.*, 1992; Zusman *et al.*, 1993; Roote and Zusman, 1995). Some are null for protein function, others retain at least some protein function (hypomorphs), and one antimorphic allele (mys^{XR04}) has been described that, in complementation tests, is worse than a null allele. We have generated new strong alleles of *myospheroid* and have characterized these and existing alleles with respect to their molecular lesions and genetic properties. Most importantly, we have identified and analyzed additional alleles that behave genetically as antimorphs, suggesting that these proteins have dominant negative properties. The properties of these antimorphic mutants are compared with those of the human splicing variant β1B, which has molecular similarities and has been shown to have dominant negative properties when assayed in transfected tissue culture cells (Altruda *et al.*, 1990; Balzac *et al.*, 1994; Retta *et al.*, 1998).

MATERIALS AND METHODS

General

All flies were grown on the food described in Condie and Brower (1989). Marker mutations not specifically referenced are described in Lindsley and Zimm (1992).

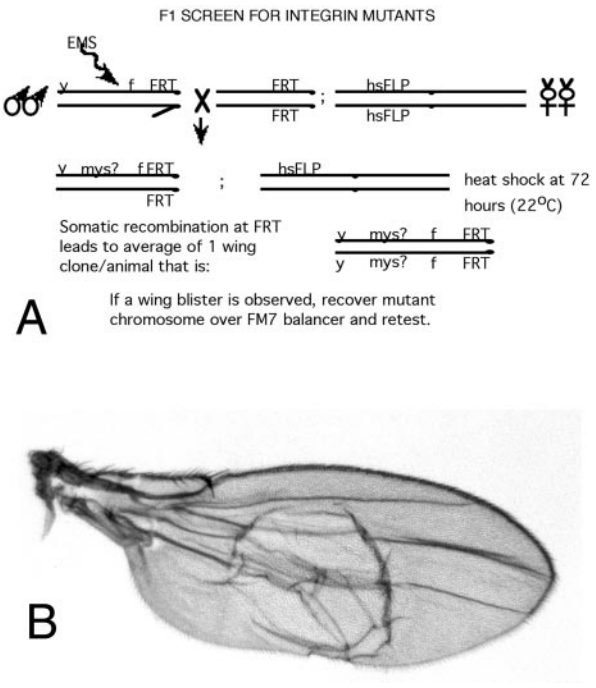

Figure 1. (A) Mosaic screen to recover new strong mutations in the *myospheroid* gene. See text for details. (B) Wing blister resulting from clone of homozygous mys^{G1} mutant cells.

Screen for New myospheroid Mutants

Some of the new strong alleles described herein were by-products of a screen for mutations in *mew*, the gene encoding αPS1, as described in Brower *et al.* (1995); both *mew* and *myospheroid* are on the X chromosome. Other alleles were from a similar screen set up specifically to identify *myospheroid* alleles (Figure 1). Briefly, males with the proximal *FRT18A* recombination site on the X chromosome (along with the cuticle markers *yellow* and $forked^{36a}$) were mutagenized with ethylmethanesulfonate (EMS) (Lewis and Bacher, 1968), and crossed to females with the same X chromosome *FRT*, as well as a heat shock-inducible *FLPase* on the second chromosome. Somatic recombination was induced by a heat shock of the F1 larvae to generate clones of cells homozygous for the mutagenized X chromosome. Animals with wing blisters from mutant clones (a known PS integrin phenotype) were selected, and those harboring *myospheroid* mutations were identified by complementation tests.

Phenotype and Genetic Assays

To score viability of various combinations of alleles, eggs were laid at the appropriate temperature, and vials were thinned to prevent overcrowding of larvae. Progeny were scored at least once a day, and if any animals from any single vial were scored, all subsequent progeny from that vial were counted, to guard against genotypic differences in developmental rates.

To quantitate the severity of dorsal herniation, eggs were collected from balanced stocks of the various mutants, and aged at 25°C to allow wild-type animals to hatch. The mutant embryos were then dechorionated in bleach (Ashburner, 1989a) and scored blind under a dissecting microscope. For each genotype, 100–200 embryos were scored. For photography, embryos were mounted in Hoyers medium and photographed with phase contrast optics.

Mutant Sequencing and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Heterozygous adults or hemizygous mutant embryos were homogenized and genomic DNA template was isolated using QIAGEN's QiaAmp tissue kit (Valencia, CA). PCR primers were designed to yield three overlapping PCR products, covering the *myospheroid* coding region (exons 2–7; Yee, 1993; Zusman *et al.*, 1993). The first fragment began 139 base pairs before the initiating AUG and the third fragment continued 62 base pairs after the UAG stop codon. The introns were amplified but the largest (between exons 3 and 5) were not sequenced in their entirety. The resulting PCR fragments were purified using QIAGEN's QiaQuick PCR purification kit and sequenced directly by the University of Arizona Genomic Analysis and Technology Core (Tucson, AZ).

For RT-PCR of mys^{XR04}, 30 hemizygous mutant embryos were used to generate mRNA with QIAGEN's RNeasy mini kit. The complementary strand was generated using a *myospheroid*-specific primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR primers were designed to then generate a 273-base pair fragment that included the intron between the sixth and seventh exons, as well as a potential downstream splice acceptor site. To examine the possible use of a potential downstream splice site, cDNAs from five developmental stages, embryo through adult, of wild-type flies were used for RT-PCR as described above. To maximize the likelihood of generating sufficient amounts of this novel potential fragment, amplified DNA was digested with *Hin*fI at various times during the amplification procedure, taking advantage of a restriction site specific to the common, larger PCR fragment.

To search for the presence of a potential novel splice site in mys^{P9}, mRNA was isolated from 15 mys^{P9}/*FM7i* and $y\ sn^3\ v$ *FRT18A* adults by using QIAGEN's RNeasy mini kit. The complementary strand was generated using QIAGEN's Omniscript reverse transcriptase and a specific primer located 62 base pairs after the UAG stop codon. Two sets of internal primers were used to generate a PCR fragment that spanned the splice site. The fragments from both mys^{P9}/*FM7i* and control DNA were examined on a 1.5% agarose 0.5× Tris borate-EDTA gel stained with ethidium bromide. The remainder of the PCR products was purified using QIAGEN's QIAquick PCR purification kit and sequenced.

Immunofluorescence and Protein Expression

For embryo staining, chromosomes with *myospheroid* alleles were balanced over an *FM7c* chromosome containing a *P[w$^+$, actin-lacZ]* insert (Davis *et al.*, 1996); simultaneous staining for β-galactosidase and βPS therefore allowed the unequivocal identification of hemizygous mutant embryos before overt *myospheroid* phenotypes became evident. Embryos were collected for 1.5 h at 25°C, and aged 23 h at 18°C, so that the majority of embryos were at stage 15–16, before muscles of the *myospheroid* embryos begin to detach. Embryos were then fixed and permeablized by using a protocol slightly modified from that of Tim Karr (Ashburner, 1989a). Embryos were incubated in three different antibody solutions, separated by washes. Blocking, washes, and antibody dilutions were in phosphate-buffered saline, pH 7, 1–10% fetal calf serum, 1% Triton-X 100. Antibody incubations were for 4 h at room temperature or overnight at 4°C, with constant agitation. In order, the incubations were in rabbit anti-β-galactosidase (Harlan Sera-Lab, Crawley Down, Sussex, United Kingdom), the mouse monoclonal anti-myospheroid CF.6G11 (Brower *et al.*, 1984), and finally a combination of goat anti-mouse fluorescein isothiocyanate (Jackson ImmunoResearch Labs, West Grove, PA) and goat anti-rabbit Texas Red (ICN Biomedicals, Cleveland, OH). In some experiments, an incubation in a mouse anti-Scr (Glicksman and Brower, 1988) was included as a control for antibody permeability. After the final wash, embryos (and the discs below) were mounted (in 30% 1 mM Tris pH 9.0, 70% glycerol, to which was added 2% n-propyl gallate to reduce bleaching) and examined in a standard Zeiss immunofluorescence or Leitz confocal microscope.

For staining wing imaginal discs, clones of homozygous *myospheroid* mutant cells were generated by somatic recombination in heterozygous animals. The procedure for clone induction was similar to that for the screen, except that known alleles were crossed to the *FRT, FLPase*-containing line, and heat shocks were applied daily to induce multiple clones per disk. (Clone induction with this protocol is typically so extensive that all of the animals of the correct genotype die as pupae or nonpupating larvae.) Wing discs were stained with the mouse monoclonal anti-myospheroid CF.6G11 and goat anti-mouse fluorescein isothiocyanate (Jackson ImmunoResearch Laboratories) as previously described (Brower *et al.*, 1984).

RNAi Treatment of Cells

Production and use of double-stranded, interfering RNA (RNAi) was similar to that described in Clemens *et al.* (2000). To produce a DNA fragment containing 681 base pairs of 3′-untranslated sequence specific for the endogenous *myospheroid* gene (the 3′-untranslated sequence in the transgenes has been replaced with sequences from the *Drosophila* tubulin α-1 gene; Bunch and Brower, 1992), genomic DNA from S2 cells was amplified by PCR with the primers mys3′d1 (CGGAAATCAGAAGGAACCC) and mys3′u2 (GTTAAGTATCCCAATTCTGAC). This fragment was then amplified using similar primers that also contained a 5′ T7 RNA polymerase binding site (GAATTAATACGACTCACTATAGGGAGA). The PCR products were purified using a QIAquick PCR purification kit (QIAGEN) and used as templates to produce double-stranded RNA via the MEGASCRIPT T7 transcription kit (Ambion, Austin, TX). The double-stranded RNA was ethanol precipitated and resuspended in water, incubated at 65°C for 30 min followed by slow cooling to room temperature, and then stored at −20°C until use. Preliminary experiments showed similar effects when the final concentration of RNAi in the growth medium was 3–30 μg/ml. We used a final concentration of 15 μg of RNAi/ml medium in the experiments reported herein.

For treatment of cells with RNAi, 1×10^6 cells were washed and suspended in 0.66 ml of HyQ-CCM3 serum-free medium (Hyclone Laboratories, Logan, UT) per well of a six-well culture dish. RNAi (30 μg) was added and the cells were incubated for 30 min at room temperature followed by the addition of 1.34 ml of M3 + 12.5% fetal calf serum + 0.2 μM methotrexate. Experiments were done 5 d or more after initial exposure to RNAi. Every 5 d cells were diluted in fresh medium containing 15 μg of RNAi/ml.

The effectiveness of RNAi treatment was tested on cells transfected with a gene expressing the αPS2 subunit from a heat shock promoter (Bunch and Brower, 1992), without a transgene expressing the βPS subunit. Therefore, expression of PS2 integrin is dependent solely on the endogenous βPS gene. If these cells are heat shocked to induce high expression of αPS2, RNAi treatment results in an 80–90% reduction in integrin expression levels, as measured by flow cytometry; thus, RNAi treatment does not eliminate steady-state βPS integrin expression. However, under the conditions of our cell spreading and expression experiments, where cells are cleared of preexisting integrins during the heat shock by the addition of dispase/collagenase, and then analyzed after 3–4 h of recovery, RNAi treatment results in a virtually complete inhibition of integrin expression (our unpublished data). It should also be noted that under the experimental conditions used in this article, there are also high levels of competing βPS subunits expressed from the heat shock-driven transgenes.

Cell Spreading Assays

Cell culture techniques and methods for transfection of cells have been previously described, as have Schneider's line 2 cells transfected with integrin transgenes under the regulation of the heat shock protein 70 promoter (Bunch and Brower, 1992; Zavortink *et al.*, 1993). Some of the current transformed cell lines were transfected using the CellFECTIN technique (Invitrogen). PCR was used

to generate mutations in the pHSβPS plasmid that correspond to the protein encoded by the mys^{G1} mutation and a cytoplasmic truncation that is missing exon 7 ($mys^{\Delta 7}$). In all experiments, the transgenes corresponded to the "c" isoform of αPS2 and the "4A" isoform of βPS (Graner *et al.*, 1998).

The ligand used in cell spreading assays is RBB-Tig. RBB-Tig is a bacterial fusion protein that contains 53 amino acids of the *Drosophila* extracellular matrix protein tiggrin (residues 1964–2016, including the RGD sequence and 25 amino acids upstream and downstream), fused to a histidine tag, from the pTrcHisB vector (Xpress SystemTM; Invitrogen). This fusion protein is as active in promoting cell spreading as the previously described tiggrin fusion protein and tiggrin itself (Fogerty *et al.*, 1994). Fusion protein was purified by affinity chromatography on Ni-NTA agarose (QIAexpress; QIAGEN). Ninety-six well tissue culture plates or slides were incubated with 500 ng/ml RBB-Tig diluted in phosphate-buffered saline (PBS) for either 1 h at room temperature or overnight at 4°C, blocked with 10% dried milk in PBS for 1 h at room temperature, and washed three times with PBS.

Standard cell spreading assays involved pretreatment with dispase/collagenase before integrin expression and assays for cell spreading in serum-free medium. These were done as previously described (Bunch and Brower, 1992; Zavortink *et al.*, 1993) with one change. The dispase/collagenase treatment to remove existing integrins and extracellular matrix was done at 37°C at the same time as the heat shock, which induces expression of the integrin transgenes. Staining cells for surface integrins demonstrated that this treatment quantitatively removes the existing heterodimers from the surface of the cells. For RNAi-treated cells, RNAi was included in the cell-spreading medium (M3 + 2 mg/ml bovine serum albumin). The number of spread cells was determined by microscopy 3–4 h after heat shock and plating. Results of cell spreading assays are expressed as the averages of three experiments with SEs. For the "normal growth conditions" assay, cells were suspended and heat shocked without protease treatment, and replated into regular growth medium with serum. Spreading was assayed overnight on plates without RBB-Tig, or 3–4 h postinduction on plates coated with RBB-Tig.

To examine integrin expression levels, cells were incubated with biotinylated anti-αPS2 integrin monoclonal antibodies (CF.2C7) and R-phycoerythrin-streptavidin (Molecular Probes, Eugene, OR) in M3 medium + 10% fetal calf serum on ice, followed by dilution with 1 ml of 2% formaldehyde in PBS. Cells were analyzed by flow cytometry at the Arizon Research Labs-Biotechnology Cell Sorting Facility (University of Arizona).

RESULTS

mys^{XR04} Is a Weak Dominant Negative In Situ

A number of strong mutant alleles of *myospheroid* have been isolated previously, and some of these have been shown to make little or no βPS protein. By using these alleles, the *myospheroid* null embryonic lethal phenotype has been described in detail, along with some genetic interactions with other integrin mutants (Wright, 1960, Wieschaus *et al.*, 1984; Newman and Wright, 1981; Wieschaus and Noell, 1986; Leptin *et al.*, 1989; Bunch *et al.*, 1992; Zusman *et al.*, 1993; Roote and Zusman, 1995). In the course of these studies, it was noticed that one EMS-generated allele, mys^{XR04}, displays antimorphic (stronger than the null phenotype) genetic properties (Wilcox, 1990; Bunch *et al.*, 1992; Brabant and Brower, 1993). Specifically, a double heterozygote of mys^{XR04} and a null allele for the αPS2-encoding gene (*inflated*) can be synthetically lethal. It is also true that the embryonic lethal phenotype of mys^{XR04} hemizygous embryos (from heterozygous mothers) can be different from zygotic null embryos; most obviously, they often have a "tail up" phenotype, and the dorsal herniation characteristic of βPS loss is often more severe. This phenotype has also been noted in embryos that lack both maternal and zygotic βPS function (Wieschaus and Noell, 1986; Leptin *et al.*, 1989; Roote and Zusman, 1995), consistent with the hypothesis that mys^{XR04} could exert negative effects on the maternally contributed wild-type βPS.

Table 1. Antimorphic complementation behavior of mys^{XR04}, mys^{G1}, and mys^{P9}

mys^{lethal} allele	mys^{lethal}/mys^{b7} % viability[a]	$mys^{lethal}\ if^{+}/mys^{+}\ if^{null}$ % viability[a]
CC6	57 (110)	n.d.
F1	80 (96)	115 (48)
F2	104 (74)	112 (68)
G4	106 (49)	100 (159)
G12	90 (79)	96 (112)
M2	92 (95)	92 (159)
N13	115 (55)	68 (57)
U3	68 (133)	n.d.
W11	63 (102)	n.d.
XR04	0 (187)	0 (214)
G1	0 (161)	0 (159)
P9	0 (104)	0 (111)

n.d., not determined. (N), number of animals in control class. The crosses with mys^{b7} were done at 28°. Tests with the *inflated* null allele were at 25°.

[a] $\%\ viability = \frac{mys^{lethal}/mys^{b7}}{FM7/mys^{b7}}$ or $\frac{mys^{lethal}\ if^{+}/mys^{+}\ if^{null}}{FM7/mys^{+}\ if^{null}} \times 100$ from a cross in which the numerator and denominator classes should segregate in equal numbers (see MATERIALS AND METHODS).

In addition to the above-mentioned phenotypes, we have characterized another indicator of the antimorphic properties of mys^{XR04}. Following on the findings of Bunch *et al.* (1992), we have analyzed the complementation behavior of dozens of weak (hypomorphic) alleles of *myospheroid*, and find that most show significant viability alone or in combination with null alleles, especially at low temperatures, but that these typically are completely lethal when trans-heterozygous with mys^{XR04} (unpublished data). Table 1 gives complementation data for mys^{b7}, one of the weakest hypomorphic alleles.

Additional Myospheroid Alleles

We performed genetic screens that isolated additional mutant alleles of *myospheroid*. Male flies were fed the mutagen EMS and crossed to females to generate F1 animals. During larval development, somatic recombination was induced on the *myospheroid*-containing X chromosomes, to generate small clones of cells homozygous for the mutagenized X chromosome (Figure 1). The adult F1 flies were then screened for wing blisters, known to be caused by strong mutations in *myospheroid*. Potential mutations in βPS were confirmed by complementation testing with other alleles of *myospheroid*, and, as described below, by sequencing. (See MATERIALS AND METHODS and Brower *et al.*, 1995, for screen details.)

Although we have not characterized the full range of embryonic lethal phenotypes for all of the new alleles generated from this screen, they all show the dorsal herniation typical of null alleles of *myospheroid*. We have examined some alleles in more detail, including mys^{G1}, mys^{G4}, mys^{G12}, mys^{M2}, and mys^{P9}. No obvious deviations from the previously described null phenotype (Wright, 1960) have been noted, except for mys^{G1} and mys^{P9}. These alleles can display the tail up and extreme herniation phenotype (Figure 2), similar to that described for mys^{XR04}. Because the phenotypes are variable, we sought to quantify the penetrance of the severe herniation. As shown in Figure 3, embryos of mys^{G1} and mys^{P9} were both significantly more severe than the null alleles.

Embryos doubly mutant for null alleles of *myospheroid* and *inflated* (αPS2) also were much more likely to exhibit a large dorsal hole (Figure 3). Because there is no maternally contributed *inflated* gene product (Bogaert *et al.*, 1987; Roote and Zusman, 1995), the double mutant will be similar to a complete zygotic and partial maternal *myospheroid* mutant. The similarity of the double mutants and the *myospheroid* antimorphs supports the notion that the antimorphs lead to a reduction in the maternal wild-type *myospheroid* function.

All of the strong *myospheroid* alleles were tested for suggestions of dominant negative behavior in crosses with weak alleles (Table 1). Again, mys^{G1} and mys^{P9} consistently behaved similarly to the antimorphic mys^{XR04} in these assays, whereas other alleles behaved similarly to known null mutations. Like mys^{XR04}, mys^{G1} and mys^{P9} also were synthetically lethal when in a double heterozygote with an *inflated* (αPS2) null allele.

Dominant Negative Alleles Have Similar Molecular Lesions

We sequenced the coding regions of the genomic DNA for all of the new *myospheroid* alleles, as well as older alleles that displayed strong, apparently null phenotypes. As shown in Table 2, many of the alleles contain premature stop codons, deletions, frameshifts, or splicing mutations that would be expected to lead to a complete lack of functional βPS protein, and for at least some of the alleles, this expectation has been confirmed by immunofluorescence (see below) or Western analysis (Leptin *et al.*, 1989; Bunch *et al.*, 1992). Only two alleles, mys^{G4} and mys^{G12}, are characterized by missense mutations. One of these, mys^{G4}, alters the second serine of the essential DXSXS MIDAS motif, whereas mys^{G12} changes the aspartate in a DYPS(hydrophobic) motif that is highly conserved in β subunits. One other *myospheroid* allele that is 100% lethal, mys^{XN101}, also results from a missense mutation (C627>S) in the extracellular stalk domain; however, the lethal phenotype of this allele indicates that it retains some wild-type function (our unpublished data).

The three alleles showing weak dominant negative effects all have molecular lesions midway through the cytoplasmic domain of the βPS subunit. Both the mys^{XR04} and mys^{G1} chromosomes contain identical G>A mutations in the splice acceptor site before the seventh exon (Figure 4). Because these two mutations were generated independently in different genetic backgrounds (as verified by molecular polymorphisms in the two mutant strains), we can be confident that the unusual antimorphic phenotype seen in both lines results from this specific molecular lesion. The mys^{P9} chromosome contains a nonsense mutation in exon 6, in the third codon upstream of the above-mentioned splice site.

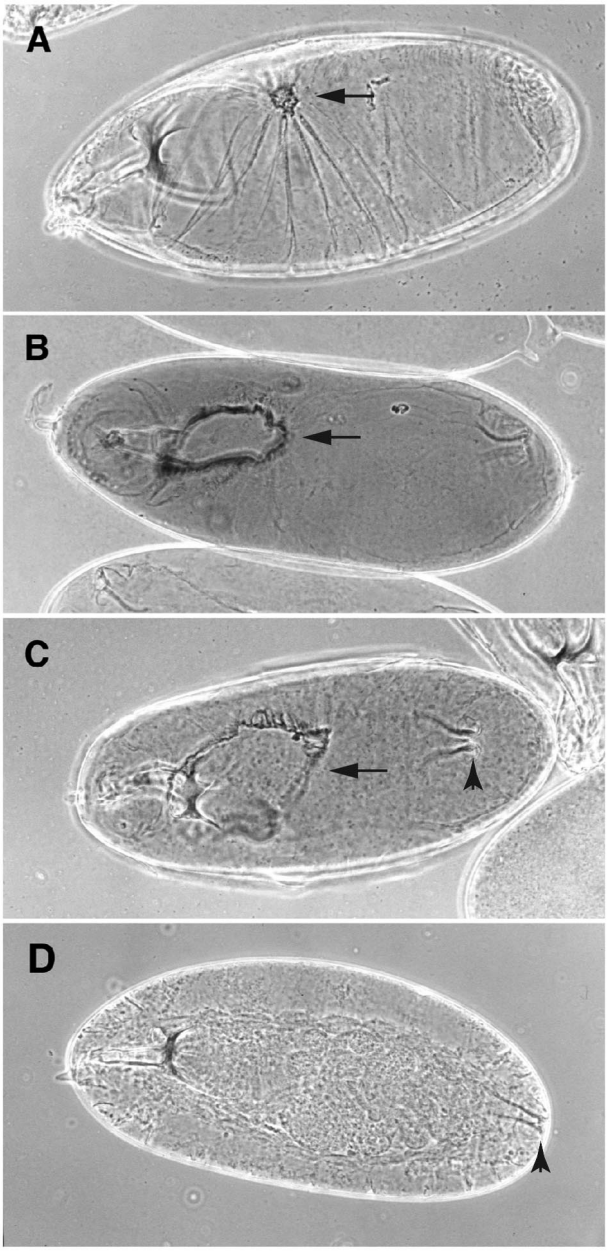

Figure 2. Embryo cuticle preparations from *myospheroid* mutants. The embryos show small, medium, and large dorsal holes (arrows) from the null mutant mys^{XG43} (A and B) and the antimorphic mys^{P9} (C) mutant. Frequencies of these phenotypes are quantitated in Figure 3. Note the anterior dorsal movement of the posterior spiracles (arrowheads) in the mys^{P9} embryo (C) relative to the wild type (D). Bar, ~0.1 mm.

Analysis of Dominant Negative Mutant mRNAs

The three cytoplasmic mutants all have mutations that have the potential to alter mRNA splicing, and so we character-

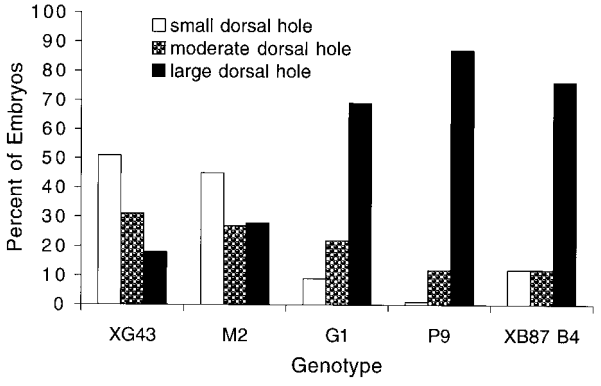

Figure 3. Dorsal herniation phenotype of *myospheroid* mutants. Hemizygous mutant embryos from the *myospheroid* alleles indicated, and the double mutant mys^{XB87} if^{B4}, were dechorionated and scored for the size of the dorsal hole. Larger holes are more frequent in the antimorphic and double mutants.

ized the transcripts that result from these lesions. The mutated guanosine in mys^{XR04} and mys^{G1} is required for proper mRNA splicing, and from examination of nearby sequences, three splicing outcomes seemed possible for the mutant mRNA: 1) The splice would shift one nucleotide to the next guanosine, creating a frameshift. 2) Splicing would occur at a nearby downstream sequence (24 nucleotides away) that resembles a consensus acceptor sequence (Figure 4); this would create an in-frame deletion of eight residues, centered on the first of the two NPXY motifs present in integrin β subunits (see DISCUSSION). 3) Splicing would not occur nearby, presumably resulting in a string of missense residues. To distinguish between these possibilities, we performed RT-PCR and sequence analyses of mRNA from mys^{XR04} mutant embryos. PCR primers were designed to generate a 273-base pair fragment (in wild type) that includes the splice junction and the potential downstream splice acceptor site. Only a single gel fragment was visualized for mys^{XR04}. The sequence of the fragment indicated a splicing shift deleting one nucleotide of the mys^{XR04} cDNA (corresponding to possibility 1 above). The frameshift introduced by the aberrant splice causes exon 7 to encode 25 novel residues (Figure 4). In mys^{G1}, a nucleotide polymorphism present in the parental strain, and which is silent in the normal reading frame, changes the alanine at the start of the new sequence to valine. Despite this minor difference, we will refer to the frameshifted βPS subunits generically as the $\beta PS^{XR04\text{-}G1}$ protein. This mutant protein is missing both NPXY motifs.

At first sight, the mys^{P9} mutation appears to be a straightforward stop codon, leading to a protein truncated three residues before the $mys^{XR04\text{-}G1}$ frameshift. However, closer examination of the nucleotide sequence reveals the possibility of a cryptic splice donor sequence just upstream, and the mys^{P9} mutation improves the potential intron consensus sequence at this site. To see whether mys^{P9} might lead to significant use of this new potential splice site, we did RT-PCR analysis of mRNA from heterozygous mys^{P9} animals. We found no trace of the smaller (263 vs. 273 base pairs) aberrantly spliced mRNA on agarose gels or upon direct sequencing of the PCR products. There also was no indication that expression level of the mutant mRNA was reduced. Thus, the mys^{P9} mutation apparently leads to a straightforward truncation (Figure 4).

As mentioned above, examination of the nucleotide sequence of the wild-type gene indicated the potential for mRNA splicing that would precisely delete eight amino acids, centered on the first NPXY motif. We were curious to

Table 2. Null and antimorphic alleles of *myospheroid*

mys allele	Nucleotide change	Predicted amino acid change	Notes
Nulls			
CC6	C224>A	S75>stop	
F1	G955>A	D319>N,	
	Δ961–975	Δ322–326	
F2	Rearrangement or deletion?		Cannot amplify 3′ fragment for sequencing
G4	C587>T	S196>F	Protein localizes properly to muscle attachments
G12	G14>A	R5>K	Protein localizes properly to muscle attachments
	G1066>A	D356>N	
M2	C196>T	Q66>stop	No protein detected
N13	Large internal deletion	Frameshift after residue 127	
U3	C2296>T	Q766>stop	
W11	C1180>T	Q394>stop	
XB87	G>A at exon 3 splice acceptor		No protein detected
XG43	Δ2110–2222	Frameshift after residue 704	No protein detected
Antimorphs			
G1	G>A at exon 7 splice acceptor	Frameshift after residue 825	All phenotypes similar in this class; protein expressed on surface
P9	G2469>A	W823>stop	
XR04	G>A at exon 7 splice acceptor	Frameshift after residue 825	

ND, not determined.

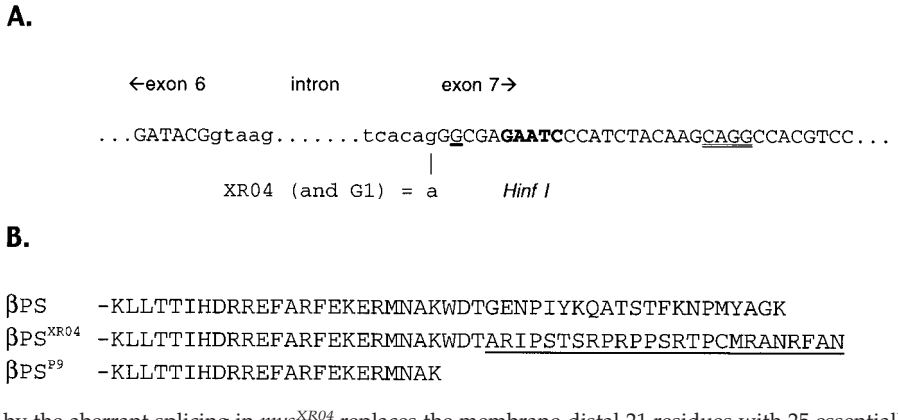

Figure 4. (A) Nucleotide sequence around the boundaries of the cytoplasmic intron of *myospheroid*. Exon sequence in caps, intron in lowercase. The G>A mutation in mys^{XR04} (and mys^{G1}) is indicated; this results in the seventh exon beginning with the underlined G. The potential downstream splice acceptor site is indicated by double underlining. To search for mRNAs that might use this site, this entire region was amplified by RT-PCR. Amplification of products using the conventional splice site was inhibited by cutting at the *Hin*fI site indicated in bold (see text). (B) Predicted amino acid sequence of the cytoplasmic domains of wild-type βPS and that from mys^{XR04} and mys^{P9}. The frameshift induced by the aberrant splicing in mys^{XR04} replaces the membrane-distal 21 residues with 25 essentially random amino acids (underlined). The first cytosine of exon 7 is a thymidine in mys^{G1}; this polymorphism is silent in the parental strain, but changes to first residue encoded by the mutant exon 7 from alanine to valine.

see whether the potential downstream splice site might be used in wild-type animals, at some restricted time or place in development. To examine this possibility, cDNA from five developmental stages, embryo through adult, of wild-type animals was used for RT-PCR as mentioned above. We anticipated that the potential alternatively spliced cDNA might be relatively rare, and PCR amplification can be a competitive process. To maximize the likelihood of generating sufficient amounts of the fragment in question, we digested the amplified DNA at various times during the amplification procedure, taking advantage of a *Hin*fI restriction site present only in the common, larger PCR fragment (Figure 4). Several variations of this scheme were tried, but a new fragment size was never seen.

Expression of Mutant Proteins

We examined a number of the mutant alleles by immunofluorescence for expression of surface integrin. As expected, the nonsense mys^{M2} mutant shows no surface PS integrin in late-stage embryos (our unpublished data); loss of PS integrins has previously been reported for the deletion and splicing mutants mys^{XG43} and mys^{XB87} (Leptin *et al.*, 1989; Bunch *et al.*, 1992). Both missense alleles, mys^{G4} and mys^{G12}, express surface integrin at levels comparable to wild-type embryos (Figure 5), and show the typical accumulation of protein at embryonic muscle attachment sites. (The embryos shown in Figure 5 are fixed before the contractions that lead to the muscle detachment characteristic of the mutant phenotype.)

The frameshifted $\beta PS^{XR04\text{-}G1}$ protein also appears to be expressed in embryos, and localized at muscle attachments. To look more thoroughly for possible expression reductions in these mutants, we examined surface integrin expression on the epithelium of larval imaginal discs containing clones of homozygous mys^{G1} cells in a heterozygous background. These clones were generated similarly to those used in the mosaic screen for mutant alleles, except that the larvae were heat shocked more extensively (daily) to induce larger numbers of clones. As shown in Figure 6, control animals with the mys^{M2} nonsense allele typically (13 of 15 wing discs) showed multiple patches of cells without detectable integrin expression. However, no patches of reduced expression were detected in mys^{G1} discs grown and heat shocked at the same time (51 wing discs examined). Thus, the weakly dominant negative allele mys^{G1} does not lead to large-scale reduction in integrin expression in the disk epithelium.

Dominant Negative Mutants Support Cell Spreading in Culture

To test the ability of mutant proteins to function in a simple cell spreading assay, we transformed *Drosophila* S2 cells with cDNAs for αPS2 (the c splice variant) and βPS (the 4A splice variant) proteins corresponding to the mys^{G1} mutant and a mutant similar to mys^{P9}, but truncated precisely at the exon 6–7 splice boundary (designated $mys^{\Delta 7}$). This latter protein is three residues longer than that encoded by mys^{P9}. All transformed genes were under the control of a heat shock promoter, and for each the endogenous 3′-untranslated sequences were replaced with tubulin α1 3′ sequence (Bunch and Brower, 1992; Graner *et al.*, 1998).

S2 cells produce small but significant amounts of endogenous βPS, and cells transformed with αPS2 alone show a significant ability to spread on RBB-Tig, an RGD-containing fragment of the PS2 ligand tiggrin (Fogerty *et al.*, 1994). This spreading is reduced by ~90% when the cells are treated with double-stranded RNAi (Carthew, 2001) against the 3′-untranslated region of the endogenous βPS transcript (Figure 7). When cells are transformed with both αPS2 and βPS genes, and subjected to the protease clearing and heat shock induction protocol, RNAi has little effect, indicating that virtually all of the spreading is due to expression from the transformed genes. This RNAi control was used for all of the results reported herein.

Both the mys^{G1} and $mys^{\Delta 7}$ proteins were expressed well on S2 cells in combination with αPS2. We tested the ability of the mutant proteins to mediate cell spreading under two different assay conditions. In one, cells are treated with protease to clear surface proteins and ECM, heat shocked to induce integrins, and allowed to spread on RBB-Tig. In this assay, both mys^{G1}- and $mys^{\Delta 7}$-containing integrins promote spreading that is comparable to that seen with wild-type βPS (Figure 7). Although slight cell line variations were

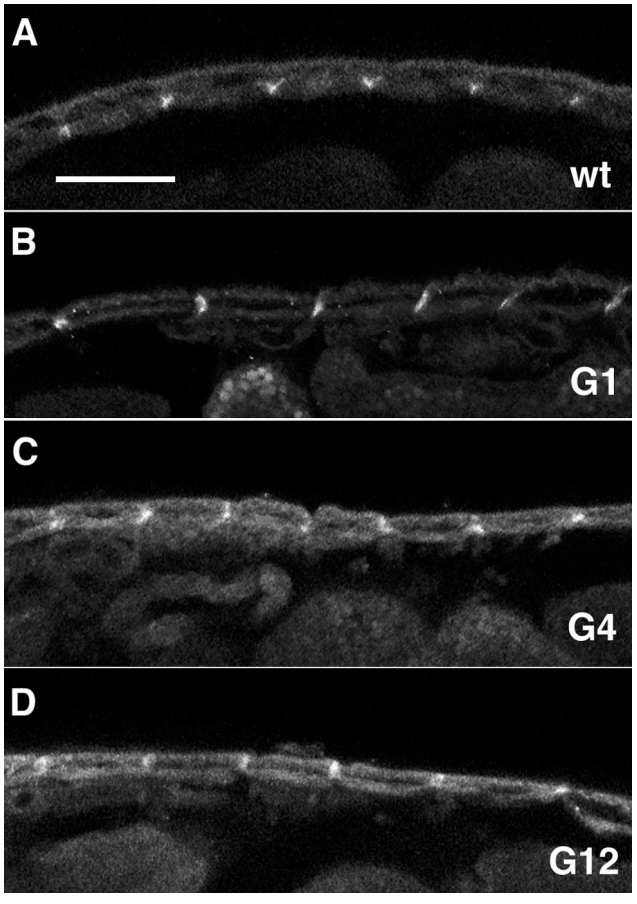

Figure 5. Confocal immunofluorescence sections of the hypoderm and underlying muscles of stage 15–16 embryos. The animals are stained with an antibody against the βPS subunit, which is seen to be concentrated in a metameric array of dots corresponding to muscle attachment sites. The wild type (wt) is a heterozygote for wild type over the nonsense allele mys^{M2}; homozygotes for mys^{M2} show no staining. Bar, ~50 μm.

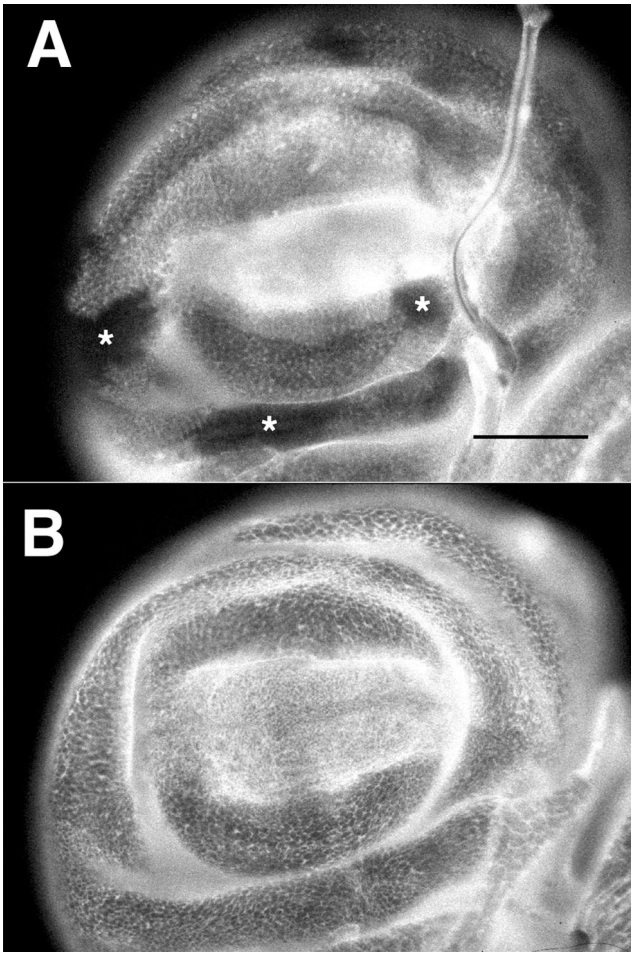

Figure 6. Third instar wing imaginal discs stained with antibody against βPS. These animals are heterozygous for *myospheroid* mutations, and have been treated to induce recombinant clones of homozygous mutant cells. (A) Heterozygote for the nonsense mutation mys^{M2}; the clonal patches show no surface expression of PS integrins (asterisks). (B) Disk from mys^{G1} heterozygote cross, which shows no patches of reduced integrin expression. Bar, ~100 μm.

observed, these variations could be ascribed to minor differences in the expression levels of the integrins (our unpublished data).

Although the mutant integrins can mediate spreading in the above-mentioned assay, we noticed that these cells spread very poorly during regular culture, relative to cells expressing wild-type β subunits. To quantitate this, we performed a second assay similar to regular growth conditions, in which the cells were not pretreated with protease but were suspended, heat shocked to induce high-level integrin expression, and allowed to spread on plates overnight in regular growth medium containing serum. (Although not shown, qualitatively similar results were obtained if uncleared cells were spread on RBB-Tig for 3–4 h.) Under these conditions, the mutant integrins promote cell spreading very poorly, relative to wild type (Figure 8), despite the fact that these cells are expressing more integrin than is seen after protease clearing and induction (our unpublished data). Interestingly, in this assay the activity of the mutant βPS subunits can be largely restored if combined with αPS2 subunits containing a mutation of the conserved cytoplasmic GFFXR motif (GFFNR>GFANA) that promotes integrin activation (O'Toole *et al.*, 1994; Hughes *et al.*, 1996).

The morphology of spread cells on RBB-Tig may also be influenced by expression of the mys^{G1}- and $mys^{\Delta 7}$-containing integrins. Although a variety of morphologies are typical even in wild type, the mutants tend to display more regions of extreme peripheral thinning (Figure 9). These are characteristics also observed in S2 cells containing mutants that affect the ability of the cell to regulate integrin activity, such as the activating αPS2 mutants (our unpublished data).

DISCUSSION

The most interesting of the mutant alleles described herein are the three cytoplasmic mutants that display dominant negative properties in flies, and we will refer to them col-

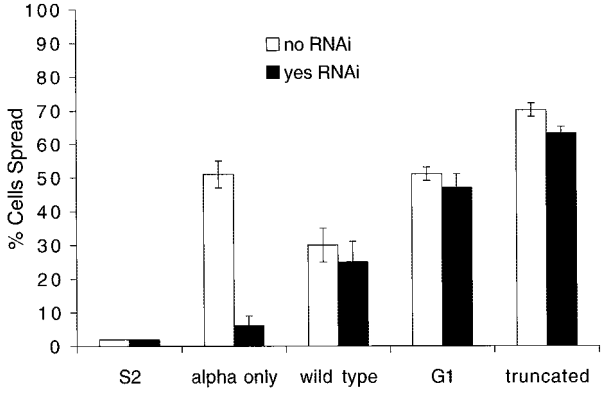

Figure 7. Cell spreading by mutant β subunits. S2 cells were transformed with αPS2 and various forms of βPS, and assayed for spreading on a fragment of the PS2 ligand tiggrin. For these experiments, cells were pretreated with protease to remove surface proteins and ECM before integrin induction. RNAi was added to inhibit expression of endogenous βPS (filled bars). Untransformed S2 cells do not spread. Cells transformed with αPS2 only can spread using the low levels of endogenous βPS, but this is reduced by RNAi treatment. Spreading of cells transformed with wild-type or mutant βPS is not significantly reduced by RNAi. The difference between wild-type and the two βPS mutants reflects differences in expression level.

lectively as mys^{DN} alleles. Although they are viable as heterozygotes, these animals have reduced integrin function relative to animals heterozygous for null alleles. This is perhaps best illustrated by the synthetic lethality of the mys^{DN} $inflated^{null}$ double heterozygotes; reducing αPS2 expression by 50% is not sufficient to generate phenotypes in a

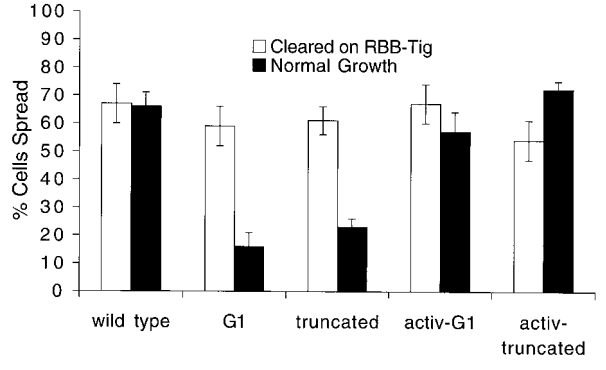

Figure 8. Cell spreading in different assay conditions. Integrin induction in transformed S2 cells followed protease treatment as before (open bars) or was in the absence of protease (filled bars). In the latter case, the spreading assay was in normal growth medium, with serum, on uncoated tissue culture plates. activ-G1 and activ-truncation indicate cells that also express a mutant αPS2 that promotes integrin activation. The mutant β subunits support spreading poorly except in conditions (cleared cells or activating αPS2 subunits) that appear to promote artificially high activation. This is true in spite of the fact that the normal growth G1 and truncated cells express integrin at higher levels than any of the cleared or activating αPS2 lines.

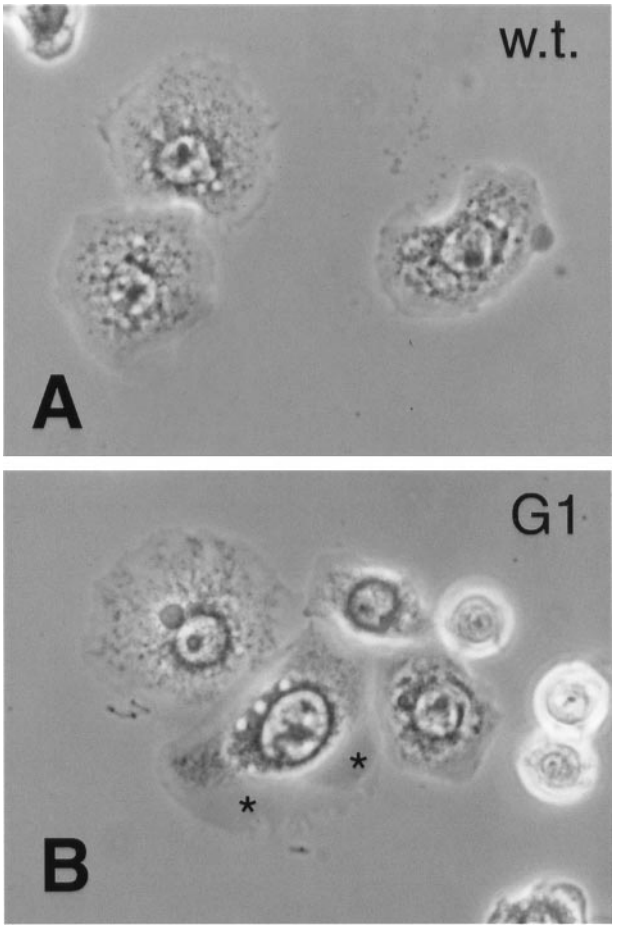

Figure 9. *Drosophila* S2 cells that were cleared, induced to express wild-type or mys^{G1} mutant βPS in association with αPS2, and allowed to spread on high concentrations of a recombinant fragment of tiggrin. The mutant cells have a tendency to display more thin, clear areas at the periphery (asterisks).

myospheroid null heterozygote. Dominant negative qualities also are indicated by crosses with weak alleles; although mys^{b7}/mys^{null} supports viability very well at high temperatures, the mys^{b7}/mys^{DN} combination is strongly lethal. Finally, the embryonic lethal phenotypes of the mutants are consistent with the idea that these alleles compromise the functioning of the maternally contributed wild-type βPS.

Similarity to Mammalian β Subunit Variants

The position of the cytoplasmic intron is conserved in most integrin β subunit genes (Schmitt and Brower, 2001), and just as mys^{XR04} and mys^{G1} create a frameshift at this site (caused by shifting the splice site one nucleotide), similar naturally occurring cytoplasmic splice variations have been described for human β1 and β3 (van Kuppevelt *et al.*, 1989; Altruda *et al.*, 1990; Languino and Ruoslahti, 1992; Kumar *et al.*, 1997; Svineng *et al.*, 1998; for review, see Melker and Sonnenberg, 1999). The β1C splice variant has been shown to result in loss of some cytoplasmic associations and sig-

naling properties, and to affect cell proliferation (Languino and Ruoslahti, 1992; Fornaro *et al.*, 1995, 1998, 2000; Meredith *et al.*, 1995; Pfaff *et al.*, 1998). Human β3B and β3C splice variants also generally show a decrease in cellular functions such as focal adhesion localization, focal adhesion kinase phosphorylation, and cell adhesion (Akiyama *et al.*, 1994; LaFlamme *et al.* 1994; Kumar *et al.*, 1997; Pfaff *et al.*, 1998). Extensive analyses of one human isoform, β1B, have uncovered dominant negative properties similar to those we see, and we will compare human β1B with our *Drosophila* mutants in greater detail.

Like mys^{XR04} and mys^{G1}, human β1B alters the protein sequence (relative to the common β1A isoform) distally from the splice site, but in this case as a result of a failure to splice and subsequent translation into the intron (Altruda *et al.*, 1990). The β1B isoform is expressed primarily in skin and liver, where it constitutes a minority of the β1 protein (Balzac *et al.*, 1993). To date, no function of β1B has been demonstrated in situ, but there have been a number of studies of the properties of the isoform in cultured cells (Balzac *et al.*, 1993, 1994; Cali *et al.*, 1998; Retta *et al.*, 1998; Armulik *et al.*, 2000). Overall, this work indicates that β1B is deficient in both "outside in" and "inside out" integrin signaling. For example, to mediate adhesion, the β1B-containing integrins typically require activation using nonphysiological treatments such as incubation in Mn^{2+} ions. And even upon adhesion or antibody cross-linking, β1B integrins are not efficient stimulators of cytoplasmic proteins such as focal adhesion kinase and paxillin.

Perhaps the most striking property of β1B is its ability to act in a dominant negative manner with respect to other cellular integrins. When transfected into cells, β1B has been reported to inhibit processes mediated by other β1 or β3 integrins, including cell spreading, motility, matrix assembly, and stimulation of specific cytoplasmic proteins (Balzac *et al.*, 1994; Cali *et al.*, 1998; Retta *et al.*, 1998; see Armulik *et al.*, 2000, for a contradictory view of the effects on αvβ3 functions). Retta *et al.* (1998) further report that these dominant negative properties are dependent on the "intron-encoded" residues; proteins that are simply truncated at the splice site do not inhibit the functions of endogenous integrins.

All of the experiments showing dominant negative effects of β1B rely on cell transfection, and the mys^{DN} mutants are the first demonstration that replacement of the C-terminal portion of the β cytoplasmic domain can have dominant negative effects in situ. And because the *Drosophila* mutants are generated in otherwise normal chromosomes, and their expression is controlled by the wild-type regulatory machinery, it is by analogy reasonable to think that physiological levels of β1B expression could have significant repercussions for human cells in situ.

Why Dominant Negative?

How do the mys^{DN} mutants (and by extension, human β1B) exert their unusual properties? We can rule out some possibilities from these studies. It has recently been noted that β1B contains a new double lysine motif that can reduce surface expression through trapping of the protein in the endoplasmic reticulum (Kee *et al.*, 2000). The βPS^{DN} proteins contain no similar double lysine, and our expression studies in situ indicate that the mutant proteins reach the cell surface without great difficulty.

Another potential explanation is that the βPS^{DN} proteins just get in the way, by adding a pool of nonfunctional integrins that compete on the cell surface with the wild-type proteins. The simplest version of this scenario does not appear to be true, because two missense alleles (mys^{G4} and mys^{G12}) that make stable but probably nonfunctional (for ligand binding) protein do not show any dominant negative genetic properties. Competition models might be workable if one proposes that the cytoplasmic mutants also lead to greatly enhanced protein stability, so that the mutant proteins would eventually be present at much higher numbers than their wild-type competitors. However, when we follow the turnover of surface integrin after a single heat pulse of our transformed S2 cells, we see no indication that the βPS^{DN} proteins are unusually stable (our unpublished data). And, in the analogous β1B system, expression of the variant can exert dominant negative effects on β1A functions when each is expressed at equivalent levels (Balzac *et al.*, 1994). Also, β1B-expressing cells can display phenotypes such as reduced αv expression (presumably in αvβ3 heterodimers) in focal adhesions, without β1B actually displacing the αv integrins directly (Retta *et al.*, 1998).

Our genetic results are consistent with the previous proposal that the dominant negative mutants create a cellular signal that leads to inactivation of other surface integrins (Retta *et al.*, 1998). This idea also is consistent with data on the activity of the dominant negative mutants themselves. In general, disruptions of β subunit NPXY motifs leads to reduced integrin activation and cytoskeletal associations, but β1B, for example, can mediate cell adhesion if activated artificially with Mn^{2+} (Retta *et al.*, 1998; Armulik *et al.*, 2000). In our S2 cell assays, we see evidence that the βPS^{DN} proteins are only poorly able to mediate cell spreading during normal growth, but that a latent activity can be uncovered under conditions that are known (mutant α subunits) or suspected (protease pretreatment) to activate integrins. This also argues that a primary affect of the βPS^{DN} proteins is related to the cells' ability to regulate integrin function.

The *Drosophila* results further allow some insights as to the structural requirements for the dominant negative properties of these cytoplasmic variants. The failure to detect inhibitory effects of subunits truncated at the splice site led Retta *et al.* (1998) to surmise that some contribution of the new residues of β1B is important. There is no sequence similarity between the C-terminal residues of the $\beta PS^{XR04\text{-}G1}$ proteins and those of β1B, making it unlikely that a sequence-specific motif is involved. Moreover, we find that a truncation, mys^{P9}, has dominant negative properties in situ similar to the frameshift mutants, in contrast to the results of Retta *et al.* (1998) in cell culture. Thus, it appears that in general there is no absolute requirement for a particular sequence, or any sequence after the cytoplasmic splice site, to induce dominant negative effects. It is possible that β1B and βPS are truly different in this respect, but it seems equally likely that if the human proteins were assayed in situ, where the entire range of integrin functions is required, dominant negative effects might emerge for the truncated β1 proteins.

Like β1B, the βPS^{DN} proteins are missing both cytoplasmic NPXY motifs, which, along with neighboring residues,

have been shown to be important for activation of extracellular ligand binding, intracellular signal transduction events, or processes such as adhesion or migration mediated by vertebrate integrins (Reszka *et al.*, 1992; Filardo *et al.*, 1995; O'Toole *et al.*, 1995; Ylänne *et al.*, 1995; Baker *et al.*, 1997; Blystone *et al.*, 1997; Chang *et al.*, 1997; Tahiliani *et al.*, 1997; Vignoud *et al.*, 1997; Loo *et al.*, 1998; Pfaff *et al.*, 1998; Romzek *et al.*, 1998; Sakai *et al.*, 1998; Schaffner-Reckinger *et al.*, 1998; Buttery *et al.*, 1999; Kaapa *et al.*, 1999; Mastrangelo *et al.*, 1999; Sakai *et al.*, 1999; Levy *et al.*, 2000; Stroeken *et al.*, 2000; Wennerberg *et al.*, 2000; Boettiger *et al.*, 2001; Ginsberg *et al.*, 2001). Numerous studies have demonstrated requirements for specific residues in these motifs, although dominant negative effects have not generally been tested or noted. However, one anecdotal piece of evidence suggests that these motifs may be critical to the unusual genetics of our mutants and βlB. Grinblat *et al.* (1994) mutagenized various residues of the cytoplasmic tail of βPS, and asked whether these mutants when transformed into flies could rescue *myospheroid* null phenotypes. (The mutant constructs contained endogenous *myospheroid* regulatory elements, and were generally expressed at levels equal to or below that of the endogenous *myospheroid* gene.) Like some others, they found that changing the tyrosines of the NPXY motifs to phenylalanines had relatively little effect. This alteration is expected to prevent potential phosphorylation of the motifs, but not alter their ability to make a predicted β-turn structure (Haas and Plow, 1997; Ginsberg *et al.*, 2001; Ulmer *et al.*, 2001). However, when Grinblat *et al.* (1994) changed these tyrosines to alanines, they were unable to recover transformants that expressed the mutant proteins. Of course, this failure could have been due to some unknown technical glitch. However considering their success with numerous other constructs, Grinblat *et al.* (1994) proposed that the pair of Y>A mutations creates a toxic protein. Our results support this proposal, and combined, the studies suggest that the disruption of NPXY motifs is the critical requirement for creating these dominant negative β subunits. Finally, it appears that most of the proximal cytoplasmic tail must be intact for strong dominant negative properties. For example, Grinblat *et al.* (1994) were able to generate transformants with a cytoplasmic truncation that is only a few residues shorter than our dominant negative mys^{P9} truncation.

Scarcity of Essential Residues of βPS

Two missense mutations of *myospheroid* display the same strong phenotype seen for alleles that produce no functional βPS. Both of the "null" missense mutations change oxygenated residues in the globular head domain of βPS. mys^{G4} (S196>F) changes the second serine of the conserved MIDAS sequence DXSXS, which is involved in the formation of a cation-binding pocket (Xiong *et al.*, 2001), and this serine has been shown to be required for function or stability for vertebrate β2 and β3 (Bajt and Loftus, 1994; Bajt *et al.*, 1995; Hogg *et al.*, 1999). The mys^{G12} chromosome contains two missense mutations, R5>K and D356>N. The latter change most likely is responsible for the mutant phenotype, because this residue is acidic in all sequenced β subunits, and has been shown to be essential for function in β1 (Puzon-McLaughlin and Takada, 1996). The arginine residue at position five is early in the βPS signal sequence. The change to lysine is conservative, and analysis by the PSORT algorithm (Nakai and Kanehisa, 1992) predicts little effect on the ability of the altered domain to function as a signal sequence. Moreover, we find that mys^{G12} (as well as mys^{G4}) subunits are expressed well at the cell surface in situ, arguing strongly that the signal sequence alteration is not responsible for the mutant phenotype.

Not surprisingly, the most common class of genetically strong mutations is nonsense mutants, represented by five alleles. Of the 14 null or antimorphic alleles, only two result from missense mutations. One other missense allele, mys^{XN101}, is known to be 100% lethal and have a dorsal herniation phenotype, but other studies suggest that it has some residual function (Wieschaus *et al.*, 1984; Bunch *et al.*, 1992; our unpublished data). Three of the strong mutations are in splice sites, and four alleles are associated with deletions or other rearrangements, mutagenic events that are expected to be relatively rare after EMS mutagenesis (Ashburner, 1989b). The most common mutation created by EMS is a G-to-A transition. If one assesses the potential results only of G-to-A transitions in the *myospheroid* gene, there are approximately 10 times as many sites that would create missense mutations as the sum of sites that would lead to nonsense mutations and changes that would eliminate correct splicing. The data are limited, but the results to date, in which the number of strong missense alleles is equaled by splice site mutations, suggest that relatively few of the 846 residues of βPS are absolutely essential for function. On the other hand, experiments to be described elsewhere indicate that *myospheroid* missense mutations that alter βPS function, but do not eliminate it, are relatively easy to generate, and just as whole animal genetics provides a sensitive assay for dominant negative properties, forward genetic screens should be useful for finer dissection of other integrin structure–function relationships.

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