

A Glutamate Receptor–Interacting Protein homolog organizes muscle guidance in *Drosophila*

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During *Drosophila* embryogenesis, developing muscles extend growth-cone–like structures to navigate toward specific epidermal attachment sites. Here, we show that the homolog of Glutamate Receptor–Interacting Proteins (DGrip) acts as a key component of proper muscle guidance. Mutations in *dgrip* impair patterning of ventral longitudinal muscles (VLMs), whereas lateral transverse muscles (LTMs) that attach to intrasegmental attachment sites develop normally. Myoblast fusion, stabilization of muscle contacts, and general muscle function are not impaired in the absence of DGrip. Instead, the proper formation of cellular extensions during guidance fails in *dgrip* mutant VLMs. DGrip protein concentrates at the ends of VLMs while these muscles guide toward segment border attachment sites. Conversely, LTMs overexpressing DGrip form ectopic cellular extensions that can cause attachment of these muscles to other muscles at segment borders. Our data suggest that DGrip participates in the reception of an attractive signal that emanates from the epidermal attachment sites to direct the motility of developing muscles. This *dgrip* phenotype should be valuable to study mechanistic principles of Grip function.

[*Keywords:* *dgrip*; muscle; muscle guidance; Glutamate Receptor–Interacting Protein; *Drosophila*]

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The establishment of specialized cell–cell junctions plays a determining role in the formation of mature patterned organs in all multicellular organisms. The most prominent examples are synaptic connections, which are formed either between neurons or between neurons and other cells, for example, muscles. Cells form extensions such as growth cones, lamellipodia, or filopodia, which they use to sense specific guidance cues and to finally anchor at the relevant target cells. In *Drosophila*, developing muscles grow growth-cone–like projections to navigate toward specific epidermal attachment sites (Baylies et al. 1998; Volk 1999). *Drosophila* muscles are grouped into two categories. One muscle type, which includes the lateral transverse muscles (LTMs), is characterized by single muscle fibers attaching to a single epidermal tendon cell. The other type, indirectly attaching muscles, including the ventral longitudinal muscles (VLMs), converges with several muscle fibers on single tendon cell, recruiting extracellular matrix, to which

they adhere (Prokop et al. 1998). Recent evidence showed that these tendon cells, also called apodemes, are the source of secreted Slit protein. Slit is sensed as a positive guidance cue by Robo receptors expressed in the nascent VLMs. Furthermore, experimentally induced overexpression of Robo receptors causes LTMs to extend toward Slit expressing tendon sites (Kramer et al. 2001). In addition to the Robo/Slit-system controlling VLM guidance, the Derailed receptor tyrosine kinase controls LTM guidance (Callahan et al. 1996). Interestingly, both systems also have firmly established roles in axonal guidance processes, suggesting a common mechanistic basis for cellular motility of muscles and neurons.

Guidance processes are controlled by a diverse array of signaling proteins, with spatiotemporal activity that is subject to subtle regulation (Dickson 2002; Huber et al. 2003). How the cellular metabolism of such supramolecular signaling complexes is organized is subject of intense investigation. Proteins containing PDZ domains, a protein–protein interaction domain of ~90 amino acids (Willott et al. 1993; Jesaitis and Goodenough 1994), recruit components of a signaling network into larger molecular complexes in order to allow rapid and specific intracellular signaling (Bilder 2001; Sheng and Sala 2001). GRIP family proteins (GRIP1 and ABP/GRIP2)

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contain six or seven PDZ domains in tandem. They were first identified via an interaction of their fifth PDZ domain with the C-terminal sequence (ESVKI) of the GluR2 AMPA receptor subunit (Dong et al. 1997; Srivastava et al. 1998; Wyszynski et al. 1999) and are suggested to participate in the synaptic localization of AMPA receptors. Interfering with the interaction between GRIPs and GluR2/3 prevents AMPA receptor recruitment to the synapse in vitro (Dong et al. 1997; Osten et al. 2000; Xia et al. 2000). GRIP has also been identified as a binding partner of both ephrin receptors and ligands (Torres et al. 1998; Brückner et al. 1999; Lin et al. 1999; Contractor et al. 2002), ARF-GAP GIT1 (Ko et al. 2003), the kinesin motor protein KIF1A, and liprin- α (Ko et al. 2003; Wyszynski et al. 2002). Despite this information, the cell biological basis of GRIP function is only poorly understood. Biochemically, GRIP1 is slightly enriched in synaptic preparations but also is strongly expressed in intracellular compartments, including putative transport vesicles for glutamate receptors (Wyszynski et al. 1998, 2002; Dong et al. 1999a). GRIPs have been suggested to mediate (1) the transport of glutamate receptors directly (Dong et al. 1997; Wyszynski et al. 2002), (2) the stabilization of receptors within postsynaptic densities (Osten et al. 2000), or (3) the stabilization of intracellular stores and/or participation in sorting decisions for the destruction or recycling of internalized receptors (Shi et al. 2001; Hirbec et al. 2003). Genetic analysis in mice has shown that GRIP1 function is already required early during development, because a GRIP1 knockout was embryonic lethal at day 12 and the embryos suffered from defects in junction formation between dermis and epidermis (Bladt et al. 2002).

In the present study, we present evidence that CG14447, the single GRIP homolog in *Drosophila* and therefore named DGrip, participates in muscle development during embryogenesis. Loss of *dgrip* function caused severe defects in VLM but not LTM patterning. DGrip is required for the guidance of developing VLMs toward the apodemes. Other processes such as myoblast fusion, stabilization of muscle attachments, and muscle function per se are not affected in *dgrip* mutant embryos and larvae. Mesodermal expression of DGrip using transgenes rescued the *dgrip* mutant phenotype. Consistent with its specific function in VLM guidance, DGrip protein progressively concentrates at the ends of these muscles as they establish contact to their target position. Furthermore, when DGrip was overexpressed within embryonic mesoderm, LTMs were guided toward ectopic attachment sites at segment borders. The DGrip protein therefore appears to be used by a subset of muscles to direct their motility, likely by transporting and/or localizing signaling components of a novel pathway.

Results

Drosophila contains a single GRIP homolog that is specifically expressed in developing muscles

As previously noted (Littleton and Ganetzky 2000), the *Drosophila* genome encodes a single GRIP homolog

(CG14447). CG14447 is represented by several embryonic cDNA isolates (see Materials and Methods; Rubin et al. 2000), which all predict the same protein sequence. Comparison of this sequence with mouse GRIP1 in respect to both position and sequence of PDZ domains clearly identifies CG14447 as a GRIP family member (Fig. 1).

To examine the expression pattern of *dgrip*, an in situ hybridization against *dgrip* was performed on *Drosophila* embryos (Fig. 1). Until germ band extension, no *dgrip* expression was detected (Fig. 1A). From stage 10 onward, staining in the posterior half of the segments (Fig. 1C–F, arrowheads) is observed, indicating expression in developing somatic muscles (Baylies et al. 1998). In fact, in stage 16 embryos, a strong *dgrip* mRNA expression is observed specifically within muscles (Fig. 1G, arrowhead), whereas at this stage neither epidermis (Fig. 1G, arrow) nor central nervous system (CNS; Fig. 1F, arrow) seem to express *dgrip* mRNA.

DGrip mutants show strong patterning defects of VLMs

The *dgrip* locus maps to position 5C10 on chromosome X (Adams et al. 2000). To generate mutations, we made use of the P-element insertion P(GT1)BG01736 (Kimmerly et al. 1996) located 2 kb downstream of the DGrip stop codon. Upon remobilization of the P-element, the small deletion *dgrip*^{ex36} was recovered, in which the whole transcription unit but no other annotated gene is deleted (Fig. 2A). Alternatively, we started from P(KG)02862 (Roseman et al. 1995), which is inserted just upstream of the *dgrip* transcription start and recovered *dgrip*^{ex122}, in which the first exon, including the predicted start codon of the *dgrip* locus, is deleted. Individuals of the genotype *dgrip*^{ex36}/Y, *dgrip*^{ex122}/Y, and *dgrip*^{ex36}/*dgrip*^{ex122} were semilethal. Precise excisions of the parental P-lines instead were fully viable and did not present any of the phenotypes observed in *dgrip*^{ex36} and *dgrip*^{ex122} (data not shown). Embryos hemizygous for both *dgrip*^{ex36} and *dgrip*^{ex122} were negative for *dgrip* mRNA in the in situ hybridization (data not shown). To examine protein expression in *dgrip* mutant embryos, a polyclonal antibody against PDZ domains 6 and 7 of the protein was affinity-purified (see Materials and Methods). The DGrip encoding cDNAs predict a protein of 112 kD. Consistently, Western blot analysis of wild-type *Drosophila* embryo extracts (stage 10–17) probed with our antibody detected a single band of ~110 to 120 kD apparent size, which comigrated with recombinant DGrip expressed in insect cells. In contrast, embryo extracts derived from a *dgrip*^{ex122} homozygous strain were negative for DGrip protein on Western blot (Fig. 2B). We therefore conclude that both *dgrip*^{ex36} and *dgrip*^{ex122} represent protein null alleles of the *dgrip* locus. Consistently, both alleles resulted in identical phenotypes (see below). Moreover, phenotypes were identical in *dgrip*^{ex122}/Y irrespective of whether animals were obtained from *dgrip*^{ex122} homozygous or heterozygous mothers. Examining this together with the in situ results

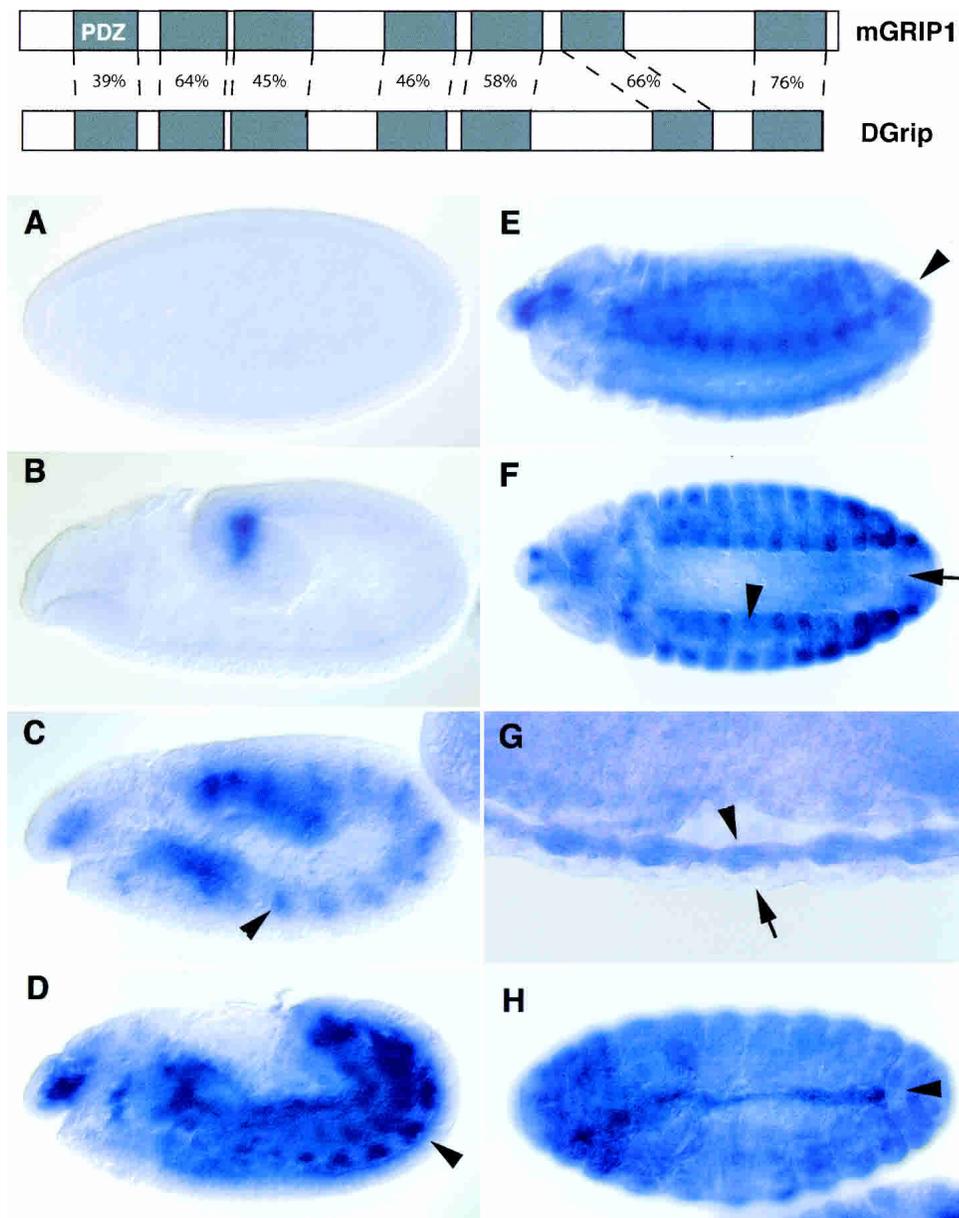


Figure 1. Embryo in situ hybridization for *dgrip*. (Top) Comparison between mouse GRIP1 and *Drosophila* protein CG14447 (DGrip), which both encode seven individually conserved PDZ domains. Sequence similarity between corresponding PDZ domains is indicated in percentages. (A–H) In situ hybridization of *dgrip*. Embryonic stages shown are as follows: 3 (A), 6 (B), 10 (C), 12 (D), 13/14 (E and F; F shows ventral view), 16 (G; dorsal view of ventral longitudinal muscles [VLMs]), and 17 (H; dorsal view). The *dgrip* mRNA accumulates in the precursors of the VLMs (C–F, arrowheads), resulting in a strong expression within VLMs after the formation of attachments (G, arrowhead). Epidermis (G, arrow) and CNS (F, arrow) do not show detectable *dgrip* expression. At stage 17 (H), cardioblasts of the dorsal vessel show strong expression of *dgrip* mRNA (arrowhead) and of DGrip protein (data not shown). *dgrip*^{ex36}/Y embryos did not express any detectable *dgrip* mRNA (data not shown).

(Fig. 1), we therefore conclude that no maternal activity of DGrip is present and that both alleles thus establish true DGrip null situations in embryos.

To examine the embryonic development of mesoderm in the absence of DGrip activity, the somatic muscle pattern of mutant embryos was visualized by myosin stainings. Control embryos showed the typical pattern of somatic muscles (Fig. 2C, WT). However, an abnormal

patterning of the VLMs was easily detected in embryos of the genotype *dgrip*^{ex36}/Y, *dgrip*^{ex122}/Y (Fig. 2C, asterisks), and *dgrip*^{ex36}/*dgrip*^{ex122} (data not shown). The defective VLMs of the mutants appeared rounded instead of stretched between the attachment sites at the segment borders. Mesodermal expression of DGrip using the *24B-gal4* (Fig. 3D) or *twist-gal4* driver (data not shown) rescued the VLM patterning defect of *dgrip*^{ex36}/Y (Fig. 3C)

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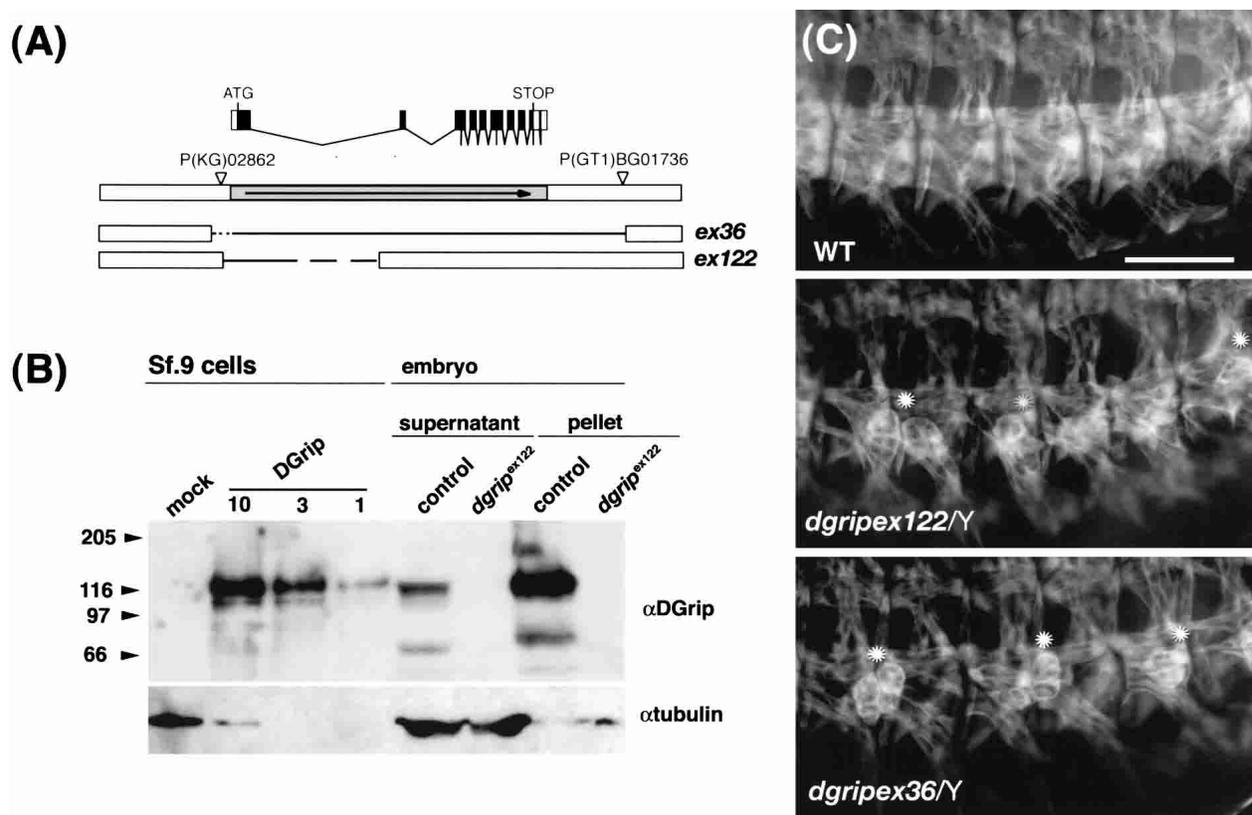


Figure 2. Specific muscle defects in *dgrip* mutants. (A) Genetic analysis of *dgrip*. By mobilization of transposon P(GT1)BG01736 2 kb downstream of the DGrip stop, deficiency *dgrip^{ex36}* eliminating the full *dgrip* ORF was recovered. P(KG)02862, located 500 bp upstream of the DGrip start codon, gave deficiency *dgrip^{ex122}*, which deletes the first exon of DGrip, including the putative start codon. Both deficiencies resulted in identical phenotypes, which were rescued by mesodermal expression of DGrip using the *24B-gal4* or *twist-gal4* driver lines. Precise excisions of the parental P-lines gave wild-type phenotypes. (B) Western blot probed with affinity purified anti-DGrip (top, see Materials and Methods) and anti-Tubulin as a loading control (bottom). Embryos (5 to 15 h) were homogenized under low-detergent conditions (“supernatant”) and the pellet was solubilized under high detergent (“pellet”); for details, see Materials and Methods. A band of ~120 kD (predicted size for DGrip 112 kD) is detected in wild-type embryo extracts, whereas no signal is present in identically produced embryonic extracts from *dgrip^{ex122}* homozygous flies, proving this band represents DGrip. Embryonic DGrip comigrated with DGrip recombinantly expressed in Sf.9 cells (mock: untransfected Sf.9 cells; 10, 3, and 1: dilution series from a *dgrip* transfected Sf.9 cell extract). DGrip was especially enriched in the pellet fraction after high-detergent extraction. (C) Muscle myosin labeling visualizing the somatic muscle pattern in several hemisegments of late stage 16 embryos. Shown are lateral images of whole-mount embryos. Muscle patterning of ventral longitudinal muscles (VLMs) is clearly defective in the two independent alleles, *dgrip^{ex122}* and *dgrip^{ex36}*. Asterisks mark groups of misattached VLMs in *dgrip* mutants. Bar, 60 μ m.

to the wild-type muscle pattern. We similarly tested the epidermal *gal4* driver lines, *engrailed-gal4* (A. Brand, University of Cambridge, Cambridge, UK) and *stripe-gal4* (G. Morata, University of Madrid, Madrid, Spain), with the latter specifically expressing in tendon cells. Driving *dgrip* expression with these both lines did not rescue muscle defects of *dgrip^{ex36}/Y* (data not shown). These results indicate that *dgrip* is required in the developing muscle but not in the epidermis for proper muscle guidance.

Muscle patterning defects in the absence of DGrip were further characterized on the cellular level by using confocal microscopy. Figure 3 shows that in *dgrip* mutants, the VLMs differed markedly from the elongated cylindrical appearance of wild-type VLMs (Fig. 3A) and consistently failed to attach at both segment borders (Fig. 3B). The mutant VLMs appeared atypically compact

and rounded. Such strongly affected VLMs position themselves randomly more at either the anterior or posterior segment end. In weaker cases, while still attached to both segment borders, the mutant VLMs appeared irregularly shaped and did not align in register at the segment borders, a defect not observed for wild-type VLMs. We observed that > 95% of all VLMs 6/7 were affected in *dgrip* mutant embryos, with 40% of the cells of this VLM type showing a full “rounding up” of the muscle cells. VLMs 12/13 were affected to 80% with ~10% fully rounded up. Defects within other muscles apart from the VLMs were less obvious in *dgrip* mutants. With a frequency of ~5%, another type of indirectly attaching muscle, muscle 8, was strongly affected (Fig. 3B, asterisk). Some defects in segment border attachment were also recognized in other muscles: muscle 4, the ventral oblique muscles 14 and 30, and the dorsal muscles 1 and

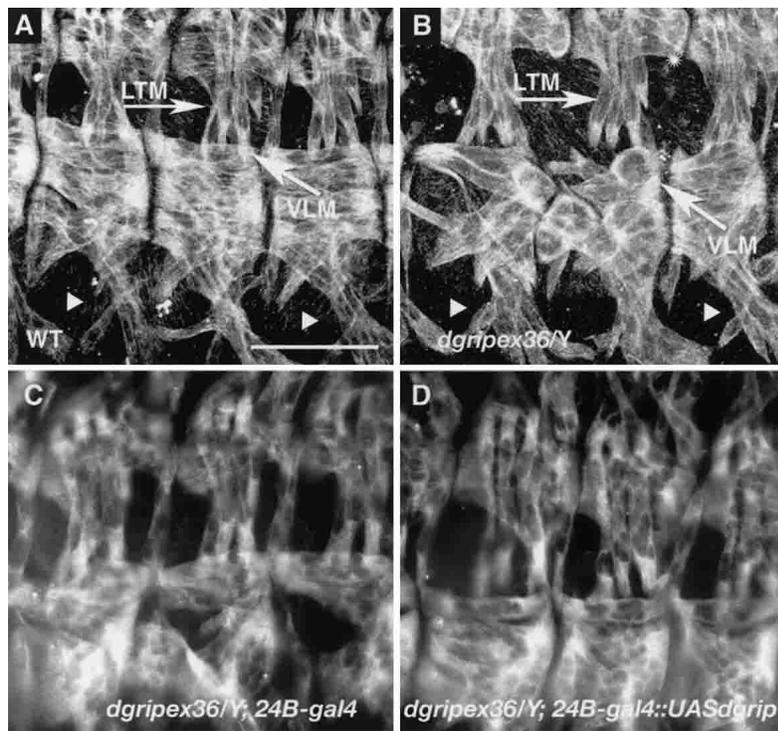


Figure 3. DGrip is essential for patterning ventral longitudinal muscles (VLMs) but not lateral transverse muscles. All images show lateral views on three hemisegments in muscle myosin stainings of late stage 16 embryos. (A, B) Confocal images projected through all muscle layers. Muscles in the VLM area (thick arrows) often round up and no longer attach to both segment borders in $dgrip^{ex36}/Y$ (B). Lateral transverse muscles (thin arrows, cf. A and B) and ventral oblique muscles (arrowheads) are not affected. Occasionally, muscle 8 is also defective (asterisk in B). Bar, 50 μ m. (C, D) Reexpression of DGrip using a weakly-expressing copy of *UAS-dgrip* in the $dgrip$ mutant background (D: $dgrip^{ex36}/Y$; $24B-gal4::UAS-dgrip$) rescues the VLM defects obvious in $dgrip$ mutants (C: $dgrip^{ex36}/Y$; $24B-gal4$).

2 showed milder defects in ~10% to 20% of the cells counted. Importantly however, we find directly attaching muscles such as the LTMs to be absolutely unaffected in $dgrip$ mutants (Fig. 3A,B, arrows). In summary, we conclude that DGrip represents an essential component needed to establish the correct patterning within VLMs and to a lesser extent in other indirectly associating muscles during *Drosophila* embryogenesis.

In the absence of DGrip, muscles differentiate properly and stably attach at ectopic positions

Defective muscle adhesion, such as that found after interfering with integrin function, often results in muscle detachment. This effect is caused in response to contractile force in the affected muscle, a condition usually fatal in late embryogenesis (MacKrell et al. 1988; Leptin et al. 1989; Brown 1994; Bökel and Brown 2002). However, $dgrip^{ex36}/Y$, $dgrip^{ex122}/Y$, and $dgrip^{ex36}/dgrip^{ex122}$ individuals develop into larvae, which maintained the defective VLM pattern observed in embryos (Fig. 4B,C). Within these animals, the embryonically affected muscles had obviously grown and elongated throughout larval development. No sign of muscle degeneration was recognizable. Affected VLMs had produced ectopic intrasegmental attachment sites (Fig. 4B,C, arrowheads) instead of the normal intersegmental attachments in wild type (Fig. 4A, arrowheads). Microscopic inspection of $dgrip$ mutant larvae showed that these attachments form at the inner layer of muscles and not to the epidermis, showing that in the absence of DGrip function, muscle–muscle junctions are formed. Such muscle–muscle junc-

tions, instead of normal tripartite muscle–muscle–epidermis junctions, have also been reported for other mutants such as *kakapo* (Prokop et al. 1998). Both in embryos and larvae, even the most strongly affected $dgrip$ mutant VLMs form multiple extensions (Fig. 4D, arrowheads), implying that DGrip-deficient muscles still seek attachments.

That $dgrip$ -deficient larvae were capable of sustained locomotion and the absence of detached muscles strongly suggest that muscle attachments were functional in this mutant. Based on phalloidin stainings (Fig. 4A–C) and electron microscopy (C. Wichmann and S.J. Sigrist, unpubl.), the defective VLMs had normal organization of the contractile apparatus as well. Moreover, escaping $dgrip^{ex36}/Y$, $dgrip^{ex122}/Y$, or $dgrip^{ex36}/dgrip^{ex122}$ adults showed a shrunken abdomen (Fig. 4J) and defective head posture, phenotypes likely due to adult muscle patterning defects. However, no other defects—in particular, no signs of a general impairment of cell adhesion—were observed in these animals.

Drosophila muscles are highly differentiated concerning their attachment site and shape (Bate 1990; Jagla et al. 2001). Fate changes among VLM founder cells (Knirr et al. 1999) could therefore be responsible for the observed muscle phenotype. To examine this possibility, we monitored the VLM pattern in $dgrip$ mutant embryos by staining with the VLM-specific differentiation marker Vestigial (Bate and Rushton 1993). Vestigial was expressed even in the most strongly affected muscles of $dgrip^{ex36}/Y$, indicating that the muscles develop according to their proper fate (data not shown). Furthermore, we made use of *5053-gal4*, which specifically drives expression in VLM 12 from stage 12 on (Ritzenthaler et al.

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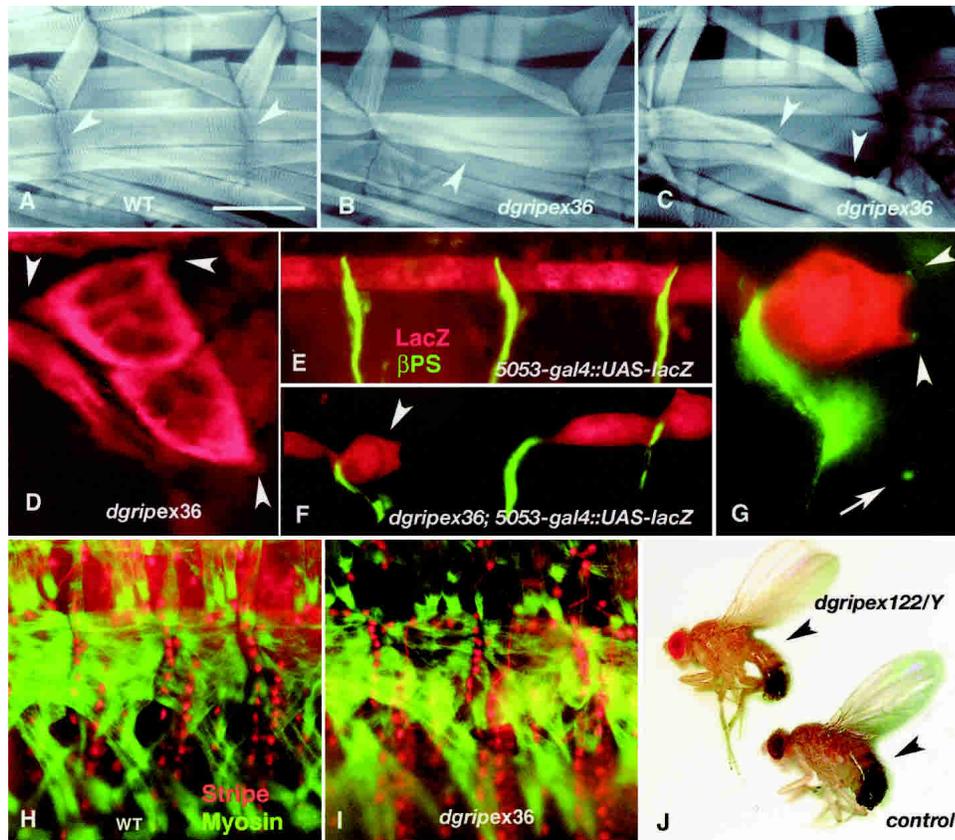


Figure 4. Muscle attachment per se is not affected in *dgrip* mutants. One hemisegment of third instar larvae in wild type (A) and *dgrip^{ex36/Y}* (B, C), fillet preparations stained with rhodamine-coupled phalloidin. In *dgrip* mutants, muscles differentiate a contractile apparatus, and no sign of detachment can be observed. Instead, ectopic muscle–muscle contacts form from misguided ventral longitudinal muscles (VLMs; B, C, arrowheads). Bar, 200 μ m. (D) Confocal image of misguided VLMs in *dgrip^{ex36/Y}*; arrowheads point to cytoplasmic extensions, indicating the formation of muscle attachments in several directions. (E–G), Stage 17 embryos stained for β -Gal to show muscle 12 and β PS-integrin in control (E) and *dgrip^{ex36}* (F, G) background. In *dgrip* mutants, one muscle 12 has not achieved segment border attachment (F, arrowhead). The ectopic muscle contacts from this misguided muscle are integrin positive (G, arrowheads; magnification of F). The lower integrin spot (arrow) in G likely represents an ectopic attachment between unlabeled muscles. (H–I) Stage 17 embryos costained with the apodeme marker Stripe (red) and with myosin (green) in *dgrip* mutants (I). Apodemes were differentiated as in wild type (H), and muscle pattern was highly impaired (I). (J) Adult *dgrip^{ex122}* male showing a shrunken abdomen (arrowheads) as typical for all *dgrip* mutant escapers. Bars, 10 μ m (D), 30 μ m (E), and 40 μ m (H).

2000). Expression of β -Galactosidase (β -Gal) driven by *5053-gal4* develops normally in *dgrip* mutant embryos (Fig. 4F,G). Collectively, these data show that VLMs still develop proper fate in the absence of *dgrip* activity. In *dgrip* mutants, muscle 12 frequently attached to ectopic positions, as shown by β PS-integrin staining (Fig. 4E–G, arrowheads in 4G), a marker of muscle attachment sites (Brown 2000). Thus, VLMs lacking DGrip activity are able to attach to ectopic intrasegmental attachment sites by contacting other muscles.

To evaluate myoblast fusion, the number of nuclei was determined in *dgrip* mutant muscles 12 of stage-17 embryos after Hoechst staining. The number of nuclei was only slightly lower in *dgrip^{ex36/Y}* than in wild type [8.7 ± 1.7 versus 10.0 ± 2.0 , respectively; $P < 0.08$]. Moreover, because correct muscle attachment is observed in the absence of myoblast fusion (Rushton et al. 1995), a defect in myoblast fusion can be excluded as the primary cause of the muscle defects observed.

Finally, we also examined possible differentiation defects within apodemes. In mutants for the transcription factor *stripe*, such defects provoke defective attachment of somatic muscles (Frommer et al. 1996; Becker et al. 1997) somewhat similar to those observed in *dgrip* mutants. However, neither *dgrip* mRNA (Fig. 1) nor protein (Fig. 6A, arrow in the inset) was detectable in the epidermis throughout the period of attachment formation. Given that DGrip is specifically expressed within the affected muscles and that the defect can be rescued by purely mesodermal expression, a direct role of DGrip in apodeme differentiation appears very unlikely. To positively exclude a role of epidermal cells with respect to the observed phenotype, we stained for the apodeme differentiation markers Delilah (data not shown) and Stripe (Fig. 4H,I) in *dgrip* mutant embryos of stage 17. Both the number and position of apodemes are unchanged in *dgrip* mutants (Fig. 4I) compared with wild type (Fig. 4H). Consistent with the formation of pure muscle–muscle junc-

tions by displaced VLMs, no sign of additional apodemes attached to VLMs was observed in *dgrip* mutants.

In summary, these results establish that muscle cell differentiation, myoblast fusion, and epidermal attachment sites are not affected by the lack of *dgrip* activity, leaving the option that the mutant muscles are misguided and fail to properly reach their target sites.

Defective guidance behavior of *dgrip* mutant muscles

To explore the possibility that DGrip may function in the guidance of developing VLMs toward their individual epidermal attachment sites, we followed the development of individual VLMs in *dgrip* mutants. For this task we labeled VLM 12 by expressing *UAS-lacZ* with the aid of the *5053-gal4* driver (Ritzenthaler et al. 2000).

The developing muscle 12 precursor was first observed at stage 12 in both control and *dgrip* mutant embryos. Throughout further development, muscles then expand by the integration of naïve myoblasts (Rushton et al. 1995). In control embryos of late stage 13, the growing muscle is still found in the posterior portion of the hemisegments, extending a single cellular extension in-

variably toward anterior (Fig. 5A–C). Already at stage 13, VLMs are apparently defective in the absence of DGrip (Fig. 5D–F). Specifically in *dgrip* mutants, we observed cellular extensions that were pointing in “wrong directions” (Fig. 5F). Moreover, muscle 12 precursor cells in *dgrip* mutant embryos often appeared bipolar, forming extensions in both anterior and posterior direction (Fig. 5D,E). In wild type, extensions extend further in stage 14 until they finally contact the epidermal attachment sites at the anterior border of the segment at late stage 14/early stage 15 (Fig. 5G,H). In *dgrip* mutants of this stage (Fig. 5I,J), cell extensions often appear collapsed, or if developed, they miss their proper target sites at the segment borders (Fig. 5J). This observation is consistent with the fact that muscles finally are often unable to form proper contact with its normal attachment site. Our results thus explain the appearance of the misattached rounded muscles that are observed in the *dgrip* mutants from stage 16 onward (cf. Fig. 5M,N, and Figs. 2C, 3B). As mentioned before, in *dgrip* mutant embryos the patterning of VLM 12 is somewhat less severely affected than that of VLMs 6/7. There was no muscle 6/7-specific driver available to explore the possibility of very likely even more penetrant guidance defects of this muscle pair

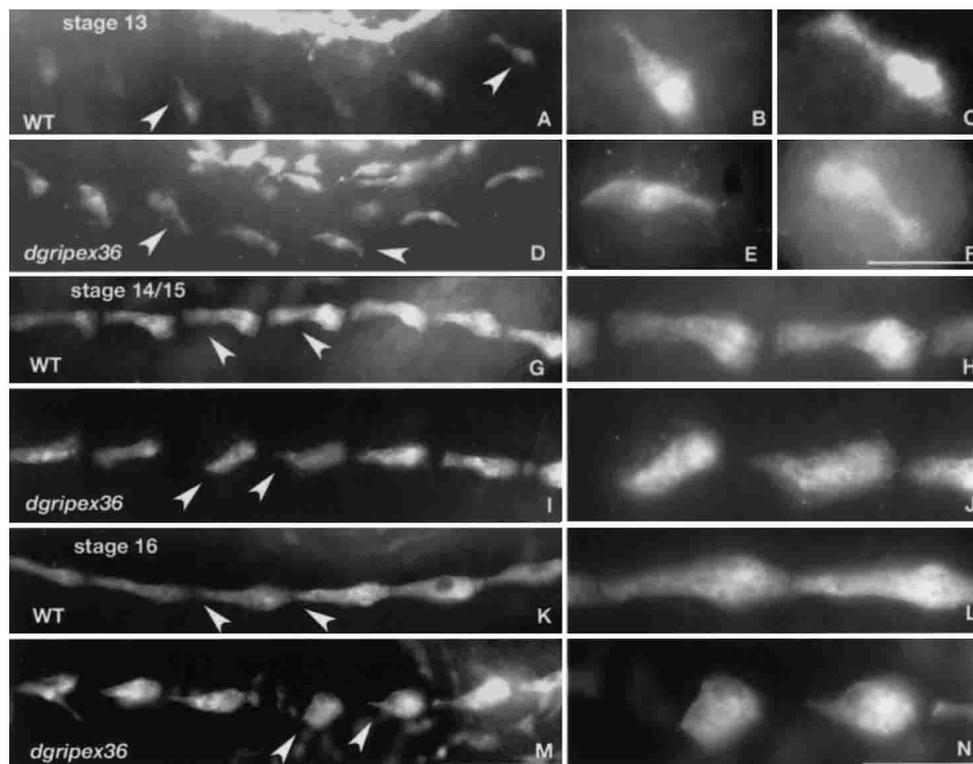


Figure 5. Defective guidance in ventral longitudinal muscles (VLMs) of *dgrip* mutant embryos. Segment border guidance of muscle 12, which is visualized in *Drosophila* embryogenesis by using *5053-gal4* to express β -Gal specifically in this somatic muscle. Out-of-focus staining derives from expression within visceral muscles. Shown are pictures from embryos in stage 13 (A–F), late stage 14/early 15 (G–J), and stage 16 (K–N); right panels show magnifications of individual muscles. In control embryos, developing muscles extend projections anteriorly starting in stage 13 (A, arrowheads, B, C) which broaden and establish first proper contact to the anterior segment border in stage 14/15 (G, arrowheads, H). In stage 16 (K, L), VLM 12 is fully attached. In *dgrip* mutant embryos, from stage 13 extensions are unusual in shape and often project in wrong directions (D–F, arrowheads). Consequently, many *dgrip* mutant muscles fail to establish proper contact (I, J; M, N). Bars, 20 μ m (F), 60 μ m (M), and 25 μ m (N).

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in the *dgrip* mutant. We conclude that the cellular behavior of *dgrip* mutant muscles becomes aberrant significantly before the developmental time point at which segment border attachment normally is established. In *dgrip* mutants, directionality of the cellular extensions normally mediating guidance seems essentially randomized. We conclude this as cells extending in anterior or posterior direction as well as “bipolar” cells are observed in similar quantities. Consistently, *dgrip* mutant VLMs are found attached to either the anterior or posterior segment border only (Figs. 2C, 3B; for an example of an anterior attachment, see Fig. 4F). We also observed that VLMs in *dgrip* mutants often stretch over segment borders (data not shown), indicating that the muscles have missed their attachment sites at the segment borders. These results are consistent with our interpretation that prior to any attachment, during stage 13, *dgrip* mutant muscles fail to respond to attractive cues by directing the outgrowth of cellular extensions (Fig. 5D). Our results thus suggest a direct role of DGrip to mediate the response to an essential attractive signal within the developing muscles. This attractive signal seems to emanate from the segment border in order to direct and/or stabilize cellular extensions of developing muscles.

DGrip accumulates in discrete compartments at muscle ends

To correlate the embryonic expression with the mutant phenotype, we stained embryos for DGrip protein expression by using our anti-DGrip antibody. In immunostainings of both *dgrip^{ex36}/Y* and *dgrip^{ex122}/Y* embryos, no signal was observed (data not shown), proving the specificity of our DGrip antibody in embryo stainings.

We first looked at stage-16 embryos, in which muscles are fully attached. Here, DGrip protein is localized to both anterior and posterior edge of the VLMs, where the muscles are in contact with their attachment sites (Fig. 6A, inset, and Fig. 6B, arrowhead; for a costaining with muscle myosin, see Fig. 6F). Weaker expression of DGrip is also detected in the contact regions of more dorsal muscles attaching to the segment border (Fig. 6A, arrows), whereas no DGrip expression could be detected at the contact sites of LTMs and other directly attaching muscles. This agrees with the mRNA distribution of *dgrip*, which also shows strong staining in the VLM region (Fig. 1E,F, arrowheads). A GFP-tagged variant of DGrip expressed using *24B-gal4* rescued the *dgrip* phenotype (data not shown) and was found to accumulate at muscle edges of VLMs as well (Fig. 6G, arrowhead). We also analyzed the temporal profile of DGrip expression in embryonic muscles. Consistent with the distribution of the *dgrip* transcript, DGrip protein is absent from early embryos and is first detected in developing mesoderm from stage 13 on (data not shown). Obviously already in early stage 14, DGrip starts accumulating at both anterior and posterior muscle end (Fig. 6C). The protein then progressively concentrates (Fig. 6C–E) to become very sharply localized there in stage 16 (Fig. 6A,B,E). To examine DGrip expression early in an identified VLM, we

again used *5053-gal4* to stain muscle 12 (muscle myosin is not yet expressed in these early stages). Even before proper attachment of muscle 12 at the segment border is established, staining at both the posterior and anterior end of muscle 12 is observed (Fig. 6H,J,L, see arrowheads in J). As expected, DGrip is sharply localized in stage 16 (Fig. 6I,K,M, see arrowheads in K,M). It should be noted that DGrip staining is certainly not restricted to the labeled VLM.

DGrip staining in embryonic muscle appears as discrete punctae, suggesting that the protein accumulates in distinct intracellular compartments (Fig. 6D,E, magnifications). To learn about its subcellular distribution, we expressed DGrip in COS-7 cells. Here, DGrip was also found expressed in discrete punctae (Fig. 6N–P), which in terms of size and distribution appeared very similar to DGrip punctae of embryonic muscles. Colocalization experiments using established markers for intracellular compartments showed a substantial overlap with markers labeling the endocytic compartment (Fig. 6N). In contrast, no overlap with markers of endoplasmic reticulum (Fig. 6O), Golgi (Fig. 6P), cell membrane, or other organelles as lysosomes or mitochondria (data not shown) was observed.

The presented data show that DGrip is expressed in those muscles, which are affected by the absence of the gene product. The site of DGrip localization is in agreement with the argument that the protein participates in the process of muscle guidance, possibly executing its function in an endosomal compartment. Consistent with DGrip having a transient function needed for embryonic muscle patterning, DGrip expression at muscle ends vanishes in postembryonic development (data not shown).

Overexpression of DGrip: ectopic cellular extensions on directly attaching muscles

Our study so far shows that DGrip is essential to mediate a motility response in the VLMs toward the anterior segment border. This is based on the finding that in the absence of the DGrip, the cellular extensions of developing VLMs no longer form properly. In contrast, the motility of LTMs is undisturbed in the absence of DGrip, consistent with the observation that these muscles do not seem to normally express DGrip. We thus asked whether an ectopic activity of DGrip would influence muscle motility. To achieve *dgrip* expression in all muscle cells, we expressed the gene in response to either *24B-gal4* or *twist-gal4*, both driving expression in all myogenic cells of *Drosophila* embryos, together with two copies of *UAS-dgrip*. DGrip overexpression in muscles was confirmed by immunofluorescence stainings using the anti-DGrip antibody. In embryos overexpressing the *dgrip* gene by using either *24B-gal4* (Fig. 7A–E) or *twist-gal4* (data not shown), muscle morphology was only slightly affected in VLMs (Fig. 7B, arrowhead) and other indirectly attaching muscles. Similarly, direct overexpression of DGrip in VLM 12 using *5053-gal4* was without phenotypic consequence (data not

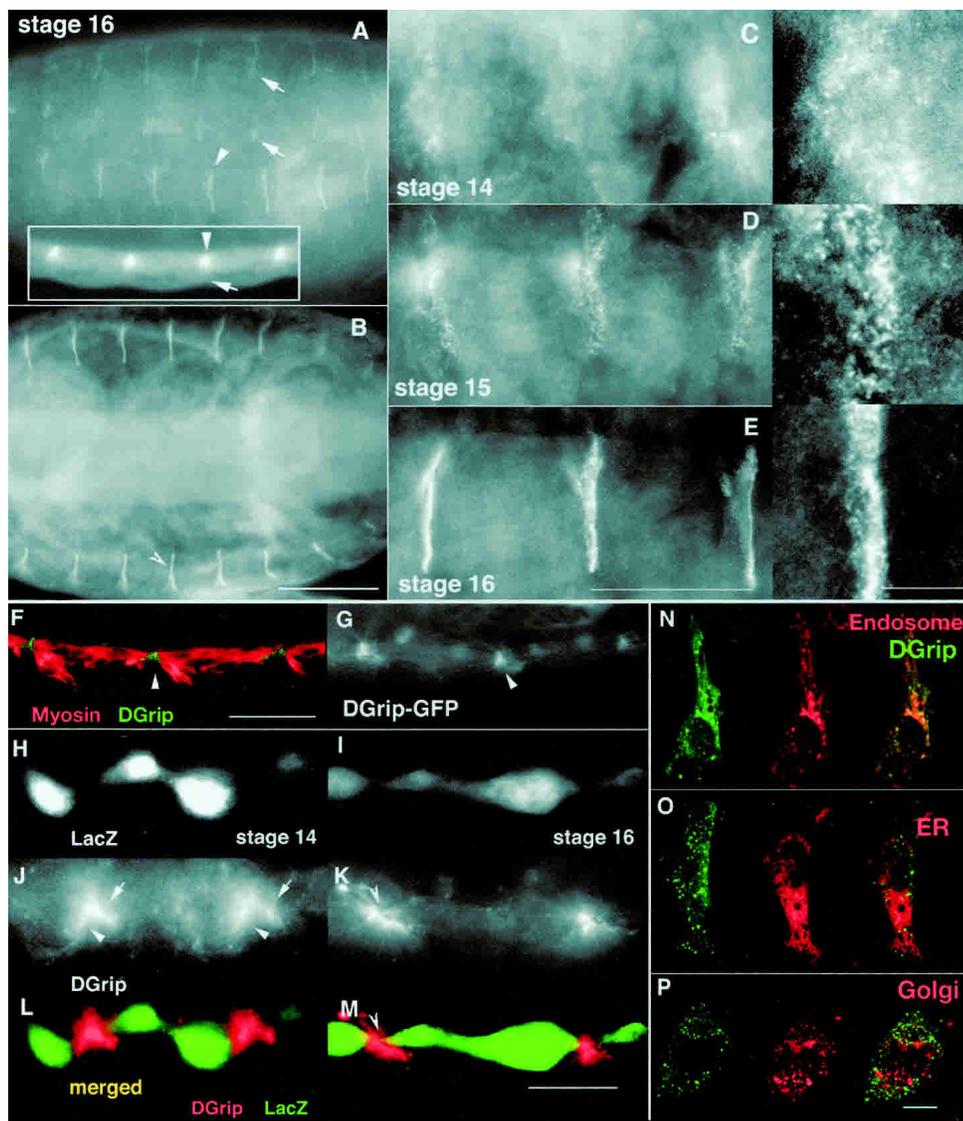


Figure 6. Expression of DGrip throughout muscle development. (A–M) Immunostainings of *Drosophila* embryos using an affinity-purified antibody against PDZ domains 6 and 7 of DGrip (see Materials and Methods). (A, B) Stage 16 wild-type embryos in lateral (A) and ventral (B) perspective. DGrip is strongly expressed at contact sites of ventral longitudinal muscles (VLMs; A, B, arrowheads) and is weaker at segmental attachment sites of dorsal muscles attaching at the segment border (A, arrows). DGrip is not observed in muscles making contacts away from the segment border. (A, inset) Vertical perspective on VLMs shows that DGrip expresses specifically in both anterior and posterior end of VLM muscles (arrowhead) but not in the epidermis (arrow). (B) Bar, 80 μ m. (C–E) Higher magnification of DGrip expression in the VLM region of stage 14 (C), 15 (D), and 16 (E). (Insets) Further magnification. DGrip progressively accumulates at the contact sites of the muscles. (E) Bar, 30 μ m; (inset) 3 μ m. (F) Costaining of DGrip with muscle myosin in stage 16. DGrip expression is confined to segment border attachment sites (arrowhead); bar, 20 μ m. (G) GFP-tagged DGrip expressed in mesoderm using *24B-gal4* is enriched at both anterior and posterior ends of muscle (arrowhead). (H–M) Costaining between endogenous DGrip (J, K) and β -Gal (H, I) specifically expressed in muscle 12 using *5053-gal4* together with *UAS-lacZ*. In stage 14, DGrip concentrates in both anterior (H, J, L, arrowheads in J) and posterior ends (H, J, L, arrows in J) of extending muscle 12, which has not yet established contact with the anterior segment border. In stage 16 (I, K, M), muscle 12 is firmly attached and DGrip expression is strongly concentrated at the segment border. (M) Bar, 30 μ m. (N–P) COS 7 cell cotransfected with DGrip and compartment markers. DGrip localizes to intracellular punctae, which in size and distribution are similar to the DGrip punctae observed in embryonic muscles (D, inset). DGrip in COS cells overlaps with endosomal markers (N). No colocalization to ER (O) or Golgi (P) was observed.

shown). In contrast, LTMs (Fig. 7A,C–E) were very sensitive with respect to DGrip overexpression. Such LTMs adopted an irregular morphology (Fig. 7A; for controls, see Fig. 3A), bent to attach at the segment border (Fig.

7A, arrowheads), or produced thin cellular extensions, which connected them to the segment borders (Fig. 7C,D). In controls, such cellular extensions formed by LTMs or bending of whole LTMs toward segment bor-

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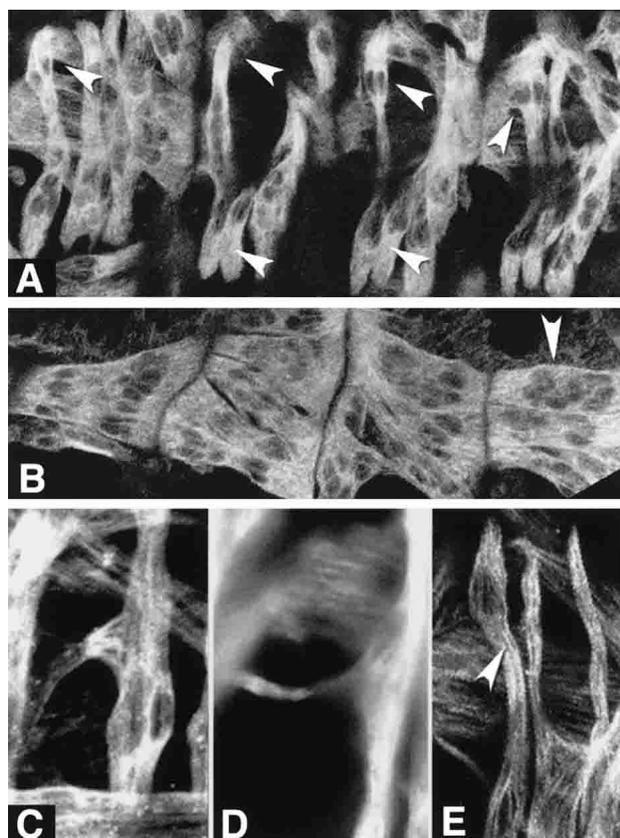


Figure 7. DGrp overexpression provokes segment border attachment of lateral transverse muscles (LTMs). All images show anti-muscle myosin stainings at stage 17 of DGrp overexpressing embryos (*24B-gal4::2xUAS-dgrlp*). (A, B) Confocal projections of hemisegments A1 to A4 from one individual embryo showing the LTM (A) or VLM (B) region. Although LTMs are normally oriented upright in the segments and appear bar-like (for a control embryo, see Fig. 3A), after DGrp overexpression LTMs often fully bend and attach at segment borders (A, arrowheads). Alternatively, these muscles form thin projections (C–E, arrowheads), which attach to the segment borders. In VLMs overexpressing DGrp, neither ectopic projections nor defective choice of attachment sites were observed (B). (A) Bar, 30 μ m.

ders were never observed, neither was formation of ectopic cellular extensions from directly attaching muscles overexpressing DGrp. Thus, ectopic expression of DGrp can efficiently provoke LTMs to attach to other muscles at the segment border. Muscles that display both direct and indirect attachment modes, such as the ventral oblique and acute muscles, display misrouted processes at the directly attaching end of the muscle, whereas the indirectly attaching ends of these muscles are essentially unaffected as for VLMs. Conversely, in the *dgrlp* mutant we specifically find the indirectly attaching ends of these muscles to be affected (data not shown). No sign of ectopic tendon cell differentiation was observed in embryos overexpressing *dgrlp* in developing muscles, indicating that misattached muscles were misguided toward preexisting segment border tendon cells (data not shown).

We conclude that DGrp is a key player in organizing different patterns of muscle attachment between different groups of muscles. The factor is both necessary and sufficient to promote the formation of muscle cell extensions, which we implicate in sensing and reacting toward a guidance signal expressed at the segment border.

Discussion

In this study we provide evidence that the only member of the GRIP family in *Drosophila* (DGrp) organizes cellular motility in order to allow proper attachment site guidance in embryogenesis. This is intriguing, as proteins of the GRIP family so far have mainly been described in the context of synapse formation and plasticity, and are considered to be involved in the clustering and/or transporting of synaptic proteins, most importantly glutamate receptors.

Drosophila Grip is both necessary and sufficient to direct developing muscles toward segment borders

The DGrp transcript and protein are strongly expressed within a specific subset of embryonic muscles of *Drosophila*, the VLMs. VLM founder cells are born in the posterior part of each segment, and they extend growth-cone-like structures in the anterior direction to target to specific attachment sites corresponding to the segment borders. At these sites, the VLMs form stable connections in a tripartite complex with apodemes and other VLMs (Fig. 5; Bate 1990; Bunch et al. 1998; Prokop et al. 1998; Martin-Bermudo and Brown 2000). In the absence of DGrp activity, cellular extensions of the VLMs are abnormal “from the beginning.” Consequently, VLMs fail to attach at the segment borders, but instead form pure muscle–muscle contacts irrespective of their position within the segment. Often the muscles fail to recognize the segment borders. All aspects of the *dgrlp* mutant phenotype were fully rescued in response to DGrp expression from a *dgrlp* cDNA-containing transgene in the developing VLMs of *dgrlp* mutant embryos. This result unambiguously establishes that the lack of *dgrlp* activity within the growing muscle is directly responsible for the defects observed.

Elimination of GRIP1 in mice results in embryonic lethality (Bladt et al. 2002) associated with defective dermoepidermal junctions. These results were interpreted to indicate that the architecture of this contact requires PDZ domain interactions mediated through GRIP1, in order to maintain proper cell adhesion. The contact between epidermis and specific muscles is not properly formed in *Drosophila* embryos mutant for *dgrlp*. This observation on first sight might hint toward a defect in the stabilization of cell adhesion in *Drosophila dgrlp* mutants as well. However, escaping adult *Drosophila* from *dgrlp* null alleles showed no signs of adhesion loss (Fig. 4J). Moreover, defects in *dgrlp* mutants are limited to one muscle group in a way that argues against cell

adhesion defects and favors a role of *dgrip* in muscle guidance, in which the pathways identified to date are found to act in a muscle subgroup-specific manner (Callahan et al. 1996; Kramer et al. 2001). Consistently, the mechanical attachment of muscles in *dgrip* mutants is unaffected, because the mutant VLMs form integrin-expressing attachment sites. Furthermore, the attachment was stable upon contraction, as the *dgrip* mutant larvae were able to locomote robustly.

Our findings exclude the possibility that the phenotypes of *dgrip* mutants are due to an effect on cell adhesion properties in the process of stabilizing the muscle attachment sites versus upcoming muscular contraction force. Instead, we provide direct evidence that the motility of VLMs is specifically affected in the absence of DGrip, by visualizing the morphological development of VLMs during guidance (Fig. 5). Wild-type VLMs form growth-cone-like extensions invariantly projecting in the anterior direction (Fig. 5A–C). However, in *dgrip* mutant muscles, the direction of cellular extensions appears randomized from the beginning, and often extensions appear collapsed (Fig. 5D–F). Furthermore, upon overexpression of DGrip, ectopic cellular extensions form specifically from LTMs, which normally do not express the protein. These aberrant extensions frequently contacted and anchored at the segment borders (Fig. 7), where obviously they became stabilized as still they are detected in late larval muscles (data not shown).

Does DGrip organize a novel signaling pathway controlling muscle motility?

Our data imply that DGrip mediates a motility response within developing muscles toward an attractive signal expressed at the segment border. It has been reported that Robo receptors are required to extend toward Slit-expressing muscle attachment sites at segment borders (Kramer et al. 2001). Loss of Robo-Slit function eliminates segment border attachment in VLMs, whereas overexpression of Robos leads to segment border attachment in LTMs. Moreover, we see that Robo receptors are expressed at the edges of developing muscles in a spatiotemporal pattern very similar to the expression profile of DGrip (data not shown). Because of these obvious parallels between Robo/Slit and DGrip, we extensively addressed a potential interaction of these factors by genetic and biochemical means. No evidence for a functional or physical interaction could be obtained. It therefore appears most likely that DGrip organizes the response to a novel signal working in parallel to the Robo/Slit-system. The finding that DGrip overexpression provokes changes in LTMs, whereas Robos are reported to be absent from these muscles (Kramer et al. 2001), also argues in this direction. In principle, DGrip could be involved in the execution of a signaling event, or alternatively, it might be important for the stabilization of first interactions pioneered, for example, by Robo/Slit signaling. Because *dgrip* mutant muscles show defective extensions early during muscle guidance and, secondly, overexpression of

DGrip directly causes the formation of cellular extensions, we favor the first alternative.

VLM-type muscles by far show the strongest defects within *dgrip* mutants, affecting ~100% of VLMs 6 and 7. However, other indirectly attaching muscles did show defects as well. Although the defects were weaker in these cells than in VLMs, they clearly were significant in comparison to control animals. Consistently, although DGrip expression seems strongest at VLM attachment sites, the contacts of more dorsal muscles, which also attach indirectly, also express the protein. A similar situation, characterized by VLMs being most affected and expressing the most DGrip between the indirectly attaching muscles, is reported for the Robo/Slit muscle guidance pathway (Kramer et al. 2001). It might be that spatiotemporal specificities in the development of the VLMs make this particular muscle group especially dependent on robust guidance signaling between the indirectly attaching muscles. *Drosophila* muscle guidance has not so far been subject to saturating genetic analysis and besides few seminal studies (Volk and VijayRaghavan 1994; Frommer et al. 1996; Becker et al. 1997; Vorbrüggen and Jäckle 1997; Kramer et al. 2001), our understanding of the process is still rather poor. In several other models of cellular motility, for example, growth cone migration, distinct pathways partially working in parallel have also been identified (for review, see Huber et al. 2003).

Mechanistic analysis of GRIP family proteins in Drosophila

Even in the complete absence of myoblast fusion, muscle founder cells still form properly attached minimuscles (Rushton et al. 1995). Hereby, the initial polarization of these specific muscle precursors seemingly does not depend on tendon cells. However, the tendon cells provide essential guidance cues that direct muscle extension (Bate 1990; Frommer et al. 1996). It is essentially unknown, how cellular polarity is organized throughout the time course of guidance and subsequent muscle attachment. Most likely the polarized transport of relevant proteins toward the “active muscle ends” is important already early within muscle guidance (Yarnitzky et al. 1997). In fact, developing muscles display a polarized microtubule network with the + ends facing the attachment sites (Clark et al. 1997).

DGrip appears concentrated at ends of muscle cells (Fig. 6H,J,L) before any proper attachment between the muscle and its prospective attachment site is established. As an intracellular adaptor molecule, DGrip might organize signaling processes, for example, by clustering transmembrane receptors, or it might act downstream of the actual signaling processes, for example, by executing transporting events that are essential for directed muscle cell motility. In fact, the correct targeting of the EGF receptor ligand Vein to the site of muscle tendon attachment has been shown to be an essential step in organizing proper muscle pattern (Strumpf and Volk 1998). Our data suggest that after supplying DGrip

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to muscles that normally do not express the protein, they start to sense distant guidance cue, which in turn causes the formation of cellular extensions. DGrip thus might switch on dormant receptors in muscles, for example, by mediating their transport to relevant cellular locations. Interestingly, DGrip has been suggested to control the transport of transmembrane receptors and signaling molecules, such as glutamate receptors and ephrins, from intracellular compartments to the cell surface (Torres et al. 1998; Wyszynski et al. 1998; Brückner et al. 1999; Dong et al. 1999b; Braithwaite et al. 2002; Hirbec et al. 2003). In *Drosophila* muscles we find DGrip localizing to discrete punctae similar to punctae formed by DGrip in culture cells. Colocalization experiments in culture cells showed that DGrip punctae often colocalized with endosomal markers, whereas no colocalizations with ER, Golgi, plasma membrane, lysosomal, or mitochondrial markers were observed. We thus favor the hypothesis that DGrip mediates signaling throughout muscle motility by regulating the endosomal trafficking of receptor complexes. Specialized proteins regulating signaling by endosomal trafficking have recently emerged key players in animal development (for review, see Piddini and Vincent 2003). Regulation of membrane protein composition by GRIPs might be subtle, as different receptor populations such as AMPA/Kainate receptors have been suggested to be regulated by GRIP in opposing manners (Hirbec et al. 2003). Palmitoylation close to the N-terminal end has been described for the Grip family members GRIP1b and pABP-L, and is suggested to control their intracellular distribution (DeSouza et al. 2002; Yamazaki et al. 2001). Indeed, the absolute N terminus of DGrip contains a conserved cysteine residue at position 13 and is similar to the N-terminal sequences demonstrated to mediate palmitoylation of GRIP1b and pABP-L. Our first experimental data in fact suggest post-translational modification of DGrip with palmitate.

The highly penetrant embryonic phenotype of DGrip presented in this study should thus be especially well suited to further study mechanisms of GRIP function in the genetically well-tractable *Drosophila* model.

Materials and methods

Genetics

For *dgrip* mutagenesis, P(KG)02862 (Roseman et al. 1995) or P(GT1)BG01736 (Kimmerly et al. 1996) were crossed to Δ 2-3-Transposase for P-element mobilization. Deficiencies *dgrip^{ex36}* and *dgrip^{ex122}* were identified and mapped by using genomic PCR from hemizygous mutant larvae (detailed information on demand). To identify *dgrip* mutant embryos, the corresponding alleles were balanced over *FM7-ftz-lacZ* (Heitzler 1997) and negatively identified in β -Gal and Sex-lethal (Bopp et al. 1991) costainings. For larvae, we balanced over *FM7-Act-GFP* and sorted male, non-GFP larvae under a fluorescence binocular (MZFLIII, Leica). For visualization of muscle 12 in *dgrip* mutants, recombinant *dgrip^{ex36}*, *UAS-lacZ* was balanced over *FM7-ftz-lacZ* and virgins crossed to *5053-gal4* males (Ritzenthaler et al. 2000). In rescue experiments, *dgrip^{ex36}* or *dgrip^{ex122}* was ei-

ther recombined with *twist-gal4* (Yin and Frasch 1998) or combined with *24B-gal4* (Brand and Perrimon 1993) and virgins crossed with homozygous *UAS-dgrip* males. A more weakly expressing *UAS-dgrip* line was chosen, and rescued animals were identified as above. For DGrip overexpression, a recombination of two strongly expressing *UAS-dgrip* lines (overexpression scored by anti-DGrip immunostainings; data not shown) was crossed to *24B-gal4* or *twist-gal4*.

Molecular biology

The following EST clones encoding *dgrip* have been isolated in the Berkeley *Drosophila* Genome Project: RE14068, RE32265, RE70628, and RE44067. All contain identical 5' and 3' sequence. RE14068 is fully sequenced (NCBI access no. AAL68270) and was used for conceptual translation of DGrip. For pUAST-*dgrip*, the DGrip ORF was amplified from EST RE14068 (BDGP, Research Genetics) by using primers 5'-ATACAAGATCTCAA GATGAAACTGTGGAAATCG-3' and 5'-AGTACTCGAGGC TCGGTAAAGAATACAGGA-3' and cloned BglIII/XhoI into the pUAST transgenesis vector (Brand and Perrimon 1993). To express C-terminally GFP-tagged DGrip, we amplified using 5'-ATACAAGATCTCAAGATGA AACTGTGGAAATCG-3' and 5'-CATCTCGAGAGAGCGCTGCATGATCATCTCG-3' omitting the DGrip stop codon, subcloned BglIII/XhoI into pEGFP-N1 (Clontech), and then cloned BglIII/NotI into pUAST. All constructs were confirmed by double-strand sequencing and transgenic flies produced by using standard procedures.

Antibody production, affinity purification, and immunodetection

For immunogen purification, a fragment encoding PDZ domains 6 and 7 of DGrip was amplified by using primers 5'-GC GCCTCGAGCATGGGTGCTCCCACAAGCACAG-3' and 5'-GACTCTAGACGGGCAAAGCATCACTCAG-3', subcloned into pDNR-1 (Clontech) XhoI/XbaI, and then cloned XhoI/NotI into pGex4T-3 (Pharmacia). The GST-fusion was expressed in BL21 cells and purified on GSH-agarose (Pharmacia), following the instructions of the manufacturer. Purified protein was injected into rabbits (BioGenes). For affinity-purification of sera, a XhoI/XbaI fragment from the pDNR-1-PDZ6-7 construct above was blunted at the 3' end with Klenow enzyme and ligated into the (His)₆-tag vector pQE-32 (Qiagen), cut SalI/HindIII where the HindIII was also treated with Klenow. Recombinant protein was expressed in XL1-blue cells and purified on Talon resin (Clontech). One milligram of the (His)₆-fusion was immobilized on a column and used for affinity-purification. Specificity of sera was tested by immunostaining and Western blotting on *dgrip* mutant embryos.

For immunoblotting, 5- to 15-h-old *Drosophila* embryos were dechorionated and homogenized in lysis buffer (50 mM HEPES, 60 mM NaCl, 3 mM MgCl₂, 1 mM CaCl₂, 0.2% Triton X-100, 0.2% Nonidet P-40, 10% glycerol) with protease inhibitor "complete mini" (Roche) added. After centrifugation, supernatant ("supernatant", Fig. 2B) was harvested, and the pellet ("pellet" Fig. 2B) was extracted with a more stringent lysis buffer (see above but 2% Triton X-100, 2% Nonidet P-40). Samples were run on an 8% PAA gel, transferred to nitrocellulose membrane (BioRad), and probed with the anti-DGrip antibody (1 : 500) followed by ECL-detection (Amersham).

Recombinant expression and immunohistochemistry in cell culture

For baculovirus expression, the DGrip ORF was amplified as for pUAST-*dgrip*, cloned into pFastBac1 (Invitrogen), and trans-

formed into DH5 α BAC cells. A positive baculovirus clone was selected with PCR and transfected into Sf.9 cells by using the BAC-to-BAC expression system (Invitrogen). For expression in mammalian cells, *dgrip* was cloned BglII/XhoI from pUAST-*dgrip* and inserted into pEGFP-N1 (Clontech) to produce *pdgrip*-EGFP. COS-7 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 1% penicillin/streptomycin on coverslips. For the colocalization studies, cells were transiently transfected by using Lipofectamine2000 (Invitrogen). The following plasmids were used for transfection in combination with *pdgrip*-EGFP: pEYFP-Actin, pEYFP-Endo, pEYFP-Mito, and pEYFP-ER (all Clontech). For antibody stainings, cells were fixed 24 h after transfection with 4% PFA for 5 min, incubated in -20°C methanol for 10 min, permeabilized by treatment with high-salt PBS (20 mM NaPi at pH 7.4; 500 mM NaCl) containing 0.3% Triton X100 followed by an incubation with 10% normal goat serum, and then incubated for 1 h with primary antibody. Antibodies used were as follows: anti-MPR300 antibody for Golgi-labeling (Hybridoma Bank, Hopkins University, Iowa) and mouse anti-lamp1 antibody (Stefan Höning, Zentrum für Biochemie und Molekulare Zellbiologie, Göttingen) for lysosome-labeling. Cy3 and Cy5 (Molecular Probes) were used as secondary antibodies. Cells were monitored under a confocal laser-scan microscope LSM510 with META modul (Zeiss). Live imaging was performed in Tyrode solution (150 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂ at pH 7.4).

Stainings

Immunocytochemistry on embryos and larvae was essentially performed as described (Lin and Goodman 1994). Antibodies were usually preadsorbed to 0 to 4 h *Drosophila* embryos. Primary antibodies were as follows: FMM5A muscle myosin monoclonal (Christoph Schuster, 1 : 100), mouse anti- β PS-integrin (Nigg Brown, 1 : 100), rat anti-Delilah and guinea pig anti-Stripe (Becker et al. 1997; 1 : 500), monoclonal anti-Sex Lethal (DSHB, 1 : 500), rabbit anti- β -Gal (Cappel, 1 : 500), and monoclonal anti- β -Gal (Promega, 1 : 500). Secondary antibodies were Cy3- and FITC- conjugated goat anti-mouse and goat anti-rabbit FAB (Dianova), used at 1 : 200.

Phalloidin-TRITC (Sigma) was used to visualize F-actin. Previously immunostained or freshly fixed larvae were incubated for 30 min in PBS/0.5% Triton-TX-100 and 5% normal goat serum, protected from the light. Larvae were then rinsed and mounted as normal. Confocal images were taken on a Leica TCS NT system with a 63-fold objective (1.3 NA), 4 \times frame averaging, and a ΔZ of 600 nm, and image stacks were projected in maximal intensity mode.

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