Development 122, 2761-2767 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV8335

derailed is required for muscle attachment site selection in *Drosophila*

Christopher A. Callahan[1,2], Joshua L. Bonkovsky[1], Audra L. Scully[1] and John B. Thomas[1,*]

[1]Molecular Neurobiology Laboratory, The Salk Institute for Biological Studies, PO Box 85800, San Diego, CA 92186, USA
[2]Department of Neurosciences, University of California at San Diego, La Jolla, CA 92093, USA

*Author for correspondence (e-mail: john_thomas@qm.salk.edu)

SUMMARY

During development, muscles must form and attach at highly stereotyped positions to allow for coordinated movements. In *Drosophila*, muscles grow towards and attach to specifically positioned cells within the epidermis. At the molecular level, very little is known about how muscles recognize these attachment sites. The *derailed* gene encodes a receptor tyrosine kinase family member that is essential for the pathfinding ability of expressing neurons. Here we show that the Drl RTK is also expressed by a small subset of developing embryonic muscles and neighboring epidermal cells during muscle attachment site selection. In *drl* mutants, these muscles often fail to attach at appropriate locations although their epidermal attachment cells appear unaffected. These results show that, similar to its role in neuronal pathway recognition, the Drl RTK participates in a mechanism required for muscle attachment site selection. The data suggest that both neurons and muscles use common mechanisms to recognize their paths or targets, and that Drl plays an analogous role in both developing systems.

Key words: *Drosophila*, muscle development, receptor tyrosine kinase, cell signaling, *derailed*

INTRODUCTION

The somatic musculature of the *Drosophila* embryo and larva consists of a highly stereotyped arrangement of muscles. Each muscle is a single large, multinucleate fiber that can be uniquely identified by virtue of its size, morphology, epidermal attachment sites and eventual motorneuronal innervation. Many of the cellular events underlying muscle patterning are understood (for review, see Bate, 1993). The first visible signs of somatic muscle development are the fusion of mesodermal cells midway through embryogenesis at specific locations within the embryo (Bate, 1990). There is good evidence that individual muscle founder cells assume specific identities at appropriate locations and become competent to fuse with surrounding mesodermal cells, to which they impart their unique identities (Rushton et al., 1995). Such fusion events give rise to identifiable, multinucleate muscle precursors whose subsequent differentiation can be followed throughout development (Bate, 1990).

A number of observations suggest that interactions between muscles and the ectoderm play key roles in the patterning of somatic muscles. After gastrulation, myoblasts migrate laterally from the midline to assume positions in close contact with the ectoderm. As myoblast fusion proceeds, muscle precursors extend growth-cone-like processes which physically contact the underlying epidermis while navigating along specific routes toward their attachment sites. Following this extension, the establishment of appropriate epidermal attachment sites occurs, giving rise to each muscle's characteristic orientation and length (Bate, 1990; Bier et al., 1990). Additional evidence for the importance of muscle-ectodermal interactions includes studies in which altering the developing ectoderm leads to disruptions in muscle patterning. For example, manipulation of the epidermis in the beetle, *Tenebrio molitor*, causes reproducible alterations in the pattern of somatic muscles (Williams and Caveney, 1980a,b). Likewise, mutations in *Drosophila* that disrupt normal epidermal development cause defects in muscle patterns (Bier et al., 1990; Volk and VijayRaghavan, 1994).

Although some of the molecular mechanisms underlying early mesodermal development are beginning to be uncovered (e.g., Corbin et al., 1991; Gonzalez-Crespo and Levine, 1993; Bladt et al., 1995), very little is known about how individual somatic muscles acquire their unique morphologies. In *Drosophila*, the *stripe* (*sr*), *Toll* (*Tl*) and *myospheroid* (*mys*) genes have been shown to be involved more generally in muscle attachment. During embryonic and pupal development, those epidermal cells destined to become sites for somatic muscle attachment express *sr* (Volk and VijayRaghavan, 1994; Lee et al., 1995a; Fernandes et al., 1996), which encodes a protein similar to members of the early growth response family of transcription factors (Lee et al., 1995a). *sr* appears to be required for proper muscle attachment, as loss-of-function mutations in the gene cause disruptions of muscle attachment and patterning for both larval (Volk and VijayRaghavan, 1994) and adult (Costello and Wyman, 1986; de la Pompa et al., 1989) somatic muscles. The cell adhesion molecule Toll is widely expressed within the epidermis and by a small subset of muscles (Nose et al., 1992). Loss of zygotic Toll expression results in a variety of somatic muscle abnormalities, including muscle loss, duplication and defective attachment (Halfon et al., 1995). Since these abnormalities are widespread and not

exclusive to the Toll-expressing muscles, proper muscle patterning may depend on Toll function within the epidermis. Finally, the integrity, but not the initial recognition and establishment, of muscle attachments requires the integrin β subunit encoded by the *mys* gene (MacKrell et al., 1988; Leptin et al., 1989).

We previously isolated the *derailed* (*drl*) gene in a screen for genes controlling axon guidance (Callahan et al., 1995). *drl* encodes a receptor tyrosine kinase (RTK) family member expressed on a subset of developing embryonic neurons. In *drl* mutant embryos, the *drl*-expressing neurons fail to extend along their appropriate pathways, suggesting that the Drl RTK is an essential component in the recognition between growing neurons and their pathways. Here we show that the Drl RTK is also expressed by a small subset of embryonic muscle fibers and neighboring epidermal cells during muscle growth and attachment events. In *drl* mutants, *drl* muscles attach at abnormal locations within the epidermis. These muscle attachment defects are not the result of gross alterations of the epidermis nor loss of epidermal attachment cell precursors. In contrast, our results suggest that analogous to its role in axon pathway selection within the nervous system, Drl participates in a mechanism required for muscle attachment site recognition.

MATERIALS AND METHODS

Drosophila stocks

All fly strains were grown on standard cornmeal medium at 18 or 25°C. The enhancer trap line *P1618* was generated by T. Volk. The *D82* GAL4 enhancer trap line was generated by D. Lin and C. S. Goodman. The generation and identification of the $drl^{P3.765}$ mutation was previously described (Callahan and Thomas, 1994; Callahan et al., 1995). Additional fly stocks and chromosomes are described in Lindsley and Zimm (1992). Null alleles of *drl* were obtained by imprecise excision of the $drl^{P3.765}$ P element using $P[ry^{+}\Delta 2\text{-}3](99B)$ as a source of transposase (Robertson et al., 1988). w^{1118}; $drl^{P3.765}$ homozygous females were mated to $CyO;\ y^{+}\ P[ry^{+}\Delta 2\text{-}3](99B)\ Sb/TM6$ males. From this cross, groups of $w^{1118};\ drl^{P3.765}/CyO;\ y^{+}\ P[ry^{+}\Delta 2\text{-}3](99B)\ Sb/+$ males were crossed to $w^{1118};\ CyO/T(2;3)ES$ females in 550 bottles. Approximately 150,000 flies were screened for lack of w^{+} in the subsequent generation and, from these, 122 independent excision events were isolated and balanced over *CyO, Actin-lacZ* (Bourgouin et al., 1992). DNA from each of the 122 P element excision lines was digested with *Xho*I and *Bgl*II, blotted, and probed with a 3.1 kb *Xho*I fragment that encompasses the P element insert (Callahan et al., 1995). Two excision lines, drl^{R300} and drl^{R343}, were found to completely remove the *drl* coding sequence and are homozygous viable. drl^{R300} and drl^{R343} were tested for complementation with $l(2)37Da^{1}$, *l(2) 37Cf* and *fs(2) TW1* (Wright et al., 1981; Wright, 1987; Gay and Contamine, 1993); both complemented the lethality or female sterility of these loci.

Constructs

Standard methods were used to construct all plasmids (Sambrook et al., 1989). pUAS-tau-lacZ was constructed by ligating a 5.4 kb *Eco*RI fragment of pBStau-lacZ with pUAST (Lin et al., 1994) digested with *Eco*RI. pUAS-drl was constructed by ligating a 3.1 kb *Eco*RI fragment of a *drl* cDNA (Callahan et al., 1995) with pUAST digested with *Eco*RI. P[UAS-tau-lacZ] and P[UAS-drl] were introduced into the fly germ line by standard P element transformation methods (Rubin and Spradling, 1982). For both constructs, multiple independent transformants were obtained. Lines of interest were then made homozygous if viable and fertile, or balanced over *FM7c*, *CyO* or *TM3*. A second chromosome P[UAS-drl] insertion, *UAS-drl19*, was used in the GAL4 transactivation studies. P[ME4-lacZ] was generated and generously supplied by J. Botas. pME4-drl was constructed by cloning a 1.4 kb *Xho*I fragment of the *apterous* gene present in P[ME4-lacZ], into the *Xho*I site upstream of the *hsp70* promoter of CaSpeR2/17 (Nose et al., 1994); subsequently, a 3.1 kb *Eco*RI fragment of a drl cDNA (Callahan et al., 1995) was blunt-ended and cloned into a blunted *Xba*I site downstream of the *hsp70* promoter. P[ME4-drl] was introduced into the germ line and balanced as above. Multiple independent transformants were obtained. A second chromosome insertion, *ME4-drl1*, was used in the studies described.

In situ hybridization

Whole-mount in situ hybridizations were performed as previously described (Tautz and Pfeile, 1989) with modifications (Jiang et al., 1991). Digoxigenin-labeled sense and antisense riboprobes of *drl* were prepared from the 3.1 kb *drl* cDNA cloned in Bluescript (Stratagene) using protocols from Boehringer Mannheim. After staining, embryos were mounted directly in 90% glycerol or dehydrated in an ethanol series, briefly rinsed in xylenes and mounted in Permount.

Immunohistochemistry

All collections for dissections were carried out at 25°C. Embryo dissections were performed as described (Thomas et al., 1984; Callahan and Thomas, 1994). For detection of β-gal and Tau-β-gal, embryos were incubated overnight at 4°C with a rabbit anti-β-gal polyclonal antibody (Cappel) diluted 1:10,000 in PBTN (phosphate-buffered saline containing 0.1% Triton X-100 with 1% bovine serum albumin and 4% normal goat serum). Embryos were washed and incubated for 2 hours at room temperature with either a FITC-conjugated goat anti-rabbit antibody (Cappel) diluted 1:200 in PBTN or a biotinylated goat anti-rabbit antibody (Vector) diluted 1:200 in PBTN. Preparations processed with biotinylated antibodies were washed again and incubated for 1 hour with either fluorescein streptavidin diluted 1:200 (Vector labs) or an avidin/biotin-HRP complex (Vectastain ABC Elite Kit, Vector labs) followed by a final wash. HRP staining was visualized by a standard DAB reaction. Each wash was performed for 30 minutes in PBT (phosphate-buffered saline containing 0.1% Triton X-100 with 1% bovine serum albumin).

For detection of Drl, rat polyclonal anti-Drl antibodies directed against Drl's extracellular domain (Callahan et al., 1995) were used at a dilution of 1:500. Incubation and washing were followed by a biotinylated goat anti-rat antibody (Vector labs) diluted 1:200 in PBTN, another wash, a 1 hour incubation in either fluorescein streptavidin diluted 1:200 (Vector labs) or an avidin/biotin-HRP complex (Vectastain ABC Elite Kit, Vector labs) followed by a final wash. Before analysis, embryos were either mounted in 100% glycerol or dehydrated in an ethanol series, cleared with methyl salicylate and mounted in Canada balsam. Somatic muscle patterns were examined by incubating dissected embryos in RITC-conjugated phalloidin diluted 1:1000 (Molecular Probes) in room temperature PBTN for 30 minutes, followed by washing and mounting in a solution of 10% polyvinyl alcohol (Air Products and Chemicals) containing 2.5% DABCO, 1,4-diazabicyclo[2.2.2]octane (Sigma).

Confocal microscopy

Fluorescent-labeled preparations were imaged using a BioRad MRC1000UV confocal microscope coupled to a Zeiss Axiovert 135M microscope. Images were collected using the T1/E2 block combination; each wavelength was collected using a separate and specific excitation filter and imaged on separate photomultiplier tubes using COMOS software (Biorad). Bright-field and fluorescent digital images were processed using Photoshop (Adobe Systems Inc.).

RESULTS

Drl muscle expression

In addition to its expression within the CNS, *drl* is expressed in both the mesoderm and epidermis during embryogenesis. Using antibodies directed against its extracellular domain (Callahan et al., 1995), Drl protein can be detected in a subset of somatic muscles as they grow and form attachments to the epidermis. Each hemisegment in abdominal segments A2 through A7 contains 30 muscles with predictable orientations and attachments to the epidermis (Fig. 1). Beginning at hour 10 of development, low levels of Drl are detected in the precursors for muscles 21-23 as they begin to elongate dorsoventrally along the epidermis (Fig. 2A). Expression increases as these muscles continue to grow toward their attachment sites, and is still present by hour 13 as assayed by *in situ* hybridization (Fig. 2B) or antibody staining (Fig. 2C). During the period of attachment site selection, Drl appears enriched near the tips of muscles 21-23, especially at the locations where they contact their ventral attachment sites (Fig. 2C). By hour 15, after attachment events are completed, *drl* transcripts and protein are no longer detectable.

Drl epidermal expression

Drl protein is first detected at approximately hour 6 as stripes 3-4 cells wide in each segment. During segmental groove formation, Drl is restricted to anterior cells of each segment near the segmental grooves. At hour 9.5, expression begins to expand posteriorly from the grooves at lateral positions, resulting in broad patches of Drl-expressing epidermal cells. These lateral Drl patches overlie the differentiating muscle precursors 21-23 (Fig. 3A). By hour 11, the lateral Drl patches become more restricted, forming two smaller clusters of Drl-expressing epidermal cells located near the dorsal and ventral attachment sites for muscles 21-23 (Fig. 3B). This pattern subsequently becomes more refined, such that by hour 12.5, each cluster has become restricted to approximately 15 cells which abut and partially overlap the epidermal attachment cell clusters for muscles 21-23, as revealed by double-labeling for Drl and *sr* expression (see below) (Fig. 3C). As in muscles 21-23, Drl ceases to be expressed in the epidermis by hour 15. Despite this pattern of *drl* epidermal expression, *drl* null mutants do not show any defects in segmental groove formation or in differentiation of the epidermis (see below).

Muscle attachment defects in *drl* mutants

To better understand the relationship between muscles 21-23 and their attachment sites within the epidermis, we examined muscle development in embryos carrying a single copy of *P1618*, a nuclear-targeted *lacZ* P element within the *sr* gene (kindly provided by T. Volk). Epidermal cells destined to become muscle attachment cells express *sr* before muscle attachment events (Volk and VijayRaghavan, 1994; Fernandes et al., 1996), and thus staining for β-gal expression in *P1618/+* embryos allows the identification of attachment cells during myogenesis.

At hour 10, *P1618/+* embryos begin to express β-gal at positions where muscles will eventually insert. This includes sites at segmental boundaries where longitudinal muscles will attach, ventral rows of cells parallel to the segmental grooves where ventral muscles will attach, and in small clusters of cells located laterally between segment boundaries where the 21-23 group will attach. In hour 11.5 embryos, muscles 21-23 have extended dorsoventrally and can be seen forming both their dorsal and ventral attachment sites within the lateral clusters of *sr-lacZ*-expressing epidermal cells (Fig. 4A). By hour 14, the *sr-lacZ* clusters have become restricted to 6-8 cells, and the ventral attachments for muscles 21-23 lie adjacent to one another within the cluster (Fig. 4B).

Normally, muscles 21-23 extend similar distances ventrally and attach adjacent to the dorsal border of muscle 12, always

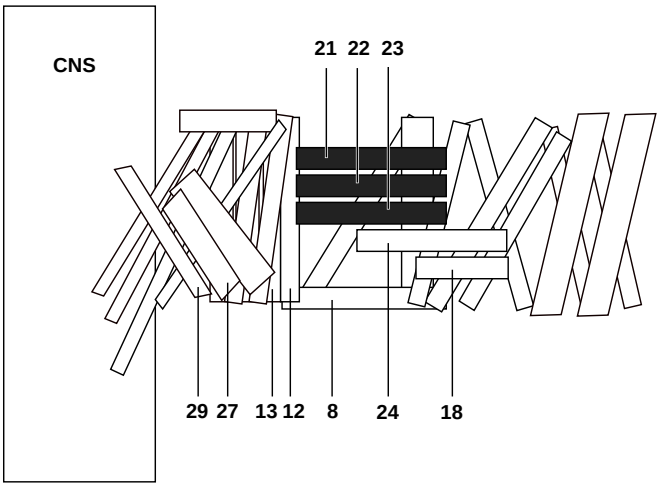

Fig. 1. Schematic diagram showing the stereotyped array of 30 muscles present in each abdominal hemisegment A2-A7. The direction of growth and positions of attachment of a developing muscle ultimately determine its length and orientation within the array. Muscles 21-23, which express *drl*, grow dorsoventrally and insert at predictable dorsal and ventral locations. The ventral inserts of these muscles are aligned adjacent to the longitudinally oriented muscle 12. Anterior is up and ventral is left.

Table 1. Muscle defects in *drl* mutants and phenotypic rescue by targeted *drl* expression

Genotype*	No. of embryos	No. of hemisegments	% (±s.e.m.) "bypass" hemisegments/ embryo†
+/+	12	138	0
drl^{R343}/+	9	100	0
drl^{R343}	22	217	20.1±3.1
drl^{R300}	9	91	19.7±6.2
drl^{R343}, *UAS-drl*^{i}	17	186	0.9±0.2‡
drl^{R343}, *D82*	14	154	19.4±3.7
drl^{R343}/drl^{R343}, *UAS-drl*	16	161	15.2±3.4
drl^{R343}, *D82*/drl^{R343}, *UAS-t-lacZ*	11	104	16.3±3.7
drl^{R343}, *D82*/drl^{R343}, *UAS-drl*	9	98	4.1±1.7‡
drl^{R343}, *ME4-drl*	32	312	0

*All genotypes were in w^{1118} background. Hr 13-15 embryos were dissected and stained with RITC-conjugated phalloidin.

†A hemisegment was classified as "bypass" if at least one muscle of the 21-23 group projected ventrally beyond muscle 13, attaching at least two longitudinal muscle widths more ventrally than normal (see Fig. 1 and text).

‡Significantly less than drl^{R343} (P<0.001 by Student's t test).

inserting within the *sr-lacZ* epidermal cluster (Figs 1, 4A,B). In *drl* mutants, muscles 21-23 are present in their normal locations and elongate dorsoventrally, but have ventral attachment site defects. We examined muscle morphology in two *drl* null alleles, drl^{R300} and drl^{R343}, both of which remove all *drl* coding sequences (Callahan et al., 1995). Both alleles show the same muscle phenotype. In 20% of hemisegments of *drl* mutants, one or more muscles of the 21-23 group pass over their normal ventral attachment sites and appear to attach far more ventrally beyond muscle 13 (Fig. 4C,D; Table 1). This dramatic phenotype we term the 'bypass' phenotype. An additional 10% of hemisegments have more subtle attachment defects where at least one muscle fails to attach within the ventral *sr-lacZ* cluster, but does not extend beyond muscle 13 (Fig. 4D).

Importantly, both the numbers and locations of the *sr-lacZ*-expressing epidermal cells in *drl* mutants are indistinguishable from drl^{+} embryos. This indicates that the muscle phenotype seen in *drl* mutants is not the result of a loss of epidermal attachment cell clusters, but instead results from the inability of the muscles and their ventral tendon cells to coordinate functional attachments.

Targeted expression of Drl to muscles rescues the attachment defects

To verify that loss of *drl* function causes the muscle attachment defects in *drl* mutants, we attempted to rescue the muscle phenotype by targeted expression of a *drl* cDNA using the GAL4 transactivation system (Fischer et al., 1988; Brand and Perrimon, 1993). One of the *UAS-drl* insertions that we generated, $UAS\text{-}drl^{i}$, due to position effect, expresses *drl* widely in the embryo independently of GAL4 (data not shown). drl^{R343} mutant embryos carrying two copies of $UAS\text{-}drl^{i}$ show nearly complete rescue of the *drl* muscle 'bypass' phenotype (Table 1), clearly demonstrating that the muscle phenotype of *drl* mutants is due to loss of *drl* function.

To address the question of where *drl* function is required, we expressed it in muscles using the GAL4 enhancer trap line *D82* (generously provided by D. Lin and C. S. Goodman). *D82* embryos express GAL4 in developing somatic muscles as assayed by the ability to transactivate *UAS-tau-lacZ* and *UAS-drl*. Embryos from a cross between *D82* and *UAS-tau-lacZ* flies (see Materials and Methods) express Tau-β-gal specifically in muscles with no detectable expression in the epidermis. Tau-β-gal expression commences at approximately hour 10, during the period when muscles 21-23 are growing and selecting attachment sites within the epidermis (Fig. 5A) and continues through the period when attachments have been established (Fig. 5B). In these *D82/UAS-tau-lacZ* embryos, we observed variability from segment to segment in the expression of the Tau-β-gal reporter. For example, within the anterior hemisegment shown in Fig. 5B, muscle 22 fails to

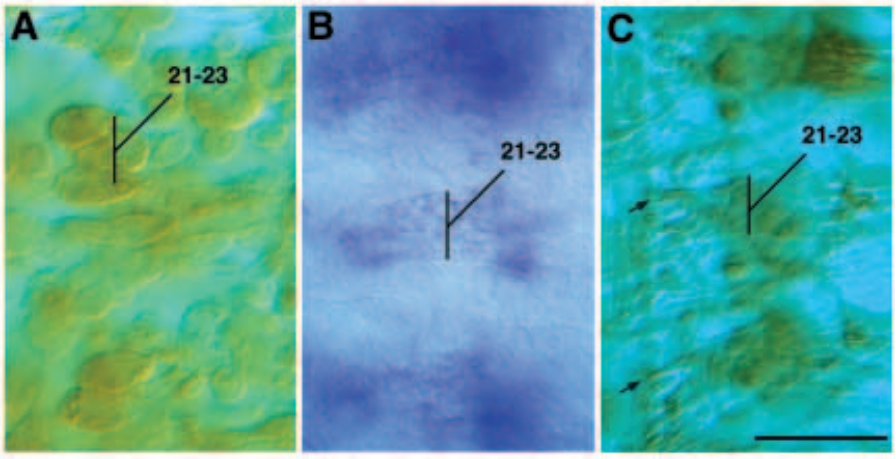

Fig. 2. Drl expression in muscles 21-23. (A) An hour 10 dissected embryo stained with an anti-Drl polyclonal antibody (Callahan et al., 1995) followed by HRP immunohistochemistry. Low levels of Drl expression are present in the precursors for muscles 21-23 as they begin growing dorsoventrally towards their attachment sites. (B) Lateral view of an hour 13 whole-mount embryo hybridized with a digoxigenin-labeled *drl* antisense riboprobe. *drl* transcripts are detected in muscles 21-23 as they form their dorsal and ventral epidermal attachments. (C) An hour 13 dissected embryo stained with Drl antibodies. Drl protein is concentrated at the ventral ends of muscles 21-23 as muscle attachment proceeds. Arrows point to the tips of muscle 21 in adjacent segments. Anterior is up and ventral is left. Scale bar is 15 μm for A, 30 μm for B,C.

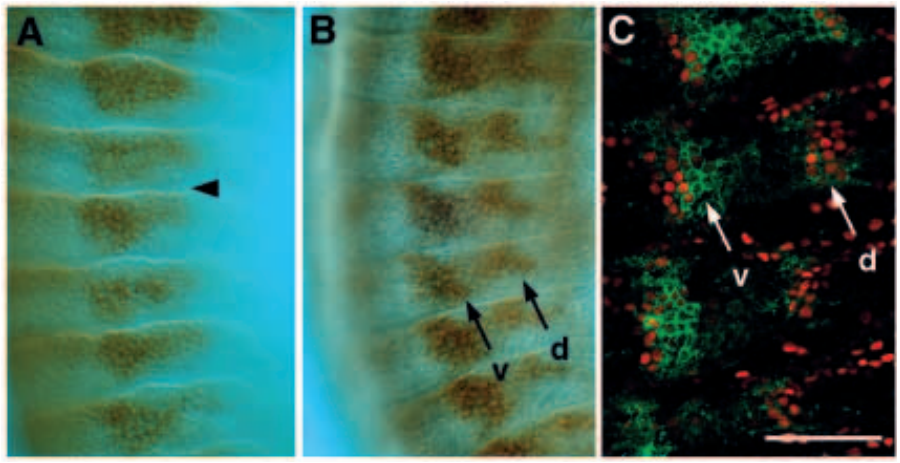

Fig. 3. Drl epidermal expression revealed with anti-Drl antibodies. (A) An hour 9.5 whole-mount embryo showing expansion of the lateral domain of Drl expression from the segmental groove (arrowhead). (B) An hour 11 embryo showing lateral epidermal expression after segregation into two smaller dorsal (d) and ventral (v) clusters which approximate the dorsal and ventral attachment sites of muscles 21-23. (C) An hour 12.5 *P1618/+* embryo double labeled with anti-Drl (green) and anti-β-gal (red). *P1618* is a *lacZ* P element within the *sr* gene, which expresses nuclear-targeted β-gal in the *sr*-expressing attachment cell clusters. Drl dorsal and ventral clusters partially overlap with *sr-lacZ* cells during muscle attachment. Anterior is up and ventral is left. Scale bar is 50 μm for A,B, 25 μm for C.

express detectable levels of the marker, while other muscles express high levels. Thus, as has been previously reported in studies using GAL4-mediated expression (Lin and Goodman, 1994), there appears to be variability in GAL4 transactivation in these embryos.

To assay the rescuing ability of *D82* GAL4-mediated expression of *drl*, we crossed drl^{R343},*D82* to drl^{R343},*UAS-drl* flies. The drl^{R343},*D82*/drl^{R343},*UAS-drl* progeny from this cross express Drl in a manner similar to that described above for Tau-β-gal (Fig. 5C). drl^{R343},*D82*/drl^{R343},*UAS-drl* embryos

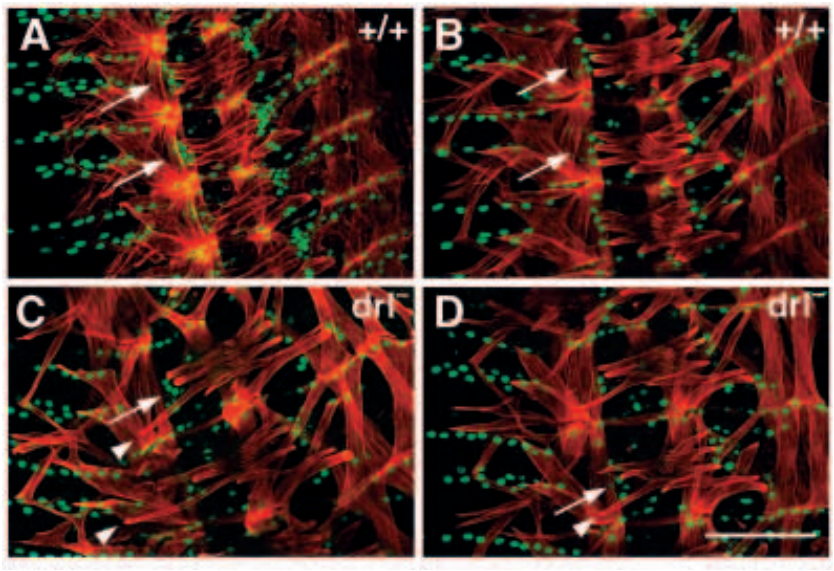

Fig. 4. Muscle attachment site defects in *drl* mutants. Dissected wild-type (A,B) and drl^{R300} (C,D) embryos carrying a single copy of *P1618* to mark the muscle attachment cell clusters. Embryos are stained with RITC-conjugated phalloidin (muscles in red) and antibodies directed against β-gal (*sr-lacZ* cell nuclei in green). (A) An hour 11.5 P1618/+ embryo showing muscles 21-23 attaching within clusters of β-gal-expressing epidermal cells. The ventral *sr-lacZ* cluster (arrows) in each hemisegment at this stage of development is composed of approximately 10 epidermal cells. (B) By hour 14, the ventral attachments for muscles 21-23 are aligned with one another within the clusters of *sr-lacZ*-expressing cells. The *sr-lacZ* clusters become reduced in size, numbering only 6-8 cells each. Arrows point to the ventral attachment cell clusters of muscles 21-23 in two adjacent segments. (C) An hour 14 drl^{R300};*P1618/+* embryo showing the muscle 'bypass' phenotype. Approximately 20% of *drl* mutant hemisegments have one or more muscles of the 21-23 group that extend beyond their appropriate ventral attachment sites, as marked by the clusters of *sr-lacZ* cells (arrow in anterior hemisegment), and insert ventral to muscle 13 (see Table 1). Arrowheads point to the ventral tips of two 'bypassing' muscles 23 in adjacent segments. (D) An hour 14 drl^{R300};*P1618/+* embryo showing an example of the more subtle phenotype seen in an additional 10% of hemisegments in which muscles project outside their attachment cell clusters (arrow), but not past muscle 13. Arrowhead points to the ventral tip of muscle 23 in the posterior of the two hemisegments shown. Anterior is up and ventral is left. Scale bar is 50 μm for A,B, 40 μm for C,D.

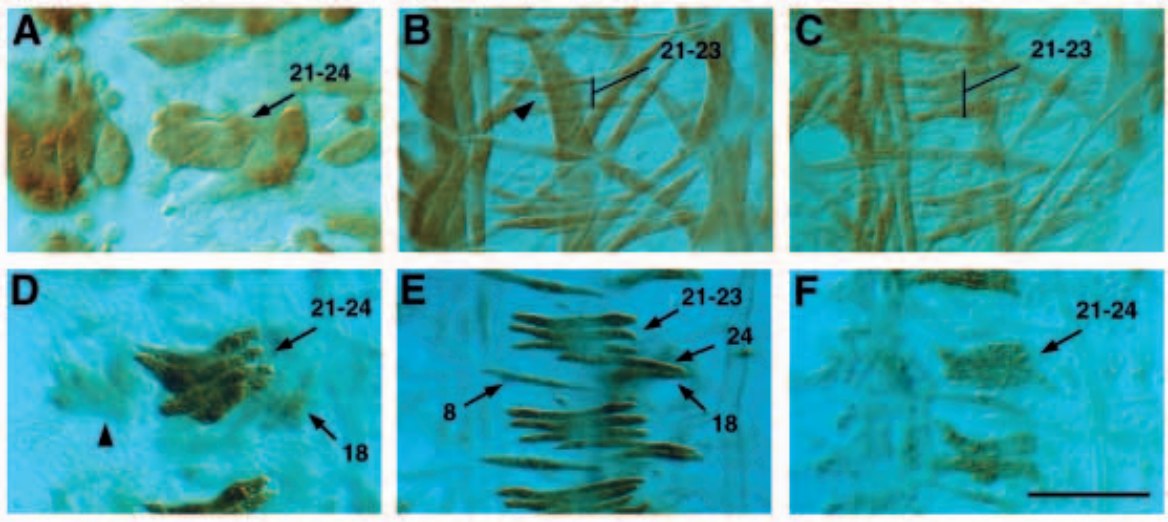

Fig. 5. Targeted expression of Drl using D82-GAL4 transactivation (A-C) and the ME4 enhancer (D-F). (A) An hour 9.5 *D82/UAS-tau-lacZ* embryo stained with an anti-β-gal antibody showing expression of Tau-β-gal in developing muscles. Muscles 21-23 are beginning to grow dorsoventrally towards their attachments sites. (B) An hour 14 *D82/UAS-tau-lacZ* embryo showing that Tau-β-gal expression continues through the period of attachment site selection. Due to the variability of the GAL4/UAS transactivation system, not all muscles consistently express the transgene. Arrowhead points to a muscle 22 that is not expressing detectable levels of Tau-β-gal. (C) An hour 14 drl^{R343},D82/drl^{R343},UAS-drl embryo stained with antibody to Drl. Drl expression is seen in muscles, similar to the expression of Tau-β-gal in *D82/UAS-tau-lacZ* embryos. In drl^{R343},*D82*/drl^{R343},*UAS-drl* embryos, the number of 'bypass' mutant hemisegments is reduced to 4% (see Table 1). (D) An hour 10 *ME4-lacZ* embryo stained with an anti-β-gal antibody showing high levels of β-gal expression in muscles 21-24 during growth towards their attachment sites. Arrowhead points to low levels of β-gal in ventral muscles 27 and 29. (E) An hour 14 *ME4-lacZ* embryo showing continued expression in muscles 21-24 and muscles 8, 18. (F) An hour 14 drl^{R343},*ME4-drl*/drl^{R343},*ME4-drl* embryo stained with an anti-Drl antibody. In these embryos, the 'bypass' mutant phenotype is completely rescued (see Table 1). Anterior is up and ventral is left. Scale bar is 25 μm for A,E, 40 μm for B,C,E,F.

show significant but partial rescue of the 'bypass' phenotype (Table 1). In contrast, drl^{R343} embryos carrying either *D82* or *UAS-drl* alone show muscle defects at frequencies similar to drl^{R343} homozygotes (Table 1). Rescue cannot be due to a cryptic interaction between GAL4 and UAS sequences because drl^{R343} embryos carrying both *D82* and *UAS-tau-lacZ* do not show any rescue of the phenotype.

To overcome the variability of GAL4 transactivation, we created a direct fusion of the *drl* cDNA to *ME4*, the muscle enhancer of the *apterous* gene (generously provided by J. Botas). When used to drive either *lacZ* or a *drl* cDNA, *ME4* directs high levels of expression in muscles 21-24 and low levels in muscles 18, 27 and 29 during the period of muscle growth and attachment site selection (Fig. 5D). Slightly later in development, *ME4* also drives expression in muscle 8 and in a small subset of tracheal cells (Fig. 5E). As with *D82*, we could not detect any *ME4*-driven expression in the epidermis. However, in contrast to *D82*-mediated expression, *ME4*-mediated expression in muscles 21-24 is highly reproducible, with high levels of expression in every segment examined. Mutant embryos homozygous for drl^{R343} and carrying two copies of *ME4-drl* show complete rescue of the *drl* muscle bypass phenotype (Table 1; Fig. 5F). Thus, Drl expression in muscles alone appears to be sufficient for rescue, strongly suggesting that during attachment site selection Drl functions in the muscles.

DISCUSSION

We have shown that the Drl RTK is expressed by a small subset of somatic muscles and neighboring epidermal cells during their attachment to the epidermis and that, in embryos homozygous for null mutations of *drl*, these muscles often fail to form ventral attachments at correct locations. Loss of *drl* function is responsible for these defects since the muscle phenotype can be rescued by expression of a *drl* cDNA.

We addressed the possibility that the ventral attachment site defects seen in *drl* mutants might be the result of a corresponding loss of the ventral epidermal tendon cells. This would not be surprising as *drl* is co-expressed in at least some *sr-lacZ*-expressing cells during attachment events and previous studies have shown that other RTK family members play roles in cell autonomous fate decisions (Hafen et al., 1987; Sprenger et al., 1989; Aroian et al., 1990). Yet, the fact that we found no differences in *sr-lacZ* expression in *drl* mutants indicates that muscle misattachment is not due to the loss of attachment cells or their precursors. Indeed, mutant muscles appear to have access to, but bypass, one or more *sr-lacZ*-expressing cells as they project ventrally towards inappropriate attachment sites.

Our results are more consistent with a role for *drl* in the muscle-epidermal interactions that underlie proper attachment site selection. One possibility is that the Drl RTK participates directly in recognition events between muscles 21-23 and their ventral attachment cells. Drl itself may mediate specific contacts between muscles 21-23 and their appropriate attachment site targets. Alternatively, Drl may be more indirectly involved in muscle-epidermal recognition events. For instance, Drl function may be required in muscles 21-23 before attachment site recognition events, serving to regulate or modify other gene products that are more directly involved in recognition processes. Such a possible modulatory role for Drl in muscle-epidermal recognition would be similar to the proposed role for Drl in neuronal pathway recognition events (Callahan et al., 1995). A further possibility is that Drl is not required for muscle-epidermal recognition events, but instead is required for the subsequent cytoskeletal changes that must accompany successful attachment. In this scenario, *drl* mutant muscles would fail to attach at their appropriate sites because they are unable to physically anchor at any location. This possibility seems less likely considering that those muscles displaying a mutant phenotype do appear eventually to attach to the epidermis, albeit at incorrect ventral locations.

The fact that muscle defects are not 100% penetrant in *drl* null mutants illustrates that there are additional genes involved in muscle 21-23 attachment site selection. At present, we do not know what relationship these additional genes have to *drl*. One possibility is that the products of these genes are somehow modified by activation of Drl, yet are not completely dependent on Drl for their function. Alternatively, the fidelity of muscle 21-23 attachment site selection may arise from the combinatorial functioning of several distinct recognition processes of which Drl controls only one. Interestingly, misexpression of Drl in muscles that normally do not express Drl does not cause mistargeting to the 21-23 attachment sites. This may be due to a lack of competence of inappropriate muscles to respond to the Drl signal, or may reflect a restricted localization of the Drl ligand. Misexpression of a constitutively active form of Drl may help to resolve this point.

Our finding that Drl is expressed both in muscles and at epidermal locations along which the muscles grow and attach suggests that there may be both an epidermal and a mesodermal component of Drl function. For example, Drl could participate in a homotypic interaction mediating some form of recognition during muscle growth over the epidermis. Our results argue against this possibility, since we were able to rescue the muscle attachment phenotype by targeted expression of Drl in muscles. The fact that we obtained only partial rescue with *D82* is likely due to variability of Drl expression in these embryos, since complete rescue could be achieved with more reproducible expression from the *ME4* enhancer. These results strongly suggest that Drl function in muscles 21-23 is sufficient for proper attachment site selection, although we cannot rule out the possibility that undetectable levels of Drl in the epidermis of *ME4-drl* and *D82/UAS-drl* embryos might be sufficient to rescue the mutant phenotype.

Regardless of *drl*'s exact role in muscle attachment site selection, these studies afford insights into the mechanisms underlying the precision of muscle organization. First, our results suggest that similar to axonal pathfinding in the developing nervous system, specific genes are required for discrete steps during target recognition by muscles. Second, these studies of Drl further demonstrate in vivo that RTK family members are essential for specific aspects of muscle differentiation. Other examples include the mouse c-met RTK, which is involved in myoblast migration into the limb (Bladt et al., 1995) and the ErbB4 and ErbB2 receptors, which are required for cardiac muscle differentiation (Gassmann et al., 1995; Lee et al., 1995b). Since RYK, the vertebrate homolog of Drl, is also expressed in muscles (Hovens et al., 1992), it will be of interest to determine its possible role in mesodermal development. Finally, the fact that in *drl* mutants, the neurons that normally express Drl fail to

recognize their appropriate target pathways, raises the intriguing possibility that both neurons and muscles use similar mechanisms to recognize their paths or targets, and that *drl* plays an analogous role in both developing systems.

We thank S. Lundgren for technical assistance; T. Volk for the enhancer trap line P1618; J. Botas for providing ME4; D. Peterson and F. Gage for assistance and advice with confocal imaging; D. Lin and C. S. Goodman for providing pUAST and the *D82* GAL4 enhancer trap line. We are also thank G. Lemke and S. Thor for stimulating discussion. This work was supported by grants from the NIH, a March of Dimes Basil O'Connor Scholar Research Award and a Pew Scholars Award from the Pew Memorial Trusts to J. B. T., and from NIH/NIGMS Training Grant PHSGM07198 to C. A. C., who is a student in the Medical Scientist Training Program at UC San Diego.

REFERENCES

Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y. and Sternberg, P. W. (1990). The let-23 gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-698.

Bate, M. (1990). The embryonic development of larval muscles in *Drosophila*. *Development* **110**, 791-804.

Bate, M. (1993). The mesoderm and its derivitives. In *The Development of Drosophila melanogaster*, (ed. M. Bate and A. Martinez-Ariass), pp. 1013-1090. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Bier, E., Jan, L. Y. and Jan, Y. N. (1990). *rhomboid*, a gene required for dorsoventral axis establishment and peripheral nervous system development in *Drosophila melanogaster*. *Genes Dev.* **4**, 190-203.

Bladt, F., Riethmacher, D., Isenmann, S., Aguzzi, A. and Birchmeier, C. (1995). Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376**, 768-771.

Bourgouin, C., Lundgren, S. E. and Thomas, J. B. (1992). *apterous* is a *Drosophila* LIM domain gene required for the development of a subset of embryonic muscles. *Neuron* **9**, 549-561.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Callahan, C. A., Muralidhar, M. G., Lundgren, S. E., Scully, A. L. and Thomas, J. B. (1995). Control of neuronal pathway selection by a *Drosophila* receptor protein-tyrosine kinase family member. *Nature* **376**, 171-174.

Callahan, C. A. and Thomas, J. B. (1994). Tau-β-galactosidase, an axon-targeted fusion protein. *Proc. Natl. Acad. Sci USA* **91**, 5972-5976.

Corbin, V., Michelson, A. M., Abmayr, S. M., Neel, V., Alcamo, E., Maniatis, T. and Young, M. W. (1991). A role for the Drosophila neurogenic genes in mesoderm differentiation. *Cell* **67**, 311-323.

Costello, W. J. and Wyman, R. J. (1986). Development of an indirect flight muscle in a muscle-specific mutant of *Drosophila melanogaster*. *Dev. Biol.* **118**, 247-258.

de la Pompa, J. L., Garcia, J. R. and Ferrus, A. (1989). Genetic analysis of muscle development in *Drosophila melanogaster*. *Dev. Biol.* **131**, 439-454.

Fernandes, J., Celniker, S. and VijayRaghavan, K. (1996). Development of the indirect flight muscle attachment sites in *Drosophila*: role of the ps integrins and the stripe gene. *Dev. Biol.* In press.

Fischer, J. A., Giniger, E., Maniatis, T. and Ptashne, M. (1988). GAL4 activates transcription in *Drosophila*. *Nature* **332**, 853-865.

Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R. and Lemke, G. (1995). Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394.

Gay, P. and Contamine, D. (1993). Study of the ref(2)P locus of Drosophila melanogaster II. Genetic studies of the 37DF region. *Mol. Gen. Genet.* **239**, 361-370.

Gonzalez-Crespo, S. and Levine, M. (1993). Interactions between dorsal and helix-loop-helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in *Drosophila*. *Genes Dev.* **7**, 1703-1713.

Hafen, E., Basler, K., Edstroem, J.-E. and Rubin, G. M. (1987). *Sevenless*, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science* **236**, 55-63.

Halfon, M. S., Hashimoto, C. and Keshishian, H. (1995). The *Drosophila* *Toll* gene functions zygotically and is necessary for proper motoneuron and muscle development. *Dev. Biol.* **169**, 151-167.

Hovens, C. M., Stacker, S. A., Andres, A.-C., Harpur, A. G., Ziemiecki, A. and Wilks, A. F. (1992). RYK, a receptor tyrosine kinase-related molecule with unusual kinase domain motifs. *Proc. Natl. Acad. Sci. USA* **89**, 11818-11822.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The *dorsal* morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. *Genes Dev.* **5**, 1881-1891.

Lee, J. C., VijayRaghaven, K., Celniker, S. E. and Tanouye, M. A. (1995a). Identification of a *Drosophila* muscle development gene with structural homology to mammalian early growth response transcription factors. *Proc. Natl. Acad. Sci. USA* **92**, 10344-10348.

Lee, K.-F., Simon, H., Chen, H., Bates, B., Hung, M.-C. and Hauser, C. (1995b). Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398.

Leptin, M., Bogaert, T., Lehmann, R. and Wilcox, M. (1989). The function of PS integrins during Drosophila embryogenesis. *Cell* **56**, 401-408.

Lin, D. M., Fetter, R. D., Kopczynski, C., Grenningloh, G. and Goodman, C. S. (1994). Genetic analysis of Fasciclin II in Drosophila: Defasciculation, refasciculation and altered fasciculation. *Neuron* **13**, 1055-1069.

Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motorneuron growth cone guidance. *Neuron* **13**, 507-523.

Lindsley, D. L. and Zimm, G. G. (1992). *The Genome of Drosophila melanogaster*. San Diego, California: Academic Press.

MacKrell, A. J., Blumberg, B., Haynes, S. R. and Fessler, J. H. (1988). The lethal myospheroid gene of *Drosophila* encodes a membrane protein homologous to vertebrate integrin beta subunits. *Proc. Natl. Acad. Sci. USA* **85**, 2633-2637.

Nose, A., Mahajan, V. B. and Goodman, C. S. (1992). Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in Drosophila. *Cell* **70**, 553-567.

Nose, A., Takeichi, M. and Goodman, C. S. (1994). Ectopic expression of Connectin reveals a repulsive function during growth cone guidance and synapse formation. *Neuron* **13**, 525-539.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnson-Schlitz, D., Benz, W. K. and Engels, W. R. (1988). A stable source of P-element transposase in *Drosophila melanogaster*. *Genetics* **118**, 461-470.

Rubin, G. M. and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. *Science* **218**, 348-353.

Rushton, E., Drysdale, R., Abmayr, S. M., Michelson, A. M. and Bate, M. (1995). Mutations in a novel gene, *myoblast city*, provide evidence in support of the founder cell hypothesis for *Drosophila* muscle development. *Development* **121**, 1979-1988.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Sprong Harbor Press.

Sprenger, F., Stevens, L. M. and Nusslein-Volhard, C. (1989). The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338**, 478-483.

Tautz, D. and Pfeile, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-85.

Thomas, J. B., Bastiani, M. J., Bate, C. M. and Goodman, C. S. (1984). From grasshopper to *Drosophila*: a common plan for neuronal development. *Nature* **310**, 203-207.

Volk, T. and VijayRaghavan, K. (1994). A central role for segment border cells in the induction of muscle patterning in the *Drosophila* embryo. *Development* **120**, 59-70.

Williams, G. J. A. and Caveney, S. (1980a). Changing muscle patterns in a segmental epidermal field. *J. Embryol. Exp. Morph.* **58**, 13-33.

Williams, G. J. A. and Caveney, S. (1980b). A gradient of morphogenetic information involved in muscle patterning. *J. Embryol. Exp. Morph.* **58**, 35-61.

Wright, T. R. F. (1987). The genetic and molecular organization of the dense cluster of functionally related, vital genes in the dopa decarboxylase region of the *Drosophila melanogaster* genome. In *Results and Problems in Cell Differentiation*, (ed. W. Hennigs), pp. 95-120. Heidelberg: Springer-Verlag.

Wright, T. R. F., Beermann, W., Marsh, J. L., Bishop, C. P., Steward, R., Black, B. C., Tomsett, A. D. and Wright, E. Y. (1981). The genetics of dopa decarboxylase in Drosophila melanogaster. IV. The genetics and cytology of the 37B10-37D1 region. *Chromosoma* **83**, 45-58.

(Accepted 14 June 1996)