Terminal tendon cell differentiation requires the glide/gcm complex

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Summary

Locomotion relies on stable attachment of muscle fibres to their target sites, a process that allows for muscle contraction to generate movement. Here, we show that glide/gcm and glide2/gcm2, the fly glial cell determinants, are expressed in a subpopulation of embryonic tendon cells and required for their terminal differentiation. By using loss-of-function approaches, we show that in the absence of both genes, muscle attachment to tendon cells is altered, even though the molecular cascade induced by stripe, the tendon cell determinant, is normal. Moreover, we show that glide/gcm activates a new tendon cell gene independently of stripe. Finally, we show that segment polarity genes control the epidermal expression of glide/gcm and determine, within the segment, whether it induces glial or tendon cell-specific markers. Thus, under the control of positional cues, glide/gcm triggers a new molecular pathway involved in terminal tendon cell differentiation, which allows the establishment of functional muscle attachment sites and locomotion.

Movies available online

Key words: Drosophila melanogaster, glide/gcm, glide2/gcm2, Tendon cell differentiation, Muscle attachment, Locomotion

Introduction

In invertebrates, muscles are attached to bones, whereas in arthropods they are attached to cuticle, the insect exoskeleton. In both cases, attachment is performed by tendon cells that establish a physical link between muscles and bones or cuticle. The tension induced by muscle contraction is then transmitted to cuticle or bones, which allows locomotion.

Our knowledge on the formation of junctions between muscles and tendon cells (called attachment sites) derives from fly studies (Volk, 1999). Initially, subsets of epidermal cells within each embryonic segment acquire the competence to form tendon cells. This early muscle-independent step is under the control of the Stripe transcription factor (Frommer et al., 1996; Lee et al., 1995). Competent tendon cells provide guidance cues that direct myopodia to their attachment sites. Once muscles and target tendon cells get in contact with each other, myopodia stop growing (Bate, 1990; Swan et al., 2004) and only competent tendon cells that are in contact with muscles maintain tendon cell marker expression (Becker et al., 1997). This late muscle-dependent differentiation step is characterised by the expression of delilah (Armand et al., 1994) and \( \beta \)-tubulin (Buttgereit, 1993; Buttgereit et al., 1991; Yarnitzky et al., 1997) and leads to the formation of hemiadiherens junctions (HAJs), cell-specific junctions associated with extracellular matrix proteins deposited between muscles and tendon cells (Tepass and Hartenstein, 1994). Mutations in HAJ components, as for example in genes encoding the \( \beta \)PS integrin ortholog (Brown, 1994; Leptin et al., 1989; MacKrell et al., 1988; Newman and Wright, 1981) or its ligand Tiggrin (Bunch et al., 1998), lead to complete lack of adhesion at attachment sites, which results in muscle detachment and rounding (Bokel and Brown, 2002; Prokop et al., 1998). Interestingly, although \( \beta \)PS integrin is expressed in muscles and tendon cells, most accumulation occurs in muscles and only the muscular component seems required in the process (Martin-Bermudo and Brown, 1996). Thus, establishment of HAJs triggers muscle–tendon cell interactions that are necessary for the formation of functional muscle attachment sites (Clark et al., 2003; Martin-Bermudo, 2000; Martin-Bermudo and Brown, 2000). This terminal step of tendon cell differentiation that allows muscle contraction to generate locomotion is still poorly understood.

\( \beta \)PS integrin deficient/\( \beta \)PS integrin missing (glide/gcm) and glide2/gcm2, which we will refer to as glide and glide2 throughout the text for the sake of simplicity, form a gene complex on the second chromosome and encode homologous transcription factors (Akiyama et al., 1996; Alfonso and Jones, 2002; Hosoya et al., 1995; Jones et al., 1995; Kamarer and Giangrande, 2001; Miller et al., 1998; Ragone et al., 2001; Schreiber et al., 1997; Van De Bor and Giangrande, 2002; Van De Bor et al., 2000; Vincent et al., 1996). When both genes are missing, glial cells transform into neurons, whereas overexpression of either glide or glide2 is sufficient to induce gliogenesis, indicating that the glide complex triggers the glial fate (Akiyama-Oda et al., 1998; Alfonso and Jones, 2002; Bernardoni et al., 1999; Bernardoni et al., 1998; Hosoya et al., 1994).
Here, we reveal a novel role of the glide complex during Drosophila development. We show that both glide and glide2 are expressed in a subpopulation of embryonic tendon cells. In the absence of both genes, muscles are disorganised and HAJs are abnormal, even though all known tendon cell–specific markers, including stripe itself, are still expressed. Our data also show that the glide complex controls a new molecular pathway required in terminal tendon cell differentiation and tendon cell–muscle interactions. Finally, we show that the same transcription factor is used for different purposes during development (fate choice in neurogenic region, terminal differentiation in tendon cells) in response to positional cues that control its expression and its specificity.

Materials and methods

Fly strains and transgenic lines

The wild-type strain was Sevelen. Df(2L)132 (glide-glide2 deletion) and glide<sup>N7-4</sup> are described in Kammerer and Giangrande and Vincent et al., respectively (Kammerer and Giangrande, 2001; Vincent et al., 1996). The A87 enhancer trap line carries an insertion into the glide promoter (Jones et al., 1995; Vincent et al., 1996). UAS-glide F18A (Bernardoni et al., 1998), UAS-glide2 (Kammerer and Giangrande, 2001) and UAS-stripe b (Becker et al., 1997) lines were used for ectopic expression. UAS-ncGFP (gift of C. Desplan) targets GFP to nucleus and cytoplasm. Rescue experiments were realised using the stripe-gal4 driver (gift of G. Morata). Df(3R)DG4 (stripe deletion), wingless (wg<sup>1–12</sup>, patched (ptc), patched-gal4 (ptc-gal4) and engrailed-gal4 (en-gal4) flies were obtained from the Bloomington Stock Center. glide-gal4 carries a Gal4 P-element inserted into the glide promoter (Hayashi et al., 2002). The glide<sup>DN</sup> (glide Dominant Negative) construct was obtained by inserting the Glide DNA binding domain (amino acids 1 to 304) and by cloning the chimeric gene into the CasperUAST vector. The glide<sup>N7-4</sup> mutation was inserted into the UAS-glide<sup>DN</sup> construct by PCR. The UAS-glide<sup>DN</sup> and UAS-glide<sup>N7-4DN</sup> lines were obtained by germline transformation of w<sup>1118</sup> animals.

Immunohistochemistry

Embryos were immunolabelled as in Vincent et al. (1996). The following primary antibodies were used: guinea-pig anti-Stripe (1:300) (Becker et al., 1997), mouse anti-Repo (1:100) (DSHB), rabbit anti-β gal (1:500) (Cappel), rabbit anti-Alien (1:1000) (gift of A. Paululat), rabbit anti-Myosin heavy chain (MHC) (1:1000) (gift of D. P. Kiehart), rabbit anti-GFP (1:500) (Molecular Probes). Secondary antibodies conjugated with FITC, Cy3 (Jackson) were used at 1:400. Phalloidin-TRITC was used (1:100) (Molecular Probes) to label muscle F-actin in late embryos. Embryos were mounted in Vectashield medium (Vector) and analysed using conventional (Axioskop, Zeiss) or confocal (DMRE, Leica) microscopes.

RNA analysis

In-situ hybridisation was as in Bernardoni et al. (Bernardoni et al., 1999) and Kammerer and Giangrande (Kammerer and Giangrande, 2001). stripe a and Ent2 mRNAs were detected using specific probes corresponding to nucleotide 665 to 1583 (accession number NM 079671) and 681 to 1851 (accession number NM 135205), respectively. stripe a/b mRNAs were detected using a common probe corresponding to nucleotide 422 to 1140 (accession number NM 169786).

Transmission electron microscopy

Thirty-minute egg lays were kept at 25°C until stage 16. After manual devitellinization, embryos were fixed overnight at 4°C in 0.1 M Na-phosphate buffer (pH 7.4) containing 4% paraformaldehyde and 3% glutaraldehyde, postfixed for 1 hour in 1% osmium tetroxide in 0.1 M Na-phosphate buffer (pH 7.4) and dehydrated in graduated ethanol series ending with propylene oxide treatment. Embedding used an araldite-epon (48-hour polymerization at 60°C). Semi-thin sections were stained with toluidine blue and visualised under light microscope to determine the region of interest. Ultra-thin sections (60-70 nm) were contrasted with uranyl acetate and lead citrate, and examined using a Philips EM208 electron microscope. glide-glide2 embryos were genotyped by X-Gal staining.

Larval behaviour and histology

Locomotion was measured on 3.5 cm 2.5% agarose plates marked with a 0.5 cm grid by counting the total number of squares crossed in 10 minutes by each larva [modified from Naimi et al. (Naimi et al., 2001)]. For muscle visualisation, larvae were progressively dehydrated in ethanol series ending with methyl salicylate treatment and examined under polarised light (Axioskop, Zeiss).

Results

glide and glide2 are expressed in Drosophila embryonic tendon cells

In each segment, glide and glide2 are expressed from mid-stage 12 until the end of embryogenesis in a single row of epidermal cells (black arrows in Fig. 1A,B) located at the border, posterior to en|g|railed|expressing cells (data not shown). Using a glide-gal4; UAS-GFP line (glide::GFP) (Fig. 1C), we showed that glide and stripe are coexpressed (yellow nuclei, Fig. 1E). Tendon cells are subdivided into several subpopulations, depending both on their position and on the muscles to which they attach (Fig. 1F,H) (Volk, 1999). Longitudinal muscles (Fig. 1G) attached to tendon cells present along the segment border (yellow nuclei in Fig. 1E) or parallel to it (empty lozenges in Fig. 1D). Lateral transverse muscles (Fig. 1H) attached to intrasegmental tendon cells that are organised in two clusters per hemi-segment (white bracket in Fig. 1D). glide and glide2 are expressed in segment border tendon cells, the anteriormost cells in each segment (yellow nuclei in Fig. 1E and green cells in Fig. 1F-H), providing new molecular markers to identify these cells.

The glide complex is necessary for terminal tendon cell differentiation

As tendon cells are required for muscle attachment and as both glide and glide2 are able to trigger glial differentiation, we analysed muscle organisation in embryos lacking the whole complex (glide-glide2 lof).

Somatic muscles of glide-glide2 embryos did not show any abnormality until stage 16 (11.50-14.50 hours after egg-laying (AEL) stages, according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997)) (data not shown). Moreover, late markers (delilah and βtubulin) were still expressed (data not shown), indicating that muscles had established contacts with tendon cells (Yamitzky et al., 1997), a process normally achieved by stage 15 (11.20-11.50 hours AEL) (Bate, 1990; Swan et al., 2004).

By contrast, defects became obvious by early stage 17 (14.50-16.00 hours AEL): ventral longitudinal muscles bypassed their target tendon cells, reached the midline and attached to other muscles (Fig. 2B,D). This highly penetrant phenotype (93% of embryos, n=14), present in one or more
Fig. 1. glide and glide2 are expressed in tendon cells. (A,B) glide (A) and glide2 (B) in-situ hybridisation on wild-type Drosophila embryos, lateral views at mid-late stage 12. Unless specified, anterior is to the left in this and following figures. Both genes are expressed in epidermis (black arrows) and in glial cells of the central and peripheral nervous systems (large and small white arrowheads, respectively). Note that glide2 is expressed at lower levels compared with glide. (C-E) Anti-GFP (C) and anti-Stripe (D) immunolabelling on stage 14 glide::GFP embryos. Dorso-lateral views. GFP is expressed in epidermal cells (yellow brackets in C), peripheral glia (arrowheads in C) and hemocytes (asterisks in C), which also express glide and glide2 (Alfonso and Jones, 2002; Bernardoni et al., 1997; Kammerer and Giangrande, 2001). Tendon cells are visualised by anti-Stripe, those that are parallel to segment border cells being shown by empty lozenges (D). At this stage, intrasegmental tendon cells are organised in two clusters containing ten cells each and found at the dorsal and ventral ends of lateral transverse muscles (bracket in D indicates the dorsal cluster). (E) Merge labelling shows that epidermal glide-expressing cells at the segment border also express stripe (yellow nuclei in yellow brackets). Arrows in (D) indicate non-specific, tracing, labelling. (F-H) Schematic representations of muscles and tendon cells in two abdominal segments of a Drosophila embryo at stage 16/17, lateral views, dorsal to the top (modified from Armand et al., 1994). Dotted and dashed lines represent dorsal and ventral midlines, respectively. Muscles are represented in white or red, tendon cells in green (segment border cells), yellow (parallel to segment border cells) or blue (intrasegmental tendon cells). Red muscles in (F,G) indicate longitudinal and ventral longitudinal muscles, respectively. Red muscles in H show lateral transverse muscles. Both ends of most longitudinal muscles (F,G) attach to segment borders, whereas a small subset displays only one attachment site at the border. Lateral transverse muscles (H) attach to intrasegmental tendon cells. The subpopulation of glide/glide2-expressing cells corresponds to green tendon cells. Black bracket in F indicates the region shown in C-E. Scale bars: 50 μm.

Muscle attachment sites require glide

Muscle attachment defects were mostly observed ventrally (Fig. 2), whereas the glide complex is expressed in all segment border tendon cells (Fig. 1). Two lines of evidence suggested that such a confined phenotype was due to the pleiotropic effect of the glide-glide2 deficiency. First, lethality occurred before all attachment sites were fully differentiated and functional. Second, neuromuscular defects indirectly caused by lack of glia probably affected the contractions that normally occur at the end of embryogenesis. As a consequence, mutant tendon cells were not as solicited as wild-type ones.

To assess the role of the glide complex at the attachment site in the absence of nervous system phenotypes, we produced a tissue-specific glide-glide2 loss of function using a dominant negative (DN) approach. For this purpose, we generated a fusion protein between the Glide DNA binding domain and the Engrailed repressor domain. Chimeric genes with this repressor domain have already been successfully used to produce DN mutations (Jaynes and O’Farrell, 1991; Vickers and Sharrocks, 2002). Since Glide and Glide2 DNA-binding domains are highly homologous and bind to the same site (Kammerer and Giangrande, 2001; Miller et al., 1998), the glideDN construct probably inhibits targets of both proteins and, indeed, when expressed in the central nervous system (CNS), it blocks glial differentiation (B.A. and G.M.T., unpublished).
To specifically repress the glide complex dependent programme in tendon cells, we used two drivers. No muscle phenotype was detected in stripe-gal4; UAS-glide\textsuperscript{DN} flies (data not shown), probably because the driver is expressed too late to efficiently repress glide complex activity. By contrast, the earlier driver ptc-gal4 induced muscle defects similar to those observed in embryos lacking glide-glide2 (see ptc::glide\textsuperscript{DN} in Fig. 2G). To provide further evidence that the muscle defects are specifically due to glide\textsuperscript{DN}, we also generated a point mutation in the DNA binding domain (glide\textsuperscript{N7-4DN}). The glide\textsuperscript{N7-4} allele corresponds to a null, due to the fact that the mutant protein is not able to bind DNA (Miller et al., 1998; Vincent et al., 1996). Therefore, the glide\textsuperscript{N7-4DN} construct corresponds to an inactive form of glide\textsuperscript{DN}, providing a negative control. The findings that ptc::glide\textsuperscript{N7-4DN} embryos did not show muscle defects (Fig. 2H) and were perfectly viable until the adult stage indeed validate the DN approach. Finally, possible mesodermal contribution to the observed phenotype was excluded in two ways: the ptc-gal4 driver was not expressed in muscles, and mesodermal Glide\textsuperscript{DN} expression using the twi-gal4 driver did not induce attachment defects (data available upon request).

Most ptc::glide\textsuperscript{DN} embryos died just prior to eclosion, later than glide-glide2 embryos, few escapers reaching the second instar larval stage. Such escapers displayed major locomotion defects, such as uncoordinated movements and abnormal muscle contractions (see Movie 1 at http://dev.biologists.org/cgi/content/full/131/18/4521/DC1). Moreover, ptc::glide\textsuperscript{DN} animals were sluggish (Fig. 3A and Movie 1) and tended to make turns instead of following the same direction, the stereotyped behaviour shown by wild-type animals (data not shown). Larval muscle organisation was severely altered, indicating that muscle attachment is impaired (Fig. 3C,E) and that the glide pathway is required in tendon cells during larval life as well. Finally, when analysed at the end of stage 17 (17-19 hours AEL), ptc::glide\textsuperscript{DN} embryos displayed severe muscle attachment defects in longitudinal and ventral longitudinal muscles (Fig. 3I-K) that probably account for the late embryonic lethality. By contrast, ptc::glide\textsuperscript{N7-4DN} animals displayed no locomotion defects (see Movie 1 and Fig. 3A) and showed no embryonic and larval muscle defects (Fig. 3B,D,F-H).

Altogether, these results clearly demonstrate a direct role of glide-glide2 during terminal tendon cell differentiation.

**Attachment sites are defective in glide-glide2 embryos**

glide-glide2 attachment sites were analysed by electron microscopy. Hemiadherens junctions (HAJs), which are normally found at contact sites between muscles and tendon cells (Fig. 4A), contain cell-specific integrins that link the extracellular matrix components to the cytoskeleton of each cell (Prokop et al., 1998; Tepass and Hartenstein, 1994). Adhesion of HAJs to the extracellular matrix formed an electron-dense material (arrowhead in Fig. 4B) that was absent in glide-glide2 embryos (Fig. 4C), indicating that the junction was defective. Moreover, the tight interdigitation observed between wild-type muscle and tendon cell was not present in mutant animals, strongly suggesting adhesion defects (Fig. 4C). Finally, BPS integrin accumulation at the position of attachment sites was decreased in glide-glide2 embryos (arrowheads in Fig. 4D,G). The HAJ phenotypes were probably due to the loss of both tendon cell and muscular BPS, since HAJs and muscles are

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**Fig. 2.** The glide complex affects muscle attachment. Anti-Myosin labelling reveals muscle organisation in early stage 17 embryos of the following genotypes: (A,C) wild type (WT); (B,D) glide-glide2 embryos (glide-glide2 lof); (E) glide embryos carrying one copy of glide2 (glide 1of-glide2 w/1of); (F) glide-glide2 embryos specifically expressing glide in tendon cells (stripe-gal4 driver) (called Rescue); (G) ptc::glide\textsuperscript{DN} (glide\textsuperscript{DN}); and (H) ptc::glide\textsuperscript{N7-4DN} (glide\textsuperscript{N7-4DN}) embryos. (C,D) The regions delimited by dots in A,B, respectively. All panels show ventral views. Dashed line in C-H shows the ventral midline. In wild-type embryos (A,C), ventral longitudinal fibres attach to tendon cells at a distance from the midline. In glide-glide2 embryos (B,D), ventral longitudinal fibres bypass their target sites, attaching to others muscles at the midline (arrows in D). Note that muscle fibres normally attached to tendon cells that do not express glide are also indirectly affected. (E) Embryo carrying one copy of glide2 displays less severe phenotype than the double mutant. (F) The muscle phenotype is fully rescued by specifically expressing glide in tendon cells (n=10). (G) Inhibition of glide complex activity (ptc::glide\textsuperscript{DN}) induces the midline crossing phenotype (see arrow), whereas the inactive form of the dominant negative construct (ptc::glide\textsuperscript{N7-4DN} in H) does not. Scale bars: 50 μm.
normal in animals lacking only the tendon cell-specific integrin PS1 or its ligand Laminin (Gotwals et al., 1994; Prokop et al., 1998). In conclusion, muscle attachment sites were defective in glide-glide2 embryos.

Epistatic interactions between the glide complex and the stripe-induced pathway

stripe is the earliest known gene to be expressed in presumptive tendon cells (Frommer et al., 1996; Lee et al., 1995). It encodes...
two isoforms (a and b) that differ by their transcription start site but have the same DNA-binding domain (Frommer et al., 1996; Lee et al., 1995). While the role of stripe a is unknown, stripe b ectopic expression induces the expression of tendon cell markers such as shortstop (also called kakapo) (Gregory and Brown, 1998; Strumpf and Volk, 1998; Subramanian et al., 2003), alien (Goubeaud et al., 1996), delilah (Armand et al., 1994), β-tubulin (Buttgereit, 1993; Buttgereit et al., 1991) and stripe a (Becker et al., 1997; Vorbruggen and Jackle, 1997). In stripe embryos, tendon cell differentiation does not occur and most markers are less, or not at all, expressed (Frommer et al., 1996).

We asked whether stripe exerts its function through glide and found no effect on glide transcription in stripe b gain- and loss-of-function embryos (Fig. 5E,F and Fig. 5G,H, respectively). Thus, glide represents the first identified gene that is expressed independently of stripe. To further clarify the role of the glide complex, we analysed the expression of stripe a and b in embryos either lacking or ectopically expressing glide or glide2. Loss of glide-glide2 had no effect on stripe a or b expression (data not shown). By contrast, ectopic glide expression (en:glide) specifically induced stripe b expression (Fig. 5A-D and Fig. 6B). Interestingly, while stripe b ectopic expression (en:stripe b) induced tendon cell marker expression, ectopic glide (en:glide) induced only stripe b and alien (compare Fig. 6D with Fig. 6E). This may depend on the delayed stripe b ectopic expression in glide gain-of-function experiments. These results suggest that glide and stripe act on common targets such as alien.

glide2 did not induce stripe expression (compare Fig. 6A with Fig. 6C), consistent with the observation that glide2 is normally expressed later than glide and stripe. By contrast, alien was ectopically expressed (Fig. 6F), indicating that the glide complex can activate a tendon cell gene independently of stripe.

All stripe-induced tendon cell markers (shortstop, delilah, β-tubulin, how) were still expressed in glide-glide2 embryos (data not shown), implying that the glide complex controls the expression of yet unknown targets. Functional microarray experiments based on glide overexpression in the nervous system, as well as computational analyses based on the presence of Glide binding sites (GBSs), have identified potential glide targets (Egger et al., 2002; Freeman et al., 2003). We analysed several of them for tendon cell expression and identified Ent2 (Equilibrative nucleoside transporter 2) (Fig. 6G), which is also expressed in glial cells and contains eight GBSs (Freeman et al., 2003). glide was necessary (data not shown) and sufficient (Fig. 6H) to induce Ent2 expression. Also glide2, but not stripe b, ectopic expression induced Ent2 expression (data not shown and Fig. 6I, respectively). Thus Ent2 represents a target specific to the glide complex. To summarise, the glide complex did not depend on stripe and controlled a new molecular pathway independently of stripe.

Regulation of glide expression in tendon cells
The glide complex set-up of expression precedes muscle interaction with competent tendon cells and is probably muscle-independent, as confirmed by the observation that glide was still expressed in mesoderm-lacking embryos (data not shown). Segment polarity genes such as hedgehog (hh) and wingless (wg) are involved in the determination of segment border cells (Piepenburg et al., 2000), some of which later become tendon cells (Volk and VijayRaghavan, 1994). We therefore tested whether patterning genes control glide expression. patched (ptc) encodes a transmembrane receptor involved in the repression of Hh signal transduction (Alcedo and Noll, 1997). In ptc embryos, the Hh signalling pathway is constitutively activated (Forbes et al., 1993; Ingham and Hidalgo, 1993), leading to ectopic cells with segment border identity (compare Fig. 7A with Fig. 7F) (Nusslein-Volhard and Wieschaus, 1980; Piepenburg et al., 2000) that expressed glide in tendon cells (Piepenburg et al., 2000). While the role of the glide complex, we analysed the expression of glide in tendon cells (Piepenburg et al., 2000). While the role of the glide complex, we analysed the expression of glide in tendon cells (Piepenburg et al., 2000). While the role of the glide complex, we analysed the expression of glide in tendon cells (Piepenburg et al., 2000).
Glide mode of action in the epidermis

Glide and glide2 induce glial differentiation within and outside the nervous system upon ectopic expression (Akiyama-Oda et al., 1998; Alfonso and Jones, 2002; Bernardoni et al., 1998; Hosoya et al., 1995; Jones et al., 1995; Kammerer and Giangrande, 2001; Miller et al., 1999; Vincent et al., 1996). Given our finding that glide was also sufficient to induce stripe b, we asked whether ectopic glide induces tendon cell and glial

![Image](image_url)

**Fig. 6.** Epistatic relationships between the glide complex and stripe reveal a novel molecular tendon cell-specific pathway. (A-F) Anti-Stripe or anti-Alien immunolabelling on wild-type (A,D, respectively) and on either en::glide (B,E, respectively) or en::glide2 (C,F, respectively) stage-15 embryos. Dorsal views, dorsal midline is represented by a dotted line. (B) stripe ectopic expression is indicated by arrowhead. Note that ectopic glide2 does not induce stripe expression (C). (D-F) Alien ectopic expression induced by either glide (E) or glide2 (F) is indicated by arrowheads. (G-I) Ent2 in-situ hybridisation on wild-type (G), en::glide (H, glide gof) and en::stripe b (I, stripe b gof) stage-14 embryos. Lateral views, dorsal to the top. Ent2 is expressed in segment border cells (bracket in G). Note that glide, but not stripe b, ectopic expression induces Ent2 expression (compare brackets in H,G and I,G, respectively). Scale bars: 50 μm.

![Image](image_url)

**Fig. 7.** Glide regulation in the embryonic epidermis. (A,F,K) Schematic representation of epidermal cells in a segment of wild-type (A), patched (F) and wingless (K) stage-13 embryos. Black vertical lines indicate segment borders. The single row of border cells (red cell) is posterior to each segment border (A). Ectopic border cells are present in (F) and are absent in (K). (B,C,G,H,L,M) glide in-situ hybridisation on wild-type (B,C), patched (G,H) and wingless (L,M) embryos. (D,E,I,J,N,O) Stripe expression revealed by immunolabelling on wild-type (D,E), patched (I,J) and wingless (N,O) embryos. (C,E,H,J,M,O) Regions delimited by dots in B,D,G,I,L,N, respectively. Note that in patched embryos, glide and stripe are ectopically expressed (compare G,H with B,C and I,J with D,E, respectively) whereas, in wingless embryos, neither glide nor stripe are expressed in the epidermis (compare L,M with B,C and N,O with D,E, respectively). Red bracket in C and white bracket in E show wild-type expression of glide mRNA and Stripe protein, respectively. Red and white brackets in H,J indicate ectopic expression of glide and stripe, respectively. Red and white asterisks in M,O indicate the loss of glide and stripe expression, respectively. Scale bars: 50 μm.
markers in the same or in different cells, the latter case suggesting that Glide specificity depends on the presence of cell-specific factors. We induced glide expression in the anterior region of the segment by using the ptc-gal4 driver. Co-labeling with anti-Stripe and the anti-Repo glial marker (Halter et al., 1995; Xiong et al., 1994) revealed that only Repo was ectopically present (compare Fig. 8A-C with Fig. 8D-F). By contrast, when glide was ectopically expressed in the posterior region (en::glide), the two markers were ectopically present, very few cells being positive for both of them (Fig. 8G-I). This argues in favour of positional cues dictating Glide target recognition. Interestingly, dorso/ventral patterning genes seem involved as well: most ectopic Repo labelling occurred medio/ventrally, whereas most ectopic Stripe labelling occurred dorsally (Fig. 8G-H). Moreover, glide overexpression in the nervous system, a tissue that arises from the ventralmost ectoderm, did not activate stripe expression (data not shown).

Strikingly, while glide2 did not induce stripe expression (Fig. 6C), it induced Repo labelling by using both drivers (Fig. 8J and data not shown). These results indicate that: (1) cell-specific factors dictated Glide target recognition; (2) such factors provided a coordinate grid along antero/posterior and dorso/ventral axes; and (3) while glide and glide2 had similar gain-of-function phenotypes with respect to the glial cell pathway, glide had a stronger potential than glide2 in tendon cells (Fig. 8K).

Discussion

Our study sets the basis for understanding the pathway leading to the formation of functional attachment sites. glide and glide2 represent the fly glial fate determinants and are sufficient to trigger gliogenesis. Here, we show that these two transcription factors are also expressed and required in tendon cells, where they define a new developmental pathway controlling terminal differentiation. We also show that genes patterning the embryonic segment control glide expression as well as the specificity of its target recognition. Thus, positional cues dictate whether a transcription factor acts as a cell fate determinant in the neurogenic region or as a terminal differentiation gene that allows muscle contraction to generate locomotion.

Terminal tendon cell differentiation requires the glide complex to establish functional muscle attachment sites

drive and glide2 are required in segment border tendon cells.

By contrast to the nervous system, where these genes are transiently expressed and play a role in fate choice, their transcripts are present in tendon cells until the end of embryogenesis. Moreover, the glide complex is not controlled by the stripe tendon cell fate determinant and is not necessary for the expression of stripe targets. Finally, the glide complex does not trigger tendon cell differentiation. Thus, drive and glide2 do not play a role in fate choice in the epidermis.

Several observations indicate that the glide complex controls terminal tendon cell differentiation and thereby affects attachment site integrity. First, glide/glide2 embryos do express genes (delilah and βtubulin) that are activated by the establishment of muscle-tendon cell contact (Yamitzky et al., 1997) and only show muscle defects after stage 16, once
these contacts have been established. Second, glide-glide2 tendon cells and muscles are unable to form a functional attachment site, as shown by muscle midline crossing and defective HAJs. Third, tendon cell-specific inactivation of glide complex activity results in massive muscle disorganisation and defective locomotion. Interestingly, locomotion defects have also been observed in larvae mutant for flapwing, a gene encoding a phosphatase 1β, known for its role in muscle attachment maintenance (Raghavan et al., 2000). It will be interesting to determine whether glide complex targets represent Flapwing substrates, even though it is clear that Flapwing also affects a glide-independent pathway since it is expressed and required also in tendon cells and muscles (Raghavan et al., 2000).

Although Stripe represents the tendon cell fate determinant (Frommer et al., 1996; Lee et al., 1995), it is expressed until the end of embryogenesis, suggesting an additional, late, role. Indeed, while early inactivation of the stripe pathway (stage 11) by the dominant negative approach and null mutations induce the same phenotype of detached muscles, late inactivation (stage 15) induces a muscle midline crossing phenotype similar to the one we observed in glide-glide2 embryos (Vorbruggen and Jackle, 1997). Thus, the glide complex and stripe are necessary for terminal tendon cell differentiation and probably act on common targets such as alien. Interestingly, the glide complex also controls its own pathway, independently of stripe (Fig. 9). Understanding the relative contribution of the two pathways will require the identification of the stripe and glide targets. Finally, the observation that tendon cell-specific mutations alter muscle organisation highlights the importance of cell–cell interactions throughout muscle and tendon cell development. Identifying

Regulation and mode of action of the glide complex in the epidermis

glide expression is controlled along the antero-posterior axis via wingless (wg) and hedgehog (hh) signalling pathways. Hh- and Wg-secreted proteins are expressed anterior to segment border cells (DiNardo et al., 1994; Sanson, 2001); therefore, a non-autonomous process controls glide expression in the epidermis. Hh and Wg act respectively via Cubitus interruptus (Ci) (Alexandre et al., 1996; Von Ohlen et al., 1997) and Pangolin (Pan) transcription factors (Brunner et al., 1997; van de Wetering et al., 1997), which bind to the promoter of stripe and activate its expression (Piepenburg et al., 2000). Since the first 6 kb of the glide promoter that are sufficient to induce epidermal expression (data not shown) (Jones et al., 2004) contain no predicted sites for Pan and Ci, Hh and/or Wg signalling pathways probably act on glide indirectly.

While dorso/ventral (D/V) patterning contribution to stripe expression and tendon cell differentiation is not yet elucidated, a microarray study has identified glide as a target of Dorsal (Stathopoulos and Levine, 2002), the maternally provided D/V patterning factor (Belvin and Anderson, 1996). However, we found no Dorsal binding sites in the glide promoter, suggesting that this regulation is also indirect. Altogether, our results show that stripe and glide expression is mutually independent and associated with segment border cell identity. Therefore, positional cues trigger the expression of Glide and Stripe, which in turn control several aspects of tendon cell differentiation. Detailed analyses of the glide promoter will allow us to identify the transcription factors upstream from glide in tendon cells.

glide is able to induce glial- (ventral and anterior) or tendon cell- (dorsal and posterior) specific markers depending on D/V and A/P cues, indicating that the two fates (glial versus tendon cell) are mutually exclusive and that cell-specific factors depending on patterning genes dictate Glide specificity. In line with this are the data on Abrupt, a BTB-zinc finger transcription factor that is thought to act as a repressor (Hu et al., 1995). Indeed, the loco Glide target is expressed only in glial cells in wild-type embryos (Granderath et al., 1999), but is also expressed in tendon cells in abrupt embryos (Granderath et al., 2000). Abrupt seems to be expressed throughout the epidermis (Hu et al., 1995) and does not regulate glide expression (Granderath et al., 2000), indicating that it normally represses the Glide glial pathway in tendon cells. Thus, expression of tendon cell- versus glial-specific markers depends on the balance of positive and negative factors. Whether such factors interact directly with Glide and/or act on glide targets remains to be elucidated.

Vertebrate tendons

Like fly attachment sites, the vertebrate musculoskeletal system requires the coordinated formation of distinct types of tissues. The cellular and molecular bases of vertebrate tendon development are not well understood, mostly due to lack of markers and mutations that specifically affect this process. The identification of Scleraxis, a bHLH transcription factor (Cserjesi et al., 1995) expressed in tendons and their

**Fig. 9.** Model for glide pathway in tendon cell development. While Hh and Wg signaling directly control stripe expression in border cells, they act indirectly (dotted arrow) on glide. stripe b controls the expression of several genes involved in early tendon cell differentiation (left panel) as well as its own expression (Vorbruggen and Jackle, 1997); glide-glide2 have no influence on their expression (left panel). glide, glide2, and stripe b, are able to induce alien expression, indicating that the two pathways have common targets. Whether this regulation is direct or indirect remains to be determined (broken arrows). The glide complex directly activates its own pathway (Ent2). glide participates in stripe b regulation during late tendon cell differentiation. Finally, unknown glide, glide2, stripe b, alien and Ent2 targets are also indicated by a question mark.
attachment site integrity and its interaction with the fly. Neuman et al., 2001). Interestingly, one transcript is expressed both phenotype of mice double mutant for vertebrate MTJs result in muscular dystrophy, as shown by the (Benjamin and Ralphs, 1997; Brown, 2000). Alterations of (MTJs), as, in both cases, muscle attachment to tendon cells Kammerer et al., 1999; Kanemura et al., 1999) are expressed. Moreover, the dystrophin zebrafish ortholog is necessary for attachment site integrity (Bassett et al., 2003).

A single Drosophila gene (dmDLP) displays homology with both dystrophin and utrophin and is characterised by transcripts regulated by different promoters (Greener and Roberts, 2000; Neuman et al., 2001). Interestingly, one transcript is expressed in segment border cells during embryonic development. It will be interesting to analyse the contribution of this protein to fly attachment site integrity and its interaction with the glide pathway.

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References


Egger, B., Leemans, R., Loop, T., Kammermeier, L., Fan, Y., Radimerski, N.


