Thrombospondin-mediated adhesion is essential for the formation of the myotendinous junction in Drosophila

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Organogenesis of the somatic musculature in Drosophila is directed by the precise adhesion between migrating myotubes and their corresponding ectodermally derived tendon cells. Whereas the PS integrins mediate the adhesion between these two cell types, their extracellular matrix (ECM) ligands have been only partially characterized. We show that the ECM protein Thrombospondin (Tsp), produced by tendon cells, is essential for the formation of the integrin-mediated myotendinous junction. Tsp expression is induced by the tendon-specific transcription factor Stripe, and accumulates at the myotendinous junction following the association between the muscle and the tendon cell. In tsp mutant embryos, migrating somatic muscles fail to attach to tendon cells and often form hemiadherens junctions with their neighboring muscle cells, resulting in nonfunctional somatic musculature. Talin accumulation at the cytoplasmic faces of the muscles and tendons is greatly reduced, implicating Tsp as a potential integrin ligand. Consistently, purified Tsp C-terminal domain polypeptide mediates spreading of PS2 integrin-expressing S2 cells in a KGD- and PS2-integrin-dependent manner. We propose a model in which the myotendinous junction is formed by the specific association of Tsp with multiple muscle-specific PS2 integrin receptors and a subsequent consolidation of the junction by enhanced tendon-specific production of Tsp secreted into the junctional space.

KEY WORDS: Myotendinous junction, Thrombospondin, Integrin, Muscle, Tendon, Drosophila

INTRODUCTION

The development of functional musculature depends on the correct encounter and adhesion of muscles with their corresponding tendon cells. In Drosophila the hemiadherens junctions, formed on both sides of the myotendinous junction, mediate the adhesion between muscles and their corresponding tendon cells (Bokel and Brown, 2002; Brower, 2003; Brown, 2000). The muscle-specific integrin heterodimer αPS2βPS accumulates at the muscle counterpart of this junction, and binds to its specific extracellular matrix (ECM) ligand Tigorin (Bunch et al., 1998; Fogerty et al., 1994). Correspondingly, the tendon-specific integrin heterodimer αPS1βPS accumulates at the tendon counterpart of the myotendinous junction, and is thought to associate with the laminin ligand (Gotwals et al., 1994; Prokop et al., 1998). Both hemiadherens junctions on each cell type exhibit a symmetrical distribution, raising the possibility that, although each cell utilizes a distinct integrin heterodimer, the formation of the myotendinous junction is coordinated between the two cell types. In the absence of the common βPS subunit, muscles initially interact with tendon cells; however, following muscle contraction the muscles detach from the tendon cells and round up (the myospheroid phenotype) (Bokel and Brown, 2002). Notably, lack of the muscle-specific αPS2 subunit similarly leads to muscle detachment (Brown, 1994); by contrast, however, lack of the tendon-specific αPS1 (e.g. in the mew mutant embryos) does not lead to muscle detachment (Brower et al., 1995). mew mutant embryos hatch, suggesting that occupation of the muscle-specific αPS2βPS junction by its ligand may be sufficient for the formation of embryonic myotendinous junctions. The αPS1 belongs to the laminin-binding type α family of receptors and binds to laminin (Gotwals et al., 1994). Drosophila laminin may consist of β1 and β2 subunits and either of two laminin α subunits (Garrison et al., 1991; MacKrell et al., 1993). The αPS1 is thought to associate with laminin containing the Lanα subunit (also known as α3,5), which when deleted does not exhibit significant muscle-tendon attachment defects (Prokop et al., 1998). By contrast, lack of the laminin α1,2, which associates with the αPS2βPS (Graner et al., 1998), results in a mild muscle-detachment phenotype (e.g. wing blister mutants) (Martin et al., 1999), pointing to the crucial function of the muscle-specific PS2 in the formation of the myotendinous junction.

Tigorin, a Drosophila-specific ECM component, has been shown to associate with the muscle-specific αPS2βPS integrin. However, homozygous tiggrin mutant embryos do form muscle-tendon junctions and the adult flies are only semilethal (Bunch et al., 1998; Fogerty et al., 1994). In addition to its role in the establishment of myotendinous junctions, integrin-mediated adhesion is essential for several biological processes, including dorsal closure, visceral mesoderm development and the development of the adult fly wing (Brabant et al., 1998; Brower et al., 1995; Devenport and Brown, 2004; Leptin et al., 1989; Martin-Bermudo et al., 1999; Narasimha and Brown, 2004; Stark et al., 1997). Wing epithelial cells from the dorsal and ventral aspects of the wing form specialized integrin-mediated adherens junctions required for the development of the adult fly wing. At morphogenesis dorsal wing epithelial cells expressing αPS1βPS are brought together with ventral cells that express αPS2βPS. Adhesion between these two epithelial sheets of cells is presumably mediated by specific ECM ligands. Although the involvement of the laminin α1,2 (wing blister) has been described, ligand specificity of each of the PS integrin receptors in this context has yet to be elucidated.

Tendon cells are specified in the Drosophila ectoderm as a result of the activity of the tendon-specific transcription factor Stripe. Embryos mutant for stripe do not develop normal tendon cells (Frommer et al., 1996). Conversely, ectopic expression of Stripe

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leads to ectopic development of tendon cells (Becker et al., 1997; Vorbruggen and Jackle, 1997). In a search for genes that are regulated by the tendon-specific transcription factor Stripe, we recovered Drosophila Thrombospondin.

Thrombospondins (Tsps) are a family of extracellular matrix proteins that mediate cell-cell and cell-matrix interactions by binding membrane receptors, extracellular matrix proteins and cytokines (Adams, 2001; Lawler, 2000). In vertebrates there are five tsp genes expressed in various tissues, including the brain (Tsp1 and Tsp2), bones (Tsp5) and tendons (Tsp4). Tsp1 and Tsp2 are closely related trimeric proteins that share the same set of structural and functional domains. Tsp4 and Tsp5 are pentameric and differ from Tsp1 and Tsp2 in their domain arrangement. All Tsps share a typical C-terminal domain (CTD) that contains epidermal growth factor (EGF)-like repeats, and a Ca-binding domain. The N-terminal domain contains additional conserved regions including the laminin G-like domain (which is not present in Tsp5) (Adams and Lawler, 2004). Drosophila tsp is encoded by a single gene that is spliced into four variants, among which only one (TspA) contains the conserved CTD, which in addition to the EGF repeats and Ca-binding domains also includes a putative integrin-binding KGD motif. The N-terminal domain contains a conserved heparin-binding domain and putative integrin-binding motifs RGD and KGD. Drosophila Tsp is closest in structure to vertebrate Tsp-5/COMP, which is expressed mainly in cartilage and certain other connective tissues and has a role in chondrocyte attachment, differentiation and cartilage ECM assembly (Adams et al., 2003).

A wide range of functions has been attributed to the different Tsps, including a role in platelet aggregation, inflammatory response, regulation of angiogenesis during wound healing, and tumor growth (Adams and Lawler, 2004). Recently, Tsp1 and Tsp2 were described as astrocyte-secreted components that promote synapse formation in the CNS (Christopherson et al., 2005).

The large isoform of Drosophila Tsp has been shown to form pentamers and exhibits heparin-binding activity. Its major sites of expression in the embryo are the muscle attachment sites, and also the precursors of the longitudinal visceral muscles. In larval stages it is expressed in wing imaginal discs (Adams et al., 2003).

Here we report that Drosophila Tsp is a key ECM component that is required for muscle-specific adhesion to tendon cells. In tsp mutant embryos muscles fail to attach to tendon cells, and often aggregate and form ectopic integrin-mediated junctions with neighboring muscles. This leads to nonfunctional somatic musculature and embryonic lethality. In the embryo, Tsp is required for integrin-mediated adhesion as measured by Talin-specific accumulation. Furthermore, we show that Tsp can functionally bind to αPS2βPS-integrins as the purified CTD of Tsp mediates PS2 integrin-dependent cell spreading in a KGD- and PS2-dependent manner.

Taken together, our results suggest a model whereby Tsp produced by tendon cells is required for muscle-specific adhesion to tendons by binding the muscle-specific αPS2βPS integrin receptors, and a subsequent consolidation of the junction by enhanced tendon-specific production of Tsp secreted into the junctional space.

**MATERIALS AND METHODS**

**Fly strains**
The following gal4 lines were used: engrailed-gal4, patched-gal4, 69B-gal4, Df(2)LR349 (uncovering tsp), Df(3)IGD4 (uncovering stripe), myosin-G4 and αPS2βPS (obtained from the Bloomington Drosophila Stock Center). tspα was produced by P-element-mediated transposition from P{Egyg2/Tspα}EY10724. UAS-tspA was produced in our laboratory. For the rescue experiments the following lines were created: tspα/Cyo(BB);sr-gal4/TM6(BB), tspα/Cyo(BB);me2-gal4/TM6(BB) and tspα/Cyo(BB);UAS-tspA/TM6(BB). The first or second fly strains were crossed with the third strain.

**Cloning of Tsp cDNA for expression in flies and bacteria**
Expressed sequence tags (ESTs) used for creating tsp cDNA were GH27479 (Open Biosystems, USA) and AT07402 (Drosophila Genomics Resource Center). uas-tsp-AD was prepared by fusing the ESTs GH27479 (encoding the N-terminal domain) and AT07402 (encoding the CTD) in frame and cloning the fused product into a pUAST vector. In order to fuse the ESTs two rounds of PCR were performed. In the first PCR, the respective ESTs were amplified individually with the following primers:

**GH27479:**
forward primer 5′-ATGATTTGACCCCGCGTGG-3′ and reverse primer 5′-CCCAGGAGGCACCGTTGTC-3′. The reverse primer has partial sequence similarity with the 5′ region of AT07402.

**AT07402:** forward primer 5′-CACCCGCTGCCTACACGGGGCC-ATTGCTTCAAGCTG-3′ and reverse primer 5′-TCGATCTCCTGCAACTTCCAC-3′ (tsp-R-cDNA). The products of the first round of PCRs were ligated and used as templates for PCR. Here the forward primer was the same as that used to amplify GH27479, whereas the reverse primer was a different one with an EcoRI linker to facilitate later cloning processes – 5′-GGAAGCTTTGAGATGGTTGAGATGGCGG-3′ (tspA-EcoRI-R). The fused PCR product (tspAD) was cloned into pUAST at the EcoRI site to yield the uas-tspAD clone. The sequences of the various plasmids were verified by sequencing.

The cDNA GH27479 was cloned into the pGEX vector to produce a GST fusion peptide. The protein was expressed and purified according to standard protocols.

The Gateway Cloning Technology (Invitrogen Life Technologies, USA) was used to produce the purified CTD protein fused with His tag. The CTD encoding sequence was amplified for five cycles using the forward primer 5′-AAAAAGCCCGCTTTCCTTATTTAAGAGGAGATATATTGCTACACCCTCACATTTCCTCTGCCCAGTGCTTCAAGGTT-3′, which contains part of the recombination sequence and the 6× His tag sequence in the 5′ region, and the reverse primer 5′-AGAAGTGGTCCTCACTTCTGCACCTCCTACCTTCCACCTTC-3′ that consists of the recombination sequence at the 5′ region of the insert. The product from the above PCR was used as a template for a regular PCR reaction with the forward primer 5′-GGGGACAATTTGTGACAAAAAAGGACTTGTTCACGAAAC-3′ and reverse primer 5′-GGGGACAATTTGTGACAAAAAAGGACTTGTTCACGAAAC-3′, which completes the homologous recombination sequence at the 5′ end of the insert, and the reverse primer 5′-GGGGACAATTTGTGACAAAAAAGGACTTGTTCACGAAAC-3′, which completes the homologous recombination at the 3′ end. This PCR product was used to produce an entry clone in pDONR-201, by recombination. The clone was confirmed by sequencing and was used to transfer the insert into the pDEST14 expression vector. The Tsp-His tag protein was expressed and purified according to the protocols of the Gateway Expression System and Qiagen’s Ni-NTA agarose bead system. The purified proteins were concentrated and diluted in PBS containing 2 mM CaCl2 three times. Protein concentration was determined by comparison to Coomassie Blue staining on a standard protein gel. Bovine serum albumin (BSA) was used as a concentration standard.

**Staining of embryos**
Antibody staining was performed essentially as described previously (Ashburner, 1989). Primary antibodies used were anti-Mosin heavy chain (MHC) (P. Fisher, Stony Brook, NY), anti-Striped, anti-Tsp (produced in our laboratory), anti-PSβ, anti-Talin (N. Brown, University of Cambridge, Cambridge) and anti-Tiggrin (J. and L. Fessler, UCLA). Secondary antibodies included Cy3, fluoresceine or horseradish peroxidase (HRP)-conjugated anti-guinea pig, anti-rat, anti-rabbit or anti-mouse (Jackson, USA).

Visualization of embryos was performed with a Bio-Rad Radiance 2100 confocal system coupled with a Nikon Eclipse TE300 microscope.
Cell culture, spreading and flow cytometry

*Drosophila* S2 cells were maintained and transformed as described (Ianuzzi et al., 2002). Cells were transiently transfected with the selectable marker bearing plasmid pH8CO and integrin subunit expressing plasmid pH8βPS together with either pHSoPS2m8, pHSoPS2m8-LOF or pHSoPS1 (Bunch et al., 1992; Baker et al., 2002; Li et al., 1998). αPS2m8-LOF produces a mutant αPS2m8 subunit (222-224 YWQ>AWA) that is impaired in ligand binding (Baker et al., 2002; Irie et al., 1995). Following transfection, cells were grown for 2 days in selection medium containing methotrexate. Expression of PS2 integrins was similar for both wild type and the αPS2m8-LOF as determined by staining with the αPS2-specific monoclonal antibody CF.2C7 (Brower et al., 1984) followed by flow cytometry [mean fluorescence intensities (MFI) were 320±5 and 316±14, respectively. For untransformed S2 cells MFI levels averaged 46]. High levels of PS1 integrin expression in the PS1-transfected cells was confirmed by staining with the αPS1-specific antibody DK1A4 (Brower et al., 1984) followed by flow cytometry (MFI was 1173±87; for untransformed S2 cells MFI levels averaged 110).

For cell spreading, 96-well tissue culture plates were coated overnight at 4°C with approximately 30-40 μg/ml C-terminal Tsp polypeptide or the mutant C-terminal Tsp polypeptide KGD>LGE in PBS+0.3 mM CaCl₂, then blocked with 20% dried milk in PBS+0.3 mM CaCl₂ for 1 hour at room temperature, and washed three times with PBS+0.3 mM CaCl₂. Transiently transfected cells were diluted to 2×10⁵ per ml in M3 medium, lacking serum but containing 2 mg/ml BSA, and allowed to spread on the coated wells for 1 hour. At this time the cells were fixed and observed for cell spreading using a Nikon phase-contrast microscope (Nikon Diaphot-TMD). Results are expressed as the mean and standard error of four experiments. For each experiment three fields of cells, each containing more than 100 cells, were scored.

Polyacrylamide electrophoresis of fusion proteins

Wild type (KGD) and mutant (LGE) CTD fusion proteins were electrophoresed on a 4-15% gradient polyacrylamide gel together with 1.0, 0.5 and 0.25 μg/ml BSA and prestained Precision Plus Protein All Blue standards (Bio-Rad). The gel was then stained with Coomassie Blue. Each CTD concentration appears to be between 0.1 and 0.2 μg/ml. This would indicate a concentration of approximately 150 μg/ml. A 1/4 dilution of this was used for the cell spreading assays. Preliminary observations indicated that less spreading was supported by a further 1/4 dilution, whereas no additional spreading was seen if the CTD preparations were used without dilution (T.B., unpublished).

RESULTS

The distribution of Tsp changes following the formation of the myotendinous junction

Previous studies showed that *tsp* mRNA is detected in segmental stripes that correspond to the muscle attachment sites from stage 11 of embryonic development (Adams et al., 2003). We have raised an antibody to Tsp and used it to correlate the distribution of Tsp protein with the process of somatic muscle development. The specificity of the antibody is demonstrated by its lack of reactivity with embryos homozygous for Df(2L)BSC9, which removes the *tsp* gene (Fig. 3B). At stage 12-13, prior to the formation of muscle-tendon junctions, Tsp protein is detected as scattered dots around the Stripe-expressing tendon cells. These dots appear to be external to the tendon cells, as judged by their distance from the tendon cell. At stage 16, following the establishment of the myotendinous junction, Tsp becomes highly concentrated at the muscle-tendon junction sites, as deduced from its localization between the muscle edges and the tendon cell nucleus (labeled with Stripe) (Fig. 1). The change in Tsp distribution is consistent with a dynamic process, following the formation of the myotendinous junction, Tsp associates preferentially with this site. To investigate further the relationship between Tsp distribution and muscle-tendon adherens junction formation, we examined the distribution of Tsp in *myospheroid* (mys<sup>XG43</sup>) mutant embryos lacking functional integrins. In *mys* mutant embryos, the muscles migrate normally towards their attachment sites and the muscle pattern appears normal at stage 14-15 of embryonic development. However, at stage 16, when the muscles initiate their contraction, they pull away from the attachment sites and become rounded. Tsp distribution in *mys<sup>XG43</sup>* embryos at stage 16 is not as concentrated as in wild-type embryos and often appears as scattered dots, similarly to stage 12-13 wild-type embryos (Fig. 2, arrowheads). Nevertheless, we still detect higher accumulation of Tsp at the muscle edges, suggesting partial integrin-independent association with the muscle cells (Fig. 2, arrow).
Fig. 2. Tsp levels are reduced in myospheroid mutant and are induced by Stripe. (A-D) Mutant embryos lacking functional βPS (mys\textsuperscript{VG4}) and labeled with Tsp (A, red), Stripe (B, blue) and Myosin heavy chain (C, MHC, green) are shown. The merged image is shown in D. Arrows indicate sites where muscles are still associated with tendon cells, and Tsp is higher. Arrowheads indicate sites where muscles have detached, and Tsp is significantly low. (E,F) stripe mutant embryos stained for Tsp (E, green) and MHC (red). F is the merged image. Tsp is still detected in these embryos. (G-I) Embryos overexpressing Stripe using the engrailed-gal4 driver, stained for Tsp (G, red) and anti-Stripe (H, green). Their merged image is shown in I. Tsp is highly induced by Stripe.

We have initially recovered Tsp in a microarray screen for genes that are downstream of Stripe by comparing the gene expression profile of embryos overexpressing Stripe in the ectoderm with that of wild-type embryos (A.S., unpublished). Further analysis showed that in stripe mutant embryos Tsp protein is still detected, possibly because of earlier Stripe-independent transcriptional input (Fig. 2E,F). Importantly, overexpression of Stripe using the engrailed-gal4 driver leads to a significant induction of Tsp expression, confirming the microarray results and the ability of Stripe to induce Tsp expression (Fig. 2G-I). Because Stripe expression is greatly upregulated following muscle-tendon interaction, it is assumed that Stripe-dependent Tsp induction is linked to muscle-tendon interaction. We conclude that Tsp distribution is dynamic and correlates with the biogenesis of adherens junction formation.

**Tsp is required for muscle binding to tendon cells**

The genomic organization of the tsp gene suggests that the gene produces four splice variants, of which only TspA includes the conserved CTD and the Tsp type-3 repeats (FlyBase annotation data). We have induced a mutation in the tsp locus (tsp\textsuperscript{8R}) by imprecise excision of an EP element inserted within the tsp coding region, 847 nucleotides 5’ to the stop codon of TspA. Although flies homozygous for precise excisions of this EP element are viable, the imprecise excision leads to embryonic lethality. We mapped the imprecise excision using PCR with primers flanking the insertion site and found that it removes 3457 nucleotides within the tsp gene. The deletion results in the putative production of a truncated protein, which lacks the conserved CTD, the Tsp type-3 repeats and four out of six EGF repeats (Fig. 3). The gene on the 3' flank of the tsp gene (CG11327) is not affected. Staining of the tsp\textsuperscript{8R} homozygous mutants with the anti-Tsp antibody (raised against the N-terminal domain) revealed that the truncated Tsp protein is not detected in these embryos (Fig. 3C).

To assess the contribution of Tsp to the assembly of somatic musculature, we analyzed the phenotype of tsp mutant embryos. The initial myotube fusion and the migration of muscles towards tendon cells appear normal in the tsp mutant embryos (Fig. 4). However, a large proportion of the somatic muscles of stage 16 tsp\textsuperscript{8R} mutant embryos are rounded. In addition, the muscles do not extend between their corresponding Stripe-expressing tendon cells, as in wild-type embryos (Fig. 5). This phenotype is detected in all the muscle types at stage 16, although some variability exists between the distinct muscles, and the phenotype is more severe in embryos at late stage 16. No significant difference was detected between the phenotype of direct muscle-tendon junctions, e.g. the lateral transverse muscles, and indirect junctions, e.g. ventral-lateral muscles. The residual association of the muscles with tendons in tsp mutants may reflect the redundant function of laminin (wing blister), which may contribute, although to a lesser extent, to muscle-tendon interaction. In addition, maternal tsp, which may still be present at this stage, may contribute to the residual muscle-tendon association. The rounded muscle phenotype is reminiscent of the mys mutant embryos, suggesting that in tsp mutant embryos the association of somatic muscles with tendon cells may be abrogated. The overall pattern of tendon cells was slightly aberrant, as deduced from the Stripe expression pattern, presumably reflecting the aberrant somatic muscle pattern. Essentially, a similar phenotype was observed in embryos trans-heterozygous for tsp\textsuperscript{8R} and Df(2L)BSC9, which uncovers the entire tsp gene (see Fig. 6), suggesting that tsp\textsuperscript{8R} represents a severe mutant allele of tsp. Importantly, the muscle phenotype of the tsp\textsuperscript{8R} mutant embryos is rescued by overexpressing TspA in tendon cells using the stripe-gal4 driver (Fig. 5G), but not following overexpression of TspA in the muscles using the mef-2-gal4 driver (Fig. 5H). This suggests that the tendon-specific expression of Tsp is essential for its function. To test whether the somatic muscles in the tsp mutant embryos are capable of forming integrin-mediated adherens junctions, we stained the embryos for integrin βPS. The typical integrin-positive bands...
were still detected in each segment of the tsp mutant embryo, corresponding to the ends of the ventral and dorsal longitudinal muscles (Fig. 6). However, we detected ectopic integrin staining at various locations (Fig. 6, arrowheads), which appeared to correspond to regions of muscle-muscle interactions. This phenotype was detected in all tsp mutant embryos, in at least one segment. Importantly, the edges of each of the lateral transverse muscles, which normally interact with a single tendon cell, exhibited a large reduction in integrin staining except when the lateral transverse muscle was associated with a neighboring muscle cell (Fig. 6, brackets and arrow). The positive staining of integrin at sites overlapping muscle-muscle interactions, as well as the rounding up of some of the muscles, raised the possibility that the somatic muscles of tsp mutant embryos bind primarily with neighboring muscle cells and not with tendon cells, and the relative ‘normal’ staining of βPS integrin in the tsp mutant embryos represents sites of muscle-muscle-dependent adherens junctions. Indeed, a lateral view of tsp mutant embryos shows that in some cases βPS observed at the ends of the muscles is not coupled to Stripe-expressing tendon cells (Fig. 6f, arrowhead), in contrast to wild-type embryos (Fig. 6e, arrowhead).

The muscle-muscle junctions detected by the staining for βPS may utilize the Tiggrin ligand to assemble the αPS2βPS integrins on both sides of the hemiadiherens junction. Staining with anti-Tiggrin revealed that in tsp mutant embryos Tiggrin accumulation is not observed as stripes but rather in dots, and often Tiggrin-positive dots are detected in ectopic sites (Fig. 7, arrows). The large Tiggrin dots observed between the longitudinal muscles are consistent with defects in the muscle-tendon interaction, as the tendon cells are arranged as a line of cells at this region, leading to the subsequent line of Tiggrin (and integrin) staining.

We conclude that the somatic muscles in tsp mutant embryos fail to form junctions with tendon cells, but are still capable of forming integrin-mediated junctions with neighboring muscles, presumably using Tiggrin as an ECM ligand for the muscle-specific integrin.

**Integrin-mediated adherens junctions are greatly reduced in tsp mutant embryos**

The abnormal pattern of the somatic muscles in the tsp mutant embryo raised the possibility that the muscle-tendon integrin-mediated adhesion is defective in the mutant embryos. A hallmark of appropriate integrin-mediated adhesion is the accumulation of Talin at the cytoplasmic face of the hemiadiherens junction, where it binds directly to the integrin cytoplasmic domain, modulating its ligand affinity and recruiting actin microfilaments to this site (Brown et al., 2002). A significant reduction of accumulated Talin levels in tsp mutant embryos is observed. Whereas Talin is still detected at the sites of muscle-muscle junctions, it was entirely missing at sites where individual muscles would normally form junctions with single tendon cells, in particular at the junction sites formed between the lateral transverse muscles and their corresponding tendon cells (Fig. 8, brackets). The lack of Talin at these sites corresponds with the lack of βPS-integrin staining and is consistent with the loss of
appropriate myotendinous junction. Thus, in the absence of functional Tsp, individual myotubes fail to form integrin-mediated adherens junction with tendon cells.

**Tsp promotes cell spreading in a KGD- and PS2-dependent manner**

The results so far are consistent with a model where the tendon-dependent Tsp promotes adhesion of the muscles by binding to the αPS2βPS integrin receptors. To test this model directly, we cloned the CTD into a Histidine-tag expression vector. In addition, a mutated CTD (CTD*) where the KGD site was mutated into LGE was similarly produced. Both CTDs were produced in bacteria, purified on Ni-NTA agarose beads, concentrated, and diluted in PBS containing 0.3 mM CaCl₂. The purified proteins were used at ~40 µg/ml to coat tissue culture plates. S2 cells expressing either αPS2m8βPS integrin, a mutated αPS2m8 or αPS1βPS receptors were plated on these cultured dishes and the percentage of spreaded cells was determined.

Tsp CTD induces a significant elevation in the number of αPS2m8βPS cells spreading relative to a control where no ligand was added. Importantly, the mutated CTD (CTD-LGE) did not induce cell spreading and was similar to the no-ligand control (Fig. 9, upper panel). Similarly, cells expressing a mutated αPS2m8 did not induce cell spreading on the CTD and behaved like the control cells where no ligand was added. Cells expressing αPS1βPS did not show a specific elevation in the number of cells spreading on the CTD. The relatively low percentage of spread cells on the Tsp-CTD may reflect a partial reconstitution of the Tsp-CTD produced and purified from bacteria. We were not able to produce efficient amounts of the Tsp N-terminal domain, presumably because of its instability.

These experiments are consistent with a direct binding of αPS2βPS integrin receptors with the KGD site that is included in the CTD of Tsp.

**DISCUSSION**

The formation of a functional myotendinous junction is essential for the normal function of the somatic musculature and the viability of hatched larvae. In *Drosophila* embryos this junction develops following the migration of myotubes towards their corresponding tendon cells. The signal that promotes muscles to form the junction with tendon cells, as well as the mechanism governing mutual induction of the shared junction sites, is yet to be elucidated. Here,
we show that the ECM protein Thrombospondin, produced and secreted by the tendon cells, is essential for muscle adhesion to the tendon cell through association with the muscle-specific PS2 integrin receptors. Moreover, we provide a model explaining the biogenesis of the junction and its temporal and spatial regulation.

Based on our results, we suggest that the dynamics of myotendinous junction formation involve the following sequential steps. (1) When the myotube is very close to the tendon cell, Tsp secreted continuously from the tendon cell associates with the muscle leading edge and binds to the muscle-specific αPS2βPS integrin receptors. Because Drosophila Tsp forms pentamers, each pentamer potentially associates with several PS2 receptors, leading to accumulation of αPS2βPS receptors at the myotube leading edge. This association triggers integrin-mediated adhesion and Talin accumulation at the cytoplasmic tail of the PS2 integrin receptors. (2) Tsp may bind to the tendon surfaces through an unknown ligand. (3) Stripe levels in the tendon cell are elevated following the establishment of the muscle-tendon junction, because of Vein-EGF receptor (EGFR) signaling (Yarnitzky et al., 1997). Stripe induces the elevation of Tsp levels, creating a positive feedback loop that encourages further secretion and accumulation of Tsp at the junction site, strengthening the myotendinous junction.

We showed that the KGD site in the CTD of Tsp triggers PS2 integrin-dependent cell spreading. This sequence had been shown to bind certain types of vertebrate integrin receptors (Scarborough et al., 1993). The N-terminal domain of Tsp contains an additional KGD site, and an RGD site, both implicated in integrin-binding activity. These sites may also contribute to the binding of the PS2 muscle-specific integrins. Therefore, each Tsp pentamer contains multiple binding sites for PS2 integrin receptors, and thus may...
induce receptor aggregation at the muscle leading edge. It remains to be determined whether Tsp is capable of binding to PS1 integrins or other receptors expressed by the tendon cell.

Whether Tsp functions as an integrin ligand in other tissues (e.g. midgut, salivary gland, dorsal closure and the wing epithelium) is yet to be elucidated. Our phenotypic analysis of the tsp8R mutant embryos did not reveal a major phenotype in the gut, CNS or dorsal closure. Similarly, tsp8R mutant clones induced at the larval stage did not result with wing blisters as in integrin-induced clones. Although mutants for the tsp8R allele did not show staining with the anti-Tsp antibody, it is still possible that residual Tsp activity is retained in the mutants because of the activity of the other TSP isoforms (which were not affected by the deletion of the EP excision at the CTD). In addition, we detected maternal tsp transcripts that may partially rescue the zygotic tsp phenotype in the early developmental stages.

An additional relevant ECM component at the myotendinous junction is laminin. Laminin α1,2 (encoded by wing blister) is required for the formation of the myotendinous junction (Martin et al., 1999). Laminin α1,2 contains an RGD sequence and also binds to the PS2 integrins (Graner et al., 1998), demonstrating the crucial role of these receptors in the formation of the myotendinous junctions. It is possible that laminin containing the laminin α1,2 subunit associates with Tsp in the myotendinous junctional space. Both laminin and Tsp carry a heparin-binding domain and it is possible that they interact indirectly through a putative heparin-containing proteoglycan. Because we do not detect changes in laminin distribution following overexpression of Tsp (using anti-laminin antibody), we do not consider there to be any direct Tsp-laminin interaction (data not shown). The heparan sulfate glycoprotein Syndecan is produced by the muscle cells. In syndecan...
mutant embryos the somatic muscle pattern is defective, a phenotype that is attributed to an effect of Syndecan on Slit distribution and function (Steigemann et al., 2004). However, Syndecan at the muscle cell membrane may contribute to a putative indirect interaction between Tsp and laminin through its heparin-containing domain. Such interaction may enhance the accumulation of ECM components such as Tsp and laminin at the myotendinous junction. In support of this hypothesis, vertebrate Tsp has been shown to bind Syndecan at its CTD (Adams and Lawler, 2004). However, syndecan homozygous mutant embryos do not exhibit alterations in Tsp distribution (data not shown), arguing against a central role for Syndecan in Tsp distribution. Nevertheless, it remains possible that another heparin domain-containing protein functions to promote Tsp and laminin deposition at the myotendinous junction.

We consider that the Stripe-dependent positive feedback that upregulates tsp transcription contributes significantly to the establishment of the myotendinous junction. Previous studies have shown that muscle-tendon interactions form a signaling center, which is initiated by muscle-dependent Vein secretion and accumulation at the myotendinous junction. Vein activates the EGFR pathway in the tendon cell, leading to a significant elevation of the transcription factor Stripe (Yarnitzky et al., 1997). We show that Stripe induces upregulation of Tsp. Taking these results together, we suggest that the initial formation of the hemihaedrons junction creates a self-auto-regulatory nucleation center, which leads to additional deposition of Tsp and possibly other ECM components. These, in turn, gradually strengthen the hemihaedrons junction formed between the muscle and the tendon cell.

Vertebrate Thrombospondins are essential for a variety of biological activities, including cell adhesion, migration, angiogenesis, etc. Our work reveals an intriguing similarity between the role of Tsp in the formation of the myotendinous junction and the role of vertebrate Tsp1 and Tsp2 in the induction of synapses. It was shown that Tsp provided by oligodendrocytes is a potent inducer of synapse formation on the dendrites of cultured neurons (Christopherson et al., 2005). Although these synapses are not electrically active, the Tsp-induced synapses exhibit typical synapt-like ultra-structures. The biogenesis of the myotendinous junction carries several similarities to the biogenesis of synapses, including the mutual crosstalk between the two cell types involved and the gradual formation of the junction at both cell membrane involved.

In summary, our analysis of Tsp function reveals the molecular dynamics and biogenesis of the myotendinous junction. A similar scenario may unfold during Tsp-dependent synapse formation in the development of vertebrate embryos.

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