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The PS2 integrin ligand tiggrin is required for proper muscle function in *Drosophila*

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SUMMARY

Tiggrin is a novel extracellular matrix ligand for the *Drosophila* PS2 integrins. We have used flanking P elements to generate a precise deletion of *tiggrin*. Most flies lacking tiggrin die as larvae or pupae. A few adults do emerge and these appear to be relatively normal, displaying only misshapen abdomens and a low frequency of wing defects. Examination of larvae shows that muscle connections, function and morphology are defective in *tiggrin* mutants. Muscle contraction waves that extend the length of the larvae are much slower in *tiggrin* mutants. Direct examination of bodywall muscles shows defects in muscle attachment sites, where tiggrin is specifically localized, and muscles appear thinner. Transgenes expressing tiggrin are capable of rescuing *tiggrin* mutant phenotypes. Transgenes expressing a mutant tiggrin, whose Arg-Gly-Asp (RGD) integrin recognition sequence has been mutated to Leu-Gly-Ala (LGA) show much reduced, but significant, rescuing ability. Cell spreading assays detect no interactions of this mutant tiggrin with PS2 integrins. Therefore, while the RGD sequence is critical for PS2 interactions and full activity in the whole fly, the mutant tiggrin retains some function(s) that are probably mediated by interactions with other ECM molecules or cell surface receptors

Key words: *tiggrin*, Integrin, *Drosophila*, Synthetic deletion, Muscle, Extracellular matrix, RGD

INTRODUCTION

The extracellular matrix (ECM) is an interconnected network of glycoproteins, proteoglycans and glycosaminoglycans secreted and assembled by cells. Components of the ECM serve multiple functions. A major role is transmission of the mechanical force of muscle contraction to other body parts, as in vertebrate tendons. In *Drosophila melanogaster*, the novel ECM protein tiggrin is associated with muscles at their attachment sites (Fogerty et al., 1994). At these sites the PS2 integrins function to maintain muscle attachments (Brabant and Brower, 1993; Brown, 1994) and we previously demonstrated that tiggrin is a ligand of the PS2 integrins. This suggests that tiggrin in the ECM binds to the PS2 integrins and mediates PS2-ECM interactions. Furthermore, along striated muscles tiggrin and the PS2 integrins colocalize at Z-bands (Fogerty et al., 1994; unpublished observations). Tiggrin could also have other roles as it is a component of *Drosophila* basement membranes that underlie epithelia and envelop muscles and fat cells (Fogerty et al., 1994). Such basement membrane ECM that is immediately adjacent to cells can influence them by binding or modulating the action of growth factors and by direct interaction with cells through cell surface receptors (reviewed by Adams and Watt, 1993). Here we report on the initial characterization of mutations in the *tiggrin* gene and its relationship to integrins in the whole animal.

Integrins are heterodimeric, transmembrane receptors that bind to ligands in the ECM or to other cell surface proteins and thereby link the outside of the cell to the inside. Integrins' small cytoplasmic domains interact with cellular proteins to transduce force and/or modulate intracellular signaling (Hynes, 1992). In *Drosophila*, the PS1 and PS2 integrins have been extensively studied at both the cellular and genetic level (reviewed by Brown, 1993; Gotwals et al., 1994). PS1 and PS2 integrins contain a common β_{PS} subunit that is associated with either an α_{PS1} or an α_{PS2} subunit. Thus, PS1 and PS2 integrins are respectively $\alpha_{PS1}\beta_{PS}$ and $\alpha_{PS2}\beta_{PS}$ heterodimers. Three observations suggest that tiggrin interacts with the PS2 integrins. First, though tiggrin does not show any strong sequence similarity to other proteins, it does contain the integrin recognition sequence RGD and this sequence is recognized by the PS2 integrins. Second, tiggrin is detected at muscle attachment sites that also contain PS2 integrins. Third, in a cell culture assay, tiggrin supports PS2-mediated cell spreading (Bunch and Brower, 1992; Fogerty et al., 1994).

If all functions of the PS2 integrins are mediated by their

interactions with tiggrin then we would expect *tiggrin* mutants to display the same range of phenotypes that are found in mutations that remove the PS2 integrins. This would include complete detachment of most muscles from their attachment sites, and central nervous system, wing and gut defects (Brabant and Brower, 1993; Brown, 1994; Roote and Zusman, 1995). More likely, tiggrin may be only one of several ligands used by the PS2 integrins, and tiggrin may have additional functions that are independent of its interactions with the PS2 integrins. In this case, phenotypes seen in *tiggrin* mutants may overlap but not mimic those seen in PS2 integrin mutations. To test these possibilities, we have generated mutations in *tiggrin* and tested the ability of tiggrin lacking its RGD integrin recognition sequence to function both in cell culture and the whole animal. In this study, we find that the *tiggrin* mutant phenotypes are complex, affecting some but not all of the tissues that are defective in PS2 integrin mutants. In these tissues, *tiggrin* mutants display novel phenotypes not described for PS2 integrin mutants.

MATERIALS AND METHODS

Flies

Flies were reared at 25°C on food that has been described previously (Condie and Brower, 1989) or on instant fly food (Carolina Biological). For comparison of the length of pupae or larval dissections all were reared on instant food at 22°C except that animals rescued by *tiggrin* transgenes were raised at 25°C to induce higher expression of the transgenes.

Generation of *tig^{x}*, *tig^{A1}* and *tig^{O2}* mutations

Two P elements, *P[5Tw+]* (originally named l(2)k09239 and located 2kb upstream of the transcriptional start site of *tiggrin*) and *P[3Tw+]*(originally named l(2)k07502B and located 5kb downstream of *tiggrin*) (both from the Kiss collection, Berkeley; see Török et al., 1993) were recombined onto the same chromosome. Southern blot and PCR analysis confirmed the presence of both P elements in *w*;*P[5Tw+] P[3Tw+]*/+ flies. Neither of these inserts is lethal nor do they show any phenotypes when homozygous. However, the original chromosomes that were obtained from the Kiss collection did contain lethals that were separated from *P[5Tw+]* and *P[3Tw+]* by recombination.

Both P elements were excised by crossing *w*;*P[5Tw+] P[3Tw+]*/+ to *w*;*Sp*/*CyO*;*Δ2-3 Sb*/*TM3, Ser* to obtain *w*;*P[5Tw+] P[3Tw+]*/*CyO*;*Δ 2-3 Sb*/+ dysgenic males. *Δ2-3* provides a source of transposase, which induces mobilization of P elements (Laski et al., 1986). These were then crossed to *w*;*P[5Tw+] P[3Tw+]*/*CyO*. White-eyed *CyO* non-*Sb* males, which had lost both P elements, were collected. Of 45 excision chromosomes tested 40 (90%) were lethal. Southern analysis of 5 lethals and 1 viable chromosome indicated that in the lethals DNA between the two P elements had been excised (see Fig. 1 for two of these; *tig^{x}* and *tig^{x2}*) while the viable chromosome was a result of 'precise' excisions of the two P elements. One of these deletions was selected and this is the *tig^{x}* mutation. We have seen no evidence that the lethal excisions extend outside of the limits of the two P-element insertion points, but we have not attempted to precisely localize these breakpoints. The breakpoints cannot extend more that 1 kb beyond the original insertions as our Southern blots would detect significant deletions of this DNA (Fig. 1 and data not shown).

To obtain *tiggrin* point mutants, wild-type Oregon-R males were fed EMS (Grigliatti, 1986) and mated to *SM6b*/*Sp* virgins. Individual */*SM6b* males were mated to *tig^{x} wb^{K05612}*/*In(2LR)Gla* virgins and screened for the absence of */*tig^{x} wb^{K05612}* progeny. Two lethal chromosomes were obtained that upon retesting showed a failure to complement *tig^{x}* specifically. These new mutations are *tig^{A1}* and *tig^{O2}*.

Expression of *tiggrin* transgenes

Plasmids pUASTig^{+} and pUASTig^{LGA} (*tiggrin* under the control of UAS^{GAL4}) were constructed by cloning the wild-type or mutant form (identical except the sequence encoding RGD was mutagenized to encode LGA) of the *tiggrin* cDNA into the pUAST vector (Brand and Perrimon, 1993) for injection into embryos. Multiple lines carrying the different transgenes were obtained. *UASTig^{+17}* and *UASTig^{+22}* are wild-type inserts on the second chromosome. *UASTig^{LGA5}*, *UASTig^{LGA3}*, and *UASTig^{LGA2}*, contain mutant transgenes on the 1st, 2nd and 3rd chromosomes respectively.

To drive expression of these UAS^{GAL4} constructs, GAL4 enhancer traps were used. Inserts *P[GAL4^{167Y}w+]* and *P[GAL4^{185Y}w+]* express in scattered cells and the salivary glands in the embryo (Manseau et al., 1997; our observations); *P[GAL4^{c363}w+]* shows a wide expression pattern including the eye, wing, haltere and leg discs, and the brain lobes and ventral ganglia of larvae (unpublished observations). Finally, we have used a GAL4 gene that is controlled by *elav* regulatory sequences *P[GAL4^{c155}]* that promotes expression in the nervous system (from the laboratory of Cory Goodman and available from the Bloomington Stock Center). For a general description of the use of this system, see Brand et al. (1994).

Rescue of *tiggrin* mutants with transgenes

To rescue *tiggrin* mutants, GAL4 drivers (*GAL4Dr*) *167Y, 185Y, c363* and *c155* (all are on the X chromosome) were combined with responders (*UASTig*) *UASTig^{+22}*, *UASTig^{+17}* and *UASTig^{LGA3}* (all on the second chromosome) in the following cross: *w^{1118} GAL4Dr*; *tig^{x}*/*CyO* males × *w^{1118}*; *UASTig tig^{x}*/*In(2LR)Gla* females. Female progeny carry one copy of the *GAL4Dr* and are either *UASTig tig^{x}*/*tig^{x}*, *UASTig tig^{x}*/*CyO*, *tig^{x}*/*In(2LR)Gla* or *CyO*/*In(2LR)Gla*. To calculate the percentage of rescued flies, the number of *UASTig tig^{x}*/*tig^{x}* flies could be divided by the number of *UASTig tig^{x}*/*CyO* or *tig^{x}*/*In(2LR)Gla* flies × 100. However, overexpression of tiggrin causes some lethality and so the numbers of *UASTig tig^{x}*/*CyO*, ranges from 100% to 50% of the *tig^{x}*/*In(2LR)Gla* siblings. We have chosen to use the *tig^{x}*/*In(2LR)Gla* siblings as the denominator to calculate

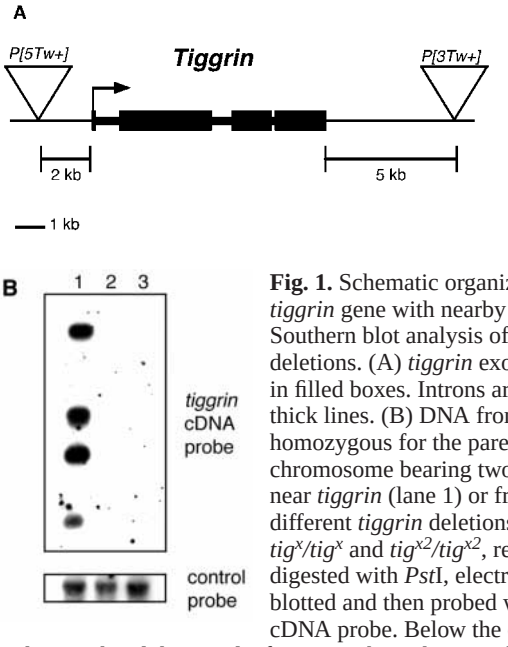

Fig. 1. Schematic organization of the *tiggrin* gene with nearby P elements and Southern blot analysis of two *tiggrin* deletions. (A) *tiggrin* exons are shown in filled boxes. Introns are indicated by thick lines. (B) DNA from flies homozygous for the parental chromosome bearing two P elements near *tiggrin* (lane 1) or from two different *tiggrin* deletions (lanes 2 and 3, *tig^{x}*/*tig^{x}* and *tig^{x2}*/*tig^{x2}*, respectively) was digested with *Pst*I, electrophoresed, blotted and then probed with a *tiggrin* cDNA probe. Below the cDNA probing is shown a band that results from reprobing the same blot with a probe derived from sequences just to the right of *P[3Tw^{+}]*.

rescue percentages in Fig. 6. Thus, our results are probably an underestimate of the rescue frequency. Using the *UASTig tig^x/CyO* siblings as the denominator does not change our conclusions. The highest degree of rescue by *UASTig^{LGA3}* in this case is 23% while it exceeds 100% for *UASTig$^{+17\ and\ +22}$*. Similar crosses and calculations were used to determine the percentage of rescue of *tig^x/tig^{A1}* and *tig^x/tig^{O2}*.

UASTig^{LGA5} is on the X chromosome and the cross to determine rescue frequency was: *w^{1118} GAL4Dr*; *tig^x/CyO* females × *w^{1118} UASTig^{LGA5}*; *tig^x/CyO* males. All females get one copy of driver and responder. Percentage rescue was calculated by dividing the number of *tig^x/tig^x* flies by half the number of *tig^x/CyO* flies. Any lethality would reduce the denominator resulting in slightly higher numbers for rescue by *UASTig^{LGA}* when using this responder relative the numbers obtained for the responders on the second chromosome. A similar situation is found in the rescue that utilizes *UASTig^{LGA2}* on the third chromosome. The cross for rescue is *w^{1118}*; *tig^x/CyO*; *UASTig^{LGA2}* females × *w^{1118} GAL4Dr*; *tig^x/CyO* males. All females get one copy of both driver and responder and the calculation of rescue frequency was the same as for *UASTig^{LGA5}*.

For examination of rescued larvae, *w^{1118} GAL4Dr*; *tig^x/In(2LR)Gla* males were crossed with *w^{1118}*; *UASTig tig^x/In(2LR)Gla* females. *In(2LR)Gla* is also marked with *Bc* and so homozygous *tig^x* larvae can be identified. Female larvae all contain one copy of the GAL4 driver while the males do not.

In the rescue experiments, the expected number of *tig^x/tig^x*, *tig^x/tig^{A1}* or *tig^x/tig^{O2}* animals was between 45 and 139 with most (l5 of the 20 shown) having expected numbers over 100. Rescue experiments were also carried out at different temperatures as expression of transgenes using the GAL4-UAS system is temperature sensitive. The results, from smaller numbers, at different temperatures were qualitatively the same as those shown for 25°C. When higher levels of expression of the transgenes were not lethal, we saw increased rescue at higher temperatures (never exceeding 30% for rescue of *tig^x/tig^x* by *UASTig^{LGA}*).

Immunostaining of embryos

Wild-type, *tig^x/tig$^+$* and *tig^x/tig^x* stage 16 to 17 embryos were fixed and immunostained (Patel, 1994) with mouse primary antibodies against tiggrin (Fogerty et al., 1994) and rabbit primary antibodies against muscle myosin (Kiehart and Feghali, 1986), the latter kindly provided by Dr D. Kiehart. Immunolocalization was visualized with biotinylated anti-mouse and anti-rabbit IgG and the avidin DH-biotinylated horseradish peroxidase H complex using diaminobenzidine and H_2O_2 as substrate (Vector Lab). Embryo fillets were prepared choosing heterozygous or wild-type embryos stained with antibodies to tiggrin and homozygous null mutants that completely lacked immunostain for tiggrin. To analyze the diaminobenzidine-peroxidase-stained muscle patterns in whole mounts of *tig^x/tig$^+$* and *tig^x/tig^x* stage 14-17 embryos, the same immunostaining technique was used, except that tiggrin was visualized by immunofluorescence, using as second antibody fluorescein-conjugated anti-mouse IgG. Specimens were examined in a Zeiss Axiophot microscope, both with and without DIC optics, photographed, scanned and reproduced without alteration with Adobe Photoshop 3 software.

Dissections, staining and measuring larvae

Wandering 3rd instar larvae were dissected as previously described (Jan and Jan, 1976; Johansen et al., 1989). Basically, they were pinned to Sylgard, dissected in Ca^{2+}-free saline (130 mM NaCl, 5 mM KCl, 36 mM sucrose, 5 mM Hepes (pH 7.3), 4 mM $MgCl_2$, 0.5 mM EGTA) and fixed in 3.5% paraformaldehyde in PBS for 30 minutes. Actin filaments were stained with rhodamine-labeled phalloidin (Rh-phalloidin) for 1 hour and then washed several times with PBS. Rh-phalloidin was freshly prepared by resuspending 20 μl of phalloidin (Molecular Probes R-415; 200U/ml methanol, which was removed by evaporation) in 400 μl of PBS + 0.15% TritonX-100.

Larvae were examined using a Zeiss Axioskop microscope and images were collected and analyzed using a cooled CCD camera (Princeton Instruments) and a MetaMorph imaging system (Universal Imaging Corporation). Distances of the gaps between muscles 9 and 10 were measured using MetaMorph. Each individual gap distance was determined by averaging the smallest and the greatest distance between phalloidin staining in adjacent muscles.

Analysis of DNA and RNA

Cosmid clones tig-c53+3, tig-c54+3 and tig-c55 were used in restriction mapping of the *tiggrin* gene and adjacent genomic DNA. tig-c54+3 contains the entire *tiggrin* gene while tig-c55, and tig-c53+3 contain inserts that begin in the second exon and extend 5′ (upstream) and 3′ (downstream) of *tiggrin*, respectively.

Analysis of DNA by restriction mapping and Southern Blotting was carried out using standard protocols described in the Genius System User's Guide (Boehringer Mannheim).

RNase protection experiments were done essentially as described in the RNase protection kit protocol (Boehringer Mannheim). For the tiggrin probe, labelled antisense RNA was synthesized (using the Promega Riboprobe System), which hybridizes to tiggrin RNA over sequences beginning at the *Pst*I site (nucleotide 6634) to the end of the transcript. A control actin antisense RNA synthesized from a DNA fragment, supplied with the Promega Riboprobe System, was added to all of the RNase protections. Though this actin antisense transcript is from human sequences, it does hybridize to a *Drosophila* transcript and is partially protected. This protected 'actin' was used to normalize differences in the levels of added RNA. In each protection assay, mRNA is hybridized to both tiggrin and actin antisense RNA in the same tube and both protected RNAs are quantitated in the same lane on the gel. All animals used had one copy of a GAL4 Driver and one copy of a UASTig responder. Additionally, they all have wild-type copies of the *tiggrin* endogenous gene. Transcripts from the endogenous gene at these time points are low (Fogerty et al., 1994) and undetectable in these assays. Protected RNAs were electrophoresed on 4% acrylamide, 7 M urea gels, dried and then exposed using a Molecular Dynamics PhosphorImager 445SI. Relative levels of tiggrin mRNA were determined by subtracting lane background signal from both the tiggrin and actin signals, and then normalizing all of the tiggrin signals using the actin controls.

Tiggrin fusion protein

An *Eco*RI fragment from pNBY, which encodes the final 333 amino acids of tiggrin's 2186 amino acids (Fogerty et al., 1994), was cloned into a pTrcHis bacterial expression vector (Xpress System^{TM}, Invitrogen). Bacteria expressing fusion protein were solublized in 8 M urea, 0.1 M sodium phosphate, 0.01 M Tris/HCl, pH 8.0 and the protein was purified by affinity chromatography on Ni-NTA agarose (QIAexpress, QIAGEN). This fusion protein TIG-RGD and a similarly produced TIG-LGA fusion protein (identical except the sequence encoding RGD was mutagenized in vitro to encode LGA) were checked by SDS-PAGE and western blotting using anti-tiggrin antibodies to ensure that equal amounts of each were present in the cell spreading assays.

Cell culture and cell spreading assays

Cell culture and spreading assays have been described previously (Bunch and Brower, 1992; Zavortink et al., 1993). *Drosophila* S2 cells (Schneider, 1972) were used that had been cotransfected with cDNAs encoding α_{PS2} and a β_{PS} subunits under the control of the HSP70 heat-shock promoter. Spreading was quantified by counting cells using a Nikon phase-contrast microscope (Nikon Diaphot-TMD). Spreading percentages were scored as number of spread cells divided by the total number of cells counted for each cell type. The percentage values

were averaged for individual experiments and the standard errors of the mean were determined.

RESULTS

Generation of *tiggrin* mutants

tiggrin has been mapped by in situ hybridization to the cytological location 26D1-2 (Fogerty et al., 1994). As this region contains no previously described mutations in, or deletions of, genes likely to encode tiggrin, we used P elements near *tiggrin* to generate a deletion that completely removes the gene. Using phage P1 and cosmid clones, we generated a map of the genomic DNA surrounding *tiggrin* (Fig. 1). Two lines of flies that contain P elements in the 26D1 region were obtained and the P elements were mapped to positions 2 kb upstream and 5 kb downstream of *tiggrin* (Fig. 1). We recombined the two P elements onto the same chromosome and then excised the P elements by genetically introducing transposase into the flies. By screening for loss of both P elements we obtained a deletion of *tiggrin*. Southern blot analysis shows that the deletion completely removes *tiggrin* (Fig. 1) and brings together DNA just to the left of the upstream P element and DNA just to the right of the downstream P element (not shown). The deletions begin at or near the P elements and cannot extend more than 1 kb beyond them (Fig. 1 and data not shown).

EMS was used to generate additional *tiggrin* mutants, tig^{A1} and tig^{O2}, which are semilethal when placed over tig^{x}. Both EMS alleles display the same phenotypes, though with different degrees of severity, as tig^{x} when placed over tig^{x}.

Deletion of the *tiggrin* gene is generally lethal

A cross of tig^{x}/*In(2LR)Gla* males and females gave 4,127 balanced adult progeny and 19 homozygous tig^{x}/tig^{x} adults. The tig^{x} deletion therefore results in about 99% lethality. In this experiment, lethality was the same at 22°C and 25°C and so the numbers were combined. The escaper frequency can be influenced by culture conditions and homozygous stocks of tig^{x} flies, though not robust, appear healthier than would be expected from flies that are 99% lethal. Thus, homozygous mutants may survive better when not competing with heterozygous siblings. Combinations of tig^{x}, tig^{A1} and tig^{O2} showed 70% to 87% lethality.

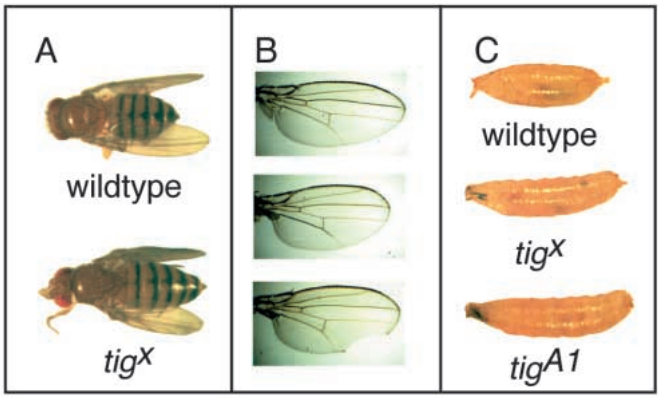

Fig. 2. Adult and pupal phenotypes of *tiggrin* mutants. (A) Wild-type (top) and tig^{x}/tig^{x} (bottom) adults. Note the elongated abdomen in the mutant. (B) Examples of tig^{x}/tig^{x} wing defects. The top two wings are from the same fly and demonstrate that absence of tiggrin can result in wings having altered shapes and sizes. This phenotype is obvious in 1-2% of the mutant flies. On the bottom is a notched wing (found in 1.5% of the mutants). Other phenotypes (not shown) include wavy posterior regions of the tig^{x}/tig^{x} wings (2.7%) and anterior wing margin defects (1.3%). Percentages are calculated from examining 440 wings from homozygous mutant adults. (C) Wild-type (top), tig^{x}/tig^{x} (middle), and tig^{A1}/tig^{A1} (bottom) pupae. The *tiggrin* mutant pupae are significantly longer than the wild-type pupae. Measurement of 20 pupae of each genotype gave average lengths of: 3.7 mm (range = 3.3-4.1 mm) for tig^{x}/tig^{x}, 3.7 mm (range = 3.3-4.0 mm) for tig^{A1}/tig^{A1} and 3.1 mm (range = 2.4-3.3 mm) for wild-type pupae.

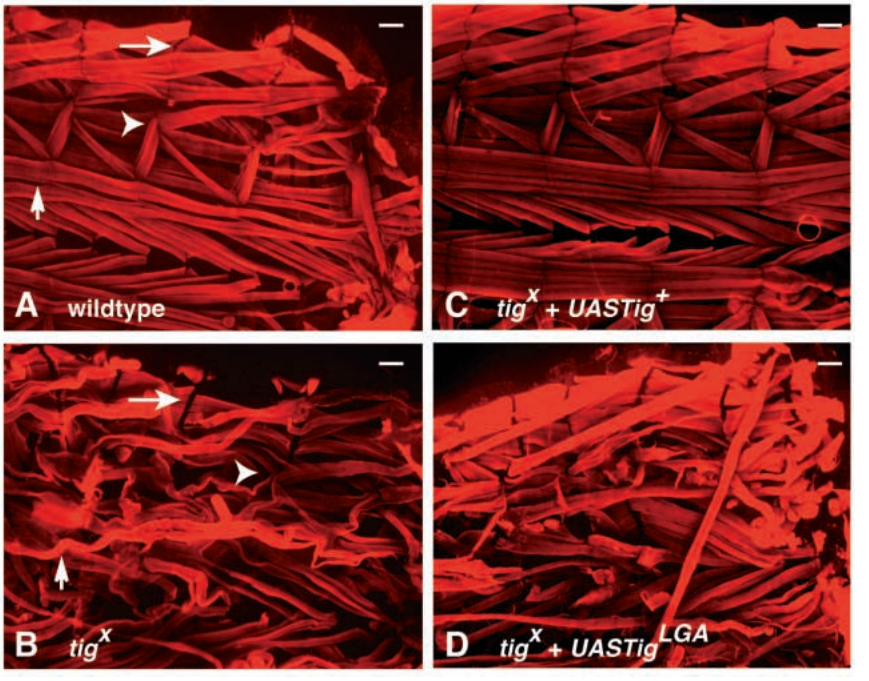

Fig. 3. Larval muscle phenotypes of *tiggrin* mutants. Dissected (A) $tig^{x}/+$ and (B) tig^{x}/tig^{x} 3rd instar wandering larvae were stained with rhodamine-labeled phalloidin to visualize actin filaments. In mutant larvae, muscles 6 and 7 appear stringy and not anchored to other muscles or the epidermis (vertical arrows). Often these muscles are missing or unrecognizable. Sites where muscles 3, 4, 5, 8 and 16 come together (arrowheads) are rarely recognizable, though one can be identified in B. Large gaps between muscles 9 and 10 are always observed in the tig^{x}/tig^{x} larvae (horizontal arrows). These gaps are shown at higher magnification in Fig. 4. Bodywall muscles from a tig^{x} mutant larva rescued by (C) a wild-type $UASTig^{+}$ transgene or (D) a $UASTig^{LGA}$ transgene are shown. For this experiment, the $GAL4^{c363}$ driver was used with the $UASTig^{+17}$ or $UASTig^{LGA3}$ responder. The $UASTig^{+}$ rescues muscles to wild-type appearance while the $UASTig^{LGA}$ shows very little rescue. Scale bars = 100 μm.

Surprisingly, the tig^x homozygous flies appear relatively normal, although escapers do display elongated, misshapen, abdomens (Fig. 2A). The abdominal phenotype is also seen in tig^{A1}/tig^x and tig^{O2}/tig^x mutant adults, though it may not be as severe (not shown). Wing defects are found in 7-8% (n=440) of the tig^x homozygous escapers (Fig. 2B). These defects include notched wings, deformed anterior margins, smaller and rounder wings, and wavy posterior regions. Flies that have just eclosed often show abnormal separation of the dorsal and ventral wing blades in the posterior region of the wing (not shown). Wing defects are also observed in tig^{A1} homozygous and tig^{A1}/tig^x animals (not shown).

Observation of over 200 eggs from a cross of tig^x/+ males and females showed that all of the eggs hatched at 22°C, 25°C and 28°C. Thus, no embryonic lethality is observed. A potential embryonic requirement is not being rescued by a maternal tiggrin contribution, as a cross of tig^x homozygous male and female escapers shows no embryonic lethality and a small percentage of the resulting larvae develop to the adult stage. To ask if *tiggrin* is a pupal lethal mutation, we examined homozygous pupae for their ability to eclose to adults. 88% of pupae (n=398) failed to eclose in this experiment. Most of *tiggrin*'s lethality can therefore be attributed to the pupal phase.

tiggrin is required in larvae for proper muscle function

At pupariation, contraction of bodywall muscles shortens the body. tig^x and tig^{A1} homozygous pupae are 16% longer than wild-type and heterozygous pupae (Fig. 2C). Pupation, as defined by the appearance of a gas bubble in the abdomen, occurs in tig^x and tig^{A1} mutant animals; however, dispersion of the bubble and head eversion fails to occur in about half of the pupae (not shown). This process also requires proper bodywall muscle function (Bodenstein, 1950).

Homozygous tig^x and tig^{A1} larvae show other behavioral abnormalities that are likely to result from muscle defects. Muscle contraction waves that pass from the posterior to anterior of larvae are responsible for locomotion. We have measured the duration of these waves in wandering 3rd instar and 1st instar larvae that have just hatched. The contraction waves are much slower in the *tiggrin* mutants, taking three times as long to pass from posterior tip to anterior tip as compared with wild-type or *tiggrin* heterozyotes in 3rd instar larvae, and twice as long in newly hatched 1st instar larvae (Table 1). Though a defect in muscle function is a reasonable cause for the slowing of the contraction waves, we have not directly measured muscle force and defects in neural function could also contribute to this phenotype. As the muscle contraction wave defect is also observed in 1st intstar larvae that have just hatched, it is unlikely to be due to a general weakened condition of the larvae caused by reduced feeding.

Direct examination of muscles in dissected larvae shows severe defects in tig^x and tig^{A1} homozygotes (Figs 3, 4). Large

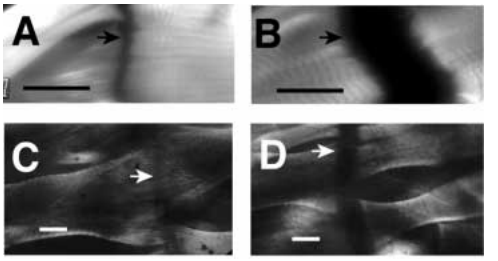

Fig. 4. Gaps between larval muscles 9 and 10 in *tiggrin* mutants. A higher magnification view of a gap between muscles 9 and 10 dissected and stained with rhodamine-labeled phalloidin is shown for (A) wild type and (B) tig^x/tig^x. Another view of the muscle gaps is seen by observing whole, fixed (C) wild-type and (D) tig^x/tig^x, larvae with polarized light. Scale bars = 50 μm.

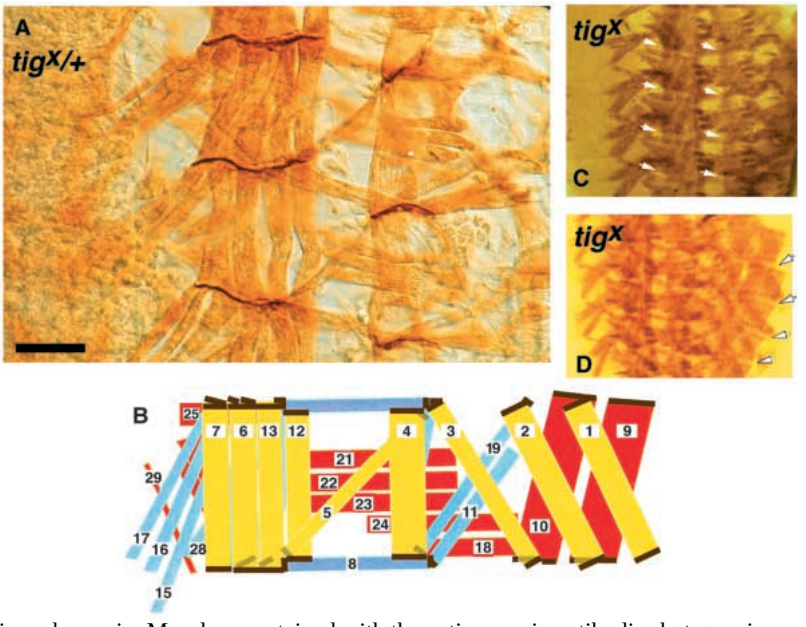

Fig 5. Bodywall musculature in heterozygous and homozygous *tiggrin* embryos. Stage 16-17 embryos from a self-cross of tig^x/+ heterozygotes were immunostained with mixed antibodies to tiggrin and myosin, sorted by tiggrin staining phenotype, and fillets were prepared. (A)The concentration of peroxidase second antibody stain at the apodemes of a heterozygote, which is indistinguishable from wild type, and coincides with tiggrin-only staining of whole embryos (not shown). (B) The pattern of muscles in a single hemisegment is shown diagrammatically as viewed from the interior with the ventral midline at the left and the dorsal midline at the right (modified from Bate, 1993). A and B are approximately aligned. A summary of the sites of tiggrin accumulations found in a series of wild-type fillets like A is shown in B as brown bars at the ends of the corresponding muscles. Notably, the transverse muscles 21-24 and ventral attachments of the ventral oblique muscles 15-17 are not associated with tiggrin accumulations in embryos. The scale bar in A corresponds to 25μm. (C,D) Fillets from two tig^x/tig^x embryos, at lower magnifications and viewed from the outside rather than the inside as in A. As in A these embryos were immunostained with mixed antibodies to tiggrin and myosin. Muscles are stained with the anti-mysosin antibodies but gaps in the staining are seen at apodemes where muscles from adjacent segments abut (arrowheads). This is due to the absence of tiggrin staining in the *tiggrin* mutants. The orientation of C is similar to A. A fillet showing the dorsal oblique muscles 9 and 10 is shown in D. Note the clear gaps that show no staining between these muscles (arrowheads).

Table 1. Time required for musclecontraction waves

Genotype	Wave time*
wt	0.6 (0.5-0.7, *n*=7) 1.1 (0.9-1.5, *n*=7)**
tig^{x}/In(2LR)Gla	0.6 (0.5-0.7, *n*=13)
tig^{x}/tig^{x}	2.1 (1.3-5.5, *n*=10) 1.9 (1.6-2.3, *n*=7)**
tig^{A1}/tig^{A1}	1.6 (1.0-2.4, *n*=10)
tig^{x}/tig^{x} + *tig^{+}* transgene	0.7 (0.7-0.7, *n*=7)
tig^{x}/tig^{x} + *tig^{LGA}* transgene	1.0 (0.9-1.5, *n*=10)

*Average time, in seconds, for muscle contraction waves . The range in average times for individual larvae is in parentheses. *n*= the number of larvae examined.

**Times for first instar larvae. All other times are for wandering 3rd instar larvae.

Table 2. Gap distance between muscles 9 and 10

Genotype	Gap distance* μm.(range)
tig^{x}/+	7.0 (5.3-10, *n*=7)
tig^{x}/tig^{x}	30.1 (20-43, *n*=18)
tig^{x}/tig^{x} UASTig^{LGA}	22.4 (10-36.8, *n*=13)
tig^{x}/*tig^{x} UASTig^{+}*	4.7 (3.5-6.5, *n*=5)

*Average distance between phalloidin staining actin in adjacent muscles (as shown in Fig. 4A,B) *n*=the number of gaps measured.

gaps are observed between the dorsal oblique muscles 9 and 10 (Figs 3, 4; Table 2) and between the ventral oblique muscles 15 and 29 (not shown). At sites where muscles 3, 4, 5, 8 and 16 come together in wildtype larvae, muscles 5 and 8 are usually missing. The muscles in *tiggrin* mutant animals often appear thinner than in wild type, and other muscles also are missing in these preparations. This is especially true of the large ventral longitudinal muscles 6 and 7. In contrast to the oblique and longitudinal muscles, examination of the transverse muscles 21-24 in *tiggrin* mutants has not revealed any defects (not shown). It should be noted that the mutant larva shown in Fig. 3 has the best preserved muscles of any mutants that we have dissected; usually more muscles are missing. During dissections of wild-type larvae, the collection of bodywall muscles appears to behave as a coherent tissue that would suggest lateral adhesion of the different muscles. During dissection of *tiggrin* mutant larvae this muscle 'tissue' appears very loose and muscles separate easily. Examination of the sarcomeric structure in these preparations has not revealed any defects in the *tiggrin* mutants (not shown).

We have also examined muscles in fixed undissected larvae using polarized light. The gaps between muscles 9 and 10 of adjacent segments are readily observed in these preparations (Fig. 4) and the muscles also appear much thinner than in wild-type animals (not shown). We do not observe as much complete loss of muscles; thin muscles 5 and 6 are usually observed in these preparations whereas they are usually absent in dissected larvae. This would indicate that the dissection procedure may cause the loss of some weakly attached muscles. Occasionally ventral oblique muscles do appear to be missing in the undissected *tiggrin* mutant larvae, though very thin muscles might not be detected in these preparations.

We have examined the distribution of tiggrin protein in muscles of embryos. In wild-type and *tig^{x}/+* animals, strong tiggrin accumulations are found at the segmentally spaced insertion sites of the major longitudinal muscles 4, 6, 7, 12 and 13, and the wide dorsal oblique muscles 9 and 10. These are the same sites that are observed to be defective in *tiggrin* larvae. Notably, only very weak staining for tiggrin was observed at the attachments of the transverse muscles 21-24 and the ventral attachments of the ventral oblique muscles 15-17. *tig^{x}/tig^{x}* animals show a complete absence of staining for tiggrin (Fig. 5C,D). Staining results were confirmed in whole mounts of stage 14-17 embryos visualizing tiggrin by immunofluorescence (not shown). *tig^{x}/tig^{+}* embryos showed strong fluorescence at muscle insertions, while *tig^{x}/tig^{x}* embryos lacked all fluorescence. Nevertheless, no abnormal muscle arrangement or structure was detected either in whole mounts or in muscle fillet preparations of *tig^{x}/tig^{x}* embryos. Thus, the lack of tiggrin does not seem to interfere with the localization pattern of the somatic embryonic musculature. This suggests that the loss of muscles seen in larvae may be due to muscles in the mutants detaching and/or degenerating during larval life.

As PS2 integrins are involved in formation of the gut, we examined *tiggrin* mutant foreguts, midguts and hindguts in dissected larvae stained with labelled phalloidin. We were not able to detect any obvious defects.

tiggrin transgenes rescue *tiggrin* mutant defects

To demonstrate that the lethality and muscle phenotypes observed in *tig^{x}/tig^{x}* animals are due to the removal of the *tiggrin* gene, we have constructed flies with transgenes that express *tiggrin*. These transgenes (*UASTig^{+}*), in combination with GAL4 drivers, rescue the lethality and muscle defects seen in *tiggrin* mutants (Tables 1, 2; Figs 3, 6). (See Materials and Methods for details of the transgenic flies and a caveat that may result in an underestimation of the percentage of flies rescued.) Adult abdominal defects and wing defects seen in *tig^{x}* homozygous flies are also rescued by the *tiggrin* transgenes (not shown).

Some functions of tiggrin are independent of its RGD integrin-binding sequence

Previous cell culture experiments showed that *Drosophila* cells expressing PS2 integrins spread on tiggrin purified from *Drosophila* cells or tiggrin fusion proteins expressed in bacteria. This spreading was inhibitable by RGD peptides, which suggested that the RGD sequence in tiggrin is critical for the integrin-tiggrin interaction (Fogerty et al., 1994). We have further tested this hypothesis in cultured cells and the whole fly. Using in vitro mutagenesis the sequence encoding RGD in tiggrin was changed to encode LGA. Fusion proteins containing the final 333 amino acids of tiggrin (of a total of 2186 amino acids) were produced in bacteria. These proteins are identical with the exception of having RGD in one case and LGA in the other (TIG-RGD and TIG-LGA respectively). A standard cell spreading assay (Fig. 7) tested the ability of each fusion protein to promote PS2 integrin-mediated cell spreading. There are four potential forms of the PS2 integrin that are generated by alternative splicing of mRNA encoding

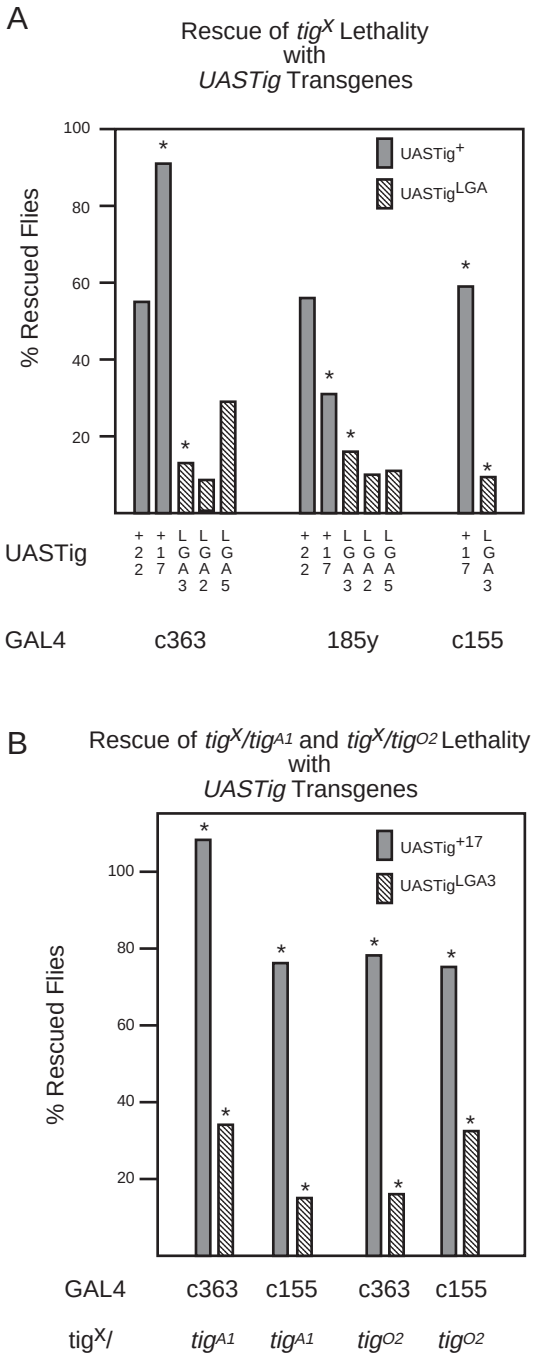

Fig. 6. Rescue of *tiggrin* mutants with *UASTig*$^{+}$ and *UASTig*^{LGA} transgenes. (A) Rescue of *tig*^{x}/*tig*^{x} adults. The percentage of *tig*^{x}/*tig*^{x} adult flies rescued by *tiggrin* transgenes as compared to the expected number (calculated from heterozygous siblings) was determined. Two different *UASTig*$^{+}$ transgenes (*UASTig*$^{+22}$, *UASTig*$^{+17}$, solid bars) and three *UASTig*^{LGA} transgenes (*UASTig*^{LGA2}, *UASTig*^{LGA3} and *UASTig*^{LGA5} crosshatched bars) were tested. Expression of these transgenes was driven by three different GAL4 drivers, *GAL4*^{185Y}, *GAL4*^{c363} and *GAL4*^{c155}(denoted C363, 185y and c155) (B) Percentage rescue of *tig*^{x}/*tig*^{A1} and *tig*^{x}/*tig*^{O2} mutant adults is shown. *UASTig*$^{+17}$ (solid bars) and *UASTig*^{LGA3} (crosshatched bars) transgenes were regulated by the *GAL4*^{c363} and *GAL4*^{c155} drivers. Results for the transgenes whose RNA expression levels have been measured, *UASTig*$^{+17}$ and *UASTig*^{LGA3}, are placed together and denoted with *. Details of the crosses and calculations of percentage rescued flies can be found in the Materials and Methods section.

the α_{PS2} and β_{PS} subunits (Bogaert et al., 1987; Brown et al., 1989; Yee, 1993; Zusman et al., 1993; M. W. G., T. A. B., S. Baumgartner, A. Kerschen and D. L. B., unpublished data). We have tested all four forms for their ability to interact with TIG-RGD and TIG-LGA (Fig. 7). As was previously demonstrated, the TIG-RGD readily promotes cell spreading (Fogerty et al., 1994; M. W. G., T. A. B., S. Baumgartner, A. Kerschen and D. L. B., unpublished data). The TIG-LGA is unable support cell spreading in this assay.

To test the importance of the RGD sequence in the whole organism, an RGD-to-LGA mutant *tiggrin* transgene (*UASTig*^{LGA}) was constructed and this was introduced into flies. This mutant transgene fails to rescue *tiggrin* mutant flies to the levels of the wild-type transgene. It shows little rescue of the muscle defects observed in dissected larvae, as muscles are often missing and gaps are observed between muscles 9 and 10 (Fig. 3D). However, measurement of these gaps indicates that they are intermediate in distance between wild-type, or *UASTig*$^{+}$ rescued, larvae (Table 2). *UASTig*^{LGA}-rescued *tig*^{x}/*tig*^{x} larvae have muscle contraction waves that are twice as fast as *tig*^{x} mutants; however, this is still much slower than wild-type or *tig*^{x} flies rescued by *UASTig*$^{+}$ (Table 1). Thus, tiggrin lacking an RGD sequence shows partial activity in these two assays. Finally, lethality can be rescued by the *UASTig*^{LGA} up to a maximum of 30% (Fig. 6A). *tig*^{x}/*tig*^{A1} and *tig*^{x}/*tig*^{O2} mutants are also rescued much more efficiently by wild-type *tiggrin* trangenes as compared with *tig*^{LGA} transgenes (Fig. 6B).

The chromosomal insertion site of the *UASTig*^{LGA} transgene might result in lower levels of expression as compared with the *UASTig*$^{+}$ transgene, which is inserted at a different site. This could explain the *UASTig*^{LGA} transgene's reduced ability to rescue *tiggrin* mutants. This possibility was addressed in two ways. First, we have tested different lines that carry the transgenes located in different chromosomal locations (three for *UASTig*^{LGA} and two for *UASTig*$^{+}$). In all cases, the *UASTig*^{LGA} was less effective at rescuing lethality, reaching a maximum of 30%, whereas *UASTig*$^{+}$ expressed from transgenes can almost completely rescue the lethality of *tig*^{x} homozygotes (Fig. 6A). Second, RNase protection assays were done to determine directly the relative levels of expression of the two *tiggrin* transgenes *UASTig*$^{+17}$ and *UASTig*^{LGA3}. The *UASTig*^{LGA} transgene is expressed at equal or higher levels than the

Table 3. RNA levels from *UASTig* transgenes

	UAS transgene			
	Larvae		Embryos	
GAL4 Driver	+17	LGA3	+17	LGA3
c363	13	22	n.d.	n.d.
185Y	20	19	n.d.	n.d.
167Y	2	9	2	5

Levels of tiggrin mRNA were determined in an RNase protection assay. Shown are levels, in arbitrary units, for wandering 3rd instar larvae and 18-21 hour embryos (transgenes with *GAL4*^{c167}only). *GAL4*^{c167} is a driver that shows significant (40%) rescue in combination with Tig^{+17}, at higher temperatures (28°C, not shown). Numbers were normalized for total RNA levels by using a separate probe for actin.

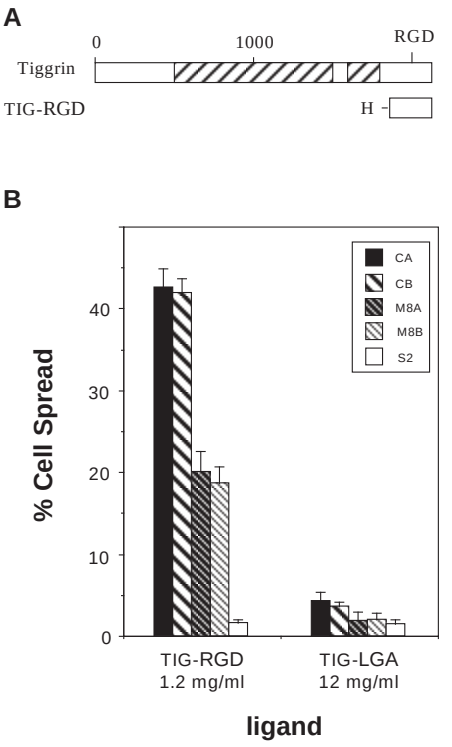

Fig. 7. PS2-mediated cell-spreading on TIG-RGD and TIG-LGA. *Drosophila* S2 cells were transformed to express the four different forms of the PS2 integrins – $\alpha_{PS2C}\beta_{PS4A}$ (CA), $\alpha_{PS2C}\beta_{PS4B}$ (CB), $\alpha_{PS2m8}\beta_{PS4A}$ (m8A) and $\alpha_{PS2m8}\beta_{PS4B}$ (m8B). These were assayed for cell spreading on a bacterially produced fusion protein (TIG-RGD) that contains 333 amino acids of tiggrin including the integrin recognition sequence RGD (A). Crosshatching in A corresponds to regions of tiggrin that is comprised of tiggrin repeats (Fogerty et al., 1994). (B) All four forms of the PS2 integrins promote cell spreading (shown here on 1.2 μg/ml TIG-RGD). These same cells do not show significant spreading on the same fusion protein whose RGD sequence has been mutated to LGA (TIG-LGA). Shown are the results for spreading on 12 μg/ml TIG-LGA (10× the amount of TIG-RGD) and they are similar for all concentrations tested both lower and higher. The numbers shown are averages with standard errors of 3 experiments.

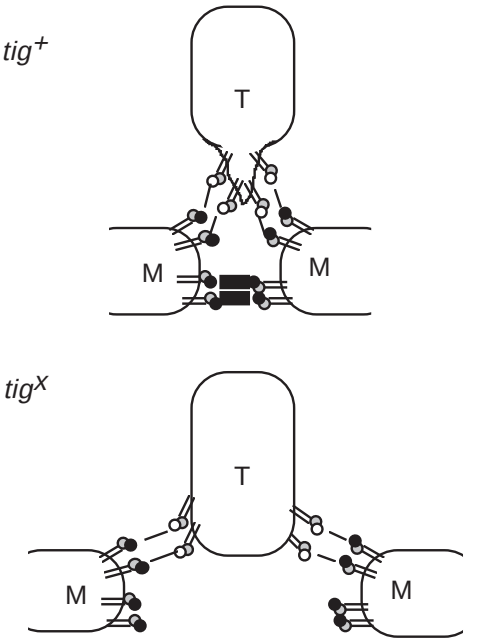

Fig. 8. Model of muscle-muscle-tendon cell attachment. PS2 integrins at muscle attachment sites are found on the muscle cell (M) and are proposed to be involved in two attachments. One attachment (thin lines) is between the muscles and the epidermal tendon cell (T) which expresses PS1 integrins. The other attachment is between the two muscle cells (thick lines). Shown is a simple attachment such as might occur at the junction of muscles 9 and 10. Tiggrin is proposed to be critical to forming or maintaining the muscle-muscle, attachments as its absence results in gaps between the muscles. Though tiggrin may function at the muscle-tendon cell attachments it appears to be not as critical; most muscles remain attached and relatively well ordered in its absence. Other ECM molecules may play roles in these attachments.

UASTig^{+} transgene (Table 3). We have tested if the mutant tiggrin protein might be less stable than wild-type tiggrin when expressed from these transgenes. Western analysis of protein levels in *tiggrin* mutant larvae expressing mutant or wild-type tiggrin from transgenes *UASTig*^{LGA3} or *UASTig*$^{+17}$, driven by either *GAL4*^{185} or *GAL4*^{363}, showed that tiggrin protein levels were within 10% of each other (not shown). Therefore, there does not appear to be a significant effect of the LGA mutation on the stability of tiggrin protein. These results suggest that it is the mutation of the RGD sequence and not merely differences in expression levels that reduce the function of tiggrin^{LGA}.

DISCUSSION

Use of P elements to remove the *tiggrin* gene

To understand the functions of tiggrin in development it is important that we examine a null mutation in the *tiggrin* gene. Null mutations could be generated by a variety of methods including mutagenesis with chemicals, X-rays or transposon insertions. These techniques also generate other types of mutations and a great deal of genetic and molecular analysis is required to confidently classify a mutation obtained by one of these traditional techniques as a null allele. One important tool used in genetically classifying a new mutation is a deletion that usually removes a region of the chromosome including the gene of interest and other neighboring loci. For the region of the second chromosome that contains *tiggrin*, we could find no such convenient deletion. For these reasons, and because we did not know what the phenotype of a *tiggrin* null mutation would be, we used a new technique to generate a precise deletion of the gene. We first created a chromosome with two P elements flanking *tiggrin* and then excised these P elements and *tiggrin* in a dysgenic cross. This technique was originally shown to be a viable method for generating large deletions (Cooley et al., 1990). Assuming that P-element insertions exist on both sides of a gene of interest it is relatively easy to generate a defined deletion. This technique of generating a null allele is a useful alternative to chemical or transposon insertional mutagenesis, imprecise excision of single P elements or X-irradiation.

tiggrin muscle phenotypes

Cell culture experiments and immunolocalization data suggest

that tiggrin is a ligand for the PS2 integrins in *Drosophila* (Fogerty et al., 1994). If PS2 integrin functions are mediated solely by its interaction with tiggrin, *tiggrin* mutants should show phenotypes similar to *inflated* mutations, which remove the α_{PS2} subunit. Mutations in *inflated* are embryonic lethal and one prominent phenotype is the detachment and rounding up of muscles (Brabant and Brower, 1993; Brown, 1994). *tiggrin* null mutations show no embryonic lethality nor detachment of muscles in the embryo. It is therefore likely that there are other ECM components that are overlapping with tiggrin in its functions as a PS2 integrin ligand. These putative ligands may be redundant with tiggrin in this function or they may be upregulated to compensate for the loss of tiggrin. Redundancy in the functions of ECM molecules is not a new concept as a number of mutations that remove genes encoding components of the ECM in mice have resulted in less severe phenotypes than those expected (George et al., 1993; Saga et al., 1992). Similar to the *tiggrin* phenotypes, mutations in the gene (*lamA*) encoding a laminin α chain in *Drosophila* show surprisingly mild embryonic phenotypes (Henchcliffe et al., 1993; Yarnitzky and Volk, 1995).

Muscle function and structure is compromised in tiggrin-deficient larvae. Muscle contraction waves that traverse the length of the larvae are much slower in *tiggrin* mutants, and bodywall muscles are generally thinner and several appear to be missing or detached in dissected specimens. Detachment of muscles in *tiggrin* mutant animals is partly due to stresses of dissection as observation of whole fixed larvae shows less muscle detachment. However, the muscle detachment seen in dissected *tiggrin* mutant larvae is not seen in heterozygous larvae and is therefore indicative of weakened attachments.

At segment borders where ends of multiple muscles attach to epidermal tendon cells, and where we find accumulations of tiggrin protein in wild-type animals, the gaps between muscles increase from 7 μm in wild-type third instar larvae to 30 μm in *tiggrin* mutant larvae. One model that could explain these gaps is that the PS2 integrins are involved in two adhesion sites when neighboring muscles make attachments to the same or neighboring epidermal tendon cells (Fig. 8). The first site is the well-documented muscle-epidermal attachment (Wright, 1960; Newman and Wright, 1981). This attachment may or may not utilize tiggrin. The second site is at a muscle-muscle attachment. Muscle-muscle attachments have not been described in detail; however, experiments that genetically remove the epidermal tendon cells result in muscles that detach from the epidermis but remain attached to each other (Martin-Bermudo and Brown, 1996). A recently isolated mutant, *rhea*, also displays muscles that detach from the epidermis but remain attached to each other (Prout et al., 1997). This strongly suggests that muscle-muscle attachments exist in normal animals. Our results would suggest that tiggrin is required to maintain and/or establish these specialized muscle-muscle junctions.

In contrast to the longitudinal and oblique bodywall muscles, transverse bodywall muscles appear to attach only to the epidermis, do not show strong tiggrin staining and do not show defects in the tiggrin mutants. This indicates that these two muscle attachment sites are different and is consistent with the model that tiggrin is involved mainly with the muscle-muscle junctions and not so critical at the muscle-epidermal junctions. Prokop et al. (1998) have observed that these two muscle junctions are ultrastructurally quite distinct. The transverse muscles display a close apposition (30-40 nm) between muscle and epidermis while at segment borders, where longitudinal and oblique muscles converge at sites on the epidermis, large accumulations of tendon matrix, including tiggrin, separate cells by several μm.

In this model, the absence of tiggrin results in muscles remaining attached to the epidermal cells but detaching from each other, resulting in the separated, but well-ordered, muscle termini (Figs 4, 8). The PS2 integrins are involved in both attachments as *inflated* mutants that remove the α_{PS2} subunit result in completely detached and rounded up muscles. This model suggests that the PS2 integrins may use different ligands for the muscle-epidermal attachment. TenM is an example of a potential ECM component that may carry out this function. It is found at attachment sites, has an RGD sequence and interacts with the PS2 integrins in cell spreading assays (Baumgartner et al., 1994; M. W. G., T. A. B., S. Baumgartner, A. Kerschen and D. L. B., unpublished data). Other extracellular molecules known to function or be located at this location include laminin, slit, masquerade, m-spondin, collagen IV and groovin (Becker et al., 1997; Borchiellini et al., 1996; Murugasu-Oei et al., 1995; Rothberg et al., 1990; Umemiya et al., 1997; Yarnitzky and Volk, 1995; our unpublished observations). By interacting directly or indirectly with the PS2 integrins and other cell surface receptors, these proteins may further support the muscle-epidermal attachment in the absence of tiggrin.

For tiggrin to mediate a direct link between cells via the PS2 integrins, it would require two PS2 integrin-binding sites, but tiggrin has only one RGD sequence. Biochemical data are consistent with tiggrin forming extended rod-like homodimers or homotrimers that are approximately 180 nm in length (Fogerty et al., 1994). If two tiggrin molecules dimerize in an anti-parallel fashion, this would place the RGD integrin-binding domain on each end of the rod and could serve as a direct link between PS2 integrins on adjacent cells. However, the 180 nm length of such a dimer or trimer is not consistent with the distance of several μm between neighboring muscles visualized by actin staining (Fig. 4; Table 2) or at the EM level (Prokop et al., 1998). Furthermore, tiggrin is anchored to the ECM by interactions with other matrix components, as evidenced by the correct localization of tiggrin in *myospheroid* mutants that lack PS2 integrins (Fogerty et al., 1994). Therefore tiggrin may provide a link to the ECM rather than a direct linkage between the PS2 integrins on neighboring cells.

In addition to muscle attachment defects, mutant muscles often appear much thinner. Though the major site of tiggrin accumulation in muscles is at the attachment sites, tiggrin is also found localized with the PS2 integrins at Z bands in jump muscles (Fogerty et al., 1994) and in larval bodywall muscles (our unpublished observations). In embryos, prior to the formation of distinctive Z bands, the basement membrane surrounding the muscles stains for tiggrin (not shown) and PS2 integrins are localized in focal hemiadherens junctions where they are responsible for adhesion of these junctions to the basement membrane (Prokop et al., 1998). Some of tiggrin's function(s) in muscle development or maintenance may be mediated along the muscle surface as well as at the muscle attachment sites.

tiggrin wing phenotypes

In the wing, we observe a low frequency of defects in *tiggrin*

mutants. Consistent with this, we have found tiggrin in the ECM that separates the dorsal and ventral epithelia of immature *Drosophila* wings (unpublished observations). In this tissue, the PS2 integrins mediate adhesion between two sheets (dorsal and ventral) of epithelial cells. Tiggrin may therefore be involved in linking PS2-mediated adhesion of these cell sheets. However, its function in the wing cannot be completely required for wing adhesion as wing defects are seen in only a small percentage of *tiggrin* mutant flies.

Function of tiggrin's integrin recognition sequence

Our results demonstrate that expression of tiggrin from transgenes rescues lethality, muscle structure and function, and wing defects. Therefore, the defects we observe in the tig^{x} deletion are due to loss of *tiggrin* and not to loss of neighboring genes that may have been affected by the deletion.

That we do not see 100% rescue in all cases is most likely due to our failure to reproduce tiggrin's spatial and temporal expression pattern. The endogenous *tiggrin* gene is expressed in embryos at high levels in hemocytes and at lower levels in muscles (Fogerty et al., 1994 and our unpublished observations). We have been unable to find GAL4 drivers that reproduce this pattern. We have used lines that express tiggrin in a variety of different tissues and times (see Materials and Methods for details). We have not noted anything exceptional about the rescuing ability of the different GAL4 drivers and their specific expression patterns. For example, the GAL4 driver c155 shows excellent rescue of lethality (Fig. 6) and muscle phenotypes (not shown). This driver results in a predominantly neural-specific expression pattern as it is driven by the *elav* regulatory sequence. Also, we do observe lethality when the *tiggrin* transgenes are overexpressed at high levels in wild-type flies. This lethality correlates more with the rescuing ability of the transgene than with the pattern of expression. We did try to rescue *tiggrin* mutants using *tiggrin* transgenes whose expression was controlled by the muscle-specific driver $GAL4^{24B}$ (Brand and Perrimon, 1993). Tiggrin expression driven by $GAL4^{24B}$ resulted in 100% lethality in wild-type flies. We do not know if this lethality is due to expression in the muscles, expression elsewhere, or just to a general high level of expression. As tiggrin is a secreted molecule, rescuing ability and lethality from misexpressed tiggrin may result from its action at sites other than the cells in which it is synthesized.

Tiggrin whose integrin recognition sequence (RGD) has been mutated to LGA shows a greatly reduced, but not eliminated, ability to rescue muscle structure and function, and lethality associated with loss of tiggrin in whole flies. Our cell culture assay does not detect any interaction between TIG-LGA and the PS2 integrins. These results would indicate that tiggrin's functions are partially mediated by its interaction with the PS2 integrins via its RGD sequence but that other interactions, perhaps with other ECM molecules and/or cell surface receptors, also serve to promote muscle structure and function. To fully understand the functions of tiggrin and the PS2 integrins, it will be important to identify these other molecules as well as ECM components that may overlap with tiggrin in mediating PS2 adhesion.

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