Drosophila myosin phosphatase and its role in dorsal closure

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SUMMARY

Myosin phosphatase negatively regulates nonmuscle myosin II through dephosphorylation of the myosin regulatory light chain (MRLC). Its regulatory myosin-binding subunit, MBS, is responsible for regulating the catalytic subunit in response to upstream signals and for determining the substrate specificity. DMBS, the Drosophila homolog of MBS, was identified to study the roles of myosin phosphatase in morphogenesis. The embryos defective for both maternal and zygotic DMBS demonstrated a failure in dorsal closure. In the mutant embryos, the defects were mainly confined to the leading edge cells which failed to fully elongate. Ectopic accumulation of phosphorylated MRLC was detected in lateral region of the leading edge cells, suggesting that the role of DMBS is to repress the activation of nonmuscle myosin II at the subcellular location for coordinated cell shape change. Aberrant accumulation of F-actin within the leading edge cells may correspond to the morphological aberrations of such cells. Similar defects were seen in embryos overexpressing Rho-kinase, suggesting that myosin phosphatase and Rho-kinase function antagonistically. The genetic interaction of DMBS with mutations in the components of the Rho signaling cascade also indicates that DMBS functions antagonistically to the Rho signal transduction pathway. The results indicate an important role for myosin phosphatase in morphogenesis.

Key words: Rho-kinase, MBS, zipper, spaghetti-squash, Rho, RhoGEF, Morphogenesis, Drosophila melanogaster

INTRODUCTION

Morphogenesis is a complex set of processes in which a number of cellular functions such as signal transduction, cell movements, rearrangements and shape changes, are involved. It is desirable to employ model systems for the investigation of such complex processes. Dorsal closure is a morphogenetic event that takes place during the late stages of embryogenesis in Drosophila melanogaster. Halfway through embryogenesis, the dorsal surface of the embryo is covered by an extraembryonic membrane, the amnioserosa. Later on, the lateral epidermis stretches dorsally and spreads over the amnioserosa. The two edges of lateral epidermis meet at the dorsal midline and fuse to close the dorsal surface of the embryo. This process is carried out by elongation of the epidermal cells without proliferation or cell recruitment (Martinez-Arias, 1993).

Dorsal closure is a process well suited to studies on the molecular and cellular basis of morphogenesis, and a number of loci involved in this process have been identified from their “dorsal open” or “dorsal hole” phenotypes, which are characterized by large holes in their dorsal cuticle (Noselli, 1998). They can be grouped into at least four classes; genes involved in the Jun amino-terminal kinase (JNK) signaling cascade, genes encoding the components of the Decapentaplegic (Dpp)-mediated signal transduction pathway, genes involved in the Rho GTPase-mediated signaling pathway, and genes encoding cytoskeletal proteins and membrane-associated molecules for cell adhesion. Activation of the JNK signaling cascade is required in the dorsal-most cells of the lateral epidermis, the leading edge cells, to induce the expression of Dpp (Noselli, 1998). One of the target genes of Dpp signaling is zipper (zip), which encodes the heavy chain of nonmuscle myosin II, and Dpp signaling in the leading edge cells activates the transcription of zip (Ariquier et al., 2001).

At the leading edge of the lateral epidermis, filamentous actin (F-actin) and nonmuscle myosin II are prominently accumulated. The supracellular purse-string composed of the actomyosin contractile apparatus provides one of the major forces for promoting dorsal closure (Young et al., 1993). An analysis of the zip mutations demonstrated an embryonic lethality due to defects in dorsal closure, indicating that nonmuscle myosin II is required for the morphogenetic processes in dorsal closure (Young et al., 1993). Genetic interactions between zip and mutations in the components of the Rho signaling pathway suggest that nonmuscle myosin II is regulated by the Rho signals (Halsell et al., 2000).

Nonmuscle myosin II is a hexamer composed of two of each of three subunits; the heavy chain, the regulatory light chain (MRLC) and the essential light chain. The force-generating activity of actomyosin is mainly regulated by phosphorylation and dephosphorylation of MRLC. Ca2+/calmodulin-dependent myosin light chain kinase (MLCK) and Rho-kinase/Rokα, one of the effectors of the Rho GTPase, are responsible for the
phosphorylation of MRLC (Tan et al., 1992; Amano et al., 1996). However, myosin phosphatase dephosphorylates MRLC, leading to the inactivation of nonmuscle myosin II. Myosin phosphatase is a heterotrimer composed of a catalytic subunit binding to protein phosphatase 1c (PP1c), the myosin-binding subunit (MBS) and M20 (Alessi et al., 1992; Hartshorne et al., 1998). MBS plays the regulatory roles of myosin phosphatase as a target of the upstream signals and as a determinant of substrate specificity. Myosin phosphatase is negatively regulated through phosphorylation of MBS by Rho-kinase/Rokα (Kimura et al., 1996; Kawano et al., 1999). Thus, Rho-kinase/Rokα doubly activates nonmuscle myosin II through direct phosphorylation of MRLC and inactivation of myosin phosphatase by phosphorylating MBS (Kaibuchi et al., 1999).

We have previously identified the Drosophila homolog of Rho-kinase that is encoded by DRhk, and have demonstrated that DRho-kinase associates with the GTP-bound DRho1 and phosphorylates the vertebrate MRLC and MBS (Mizuno et al., 1999). Recently, the same gene has been characterized genetically as Drok, and has been demonstrated to be involved in the establishment of planar polarity in adult structures such as the compound eye and wing (Winter et al., 2001). We have identified the Drosophila homolog of MBS to elucidate the functions of myosin phosphatase in morphogenesis, revealing that MBS functions in dorsal closure and that it acts antagonistically to the Rho signaling cascade and its effector Rho-kinase.

**MATERIALS AND METHODS**

**Fly stocks and culture**

{l(3)72Dd1} (DMBSP1), l(3)72DdP01802 (DMBSP2), Df(3L)jh117, DRhoGEF204291, zip+, zipG957, 69B-GAL4, arm-GAL4 and the blue balancer (TM3, P[fr+]7=H22.7;DB2) were obtained from the Bloomington Stock Center, and EPI(3)3772 (DMBSP2) was obtained from the Szeged Stock Center. DMBSP2r31 was derived from DMBSP2 by imprecise excision of the P-insertion. DRho1G2BH and DRho1720 (Strutt et al., 1997), and zipDr (Gotwals and Fristrom, 1991) were provided by M. Mlodzik and J. Fristrom, respectively. Drok2 (Winter et al., 2001) and GFP-Sqh (Sisson et al., 2000) were provided by L. Luo and R. Karess, respectively. Fly cultures and crosses were carried out at 25°C unless otherwise stated.

**Cloning of DMBS cDNA**

PCR primers were designed on the basis of the DNA sequences of two EST clones (GH27673 and GM02173) sequenced by the Berkeley Drosophila Genome Project. The primer sequences are 5'-AGC-AATAGCCGATACAAACAGCGCTAC-3' and 5'-GGTGACGTTGCTCTTATCTTTAACTAC-3'. Two embryonic cDNA libraries (Brown and Kafatos, 1988) were used as templates. Pyrobest DNA polymerase (Takara Shuzo Co., Ltd.) was used for PCR, which was performed according to the following protocol: 30 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 5 minutes. The PCR products were cloned into pBluescript and sequenced.

**Generation of an antibody to DMBS and immunoblotting**

A rabbit polyclonal anti-DMBS-C1 antibody was raised against a synthetic polypeptide, KEKESGERTSR, corresponding to the carboxyl-terminal portion of DMBS shared by DMBS-L and DMBS-S, and was affinity purified. The polyclonal antibody and the mouse monoclonal anti-Phn Antibody (Neufeld and Rubin, 1994) obtained from the Developmental Studies Hybridoma Bank were used for immunoblotting after dilution to 1/700 and 1/20, respectively. Sqh, the Drosophila homolog of MRLC, and its phosphorylated form were detected using the anti-Sqh antibody (Jordan and Karess, 1997) and rabbit polyclonal anti-phospho-MRLC antibody (Matsumura et al., 1998), respectively, as described (Winter et al., 2001). The latter antibody has been raised against a synthetic peptide based on the mouse sequence, and was provided by F. Matsumura. It reacts with the Drosophila protein.

**Generation of transgenic lines and rescue of DMBS mutants**

The DMBS-L and -S cDNAs were cloned into pP[CaSpeR-hs] and hs-DMBS-L and hs-DMBS-S transgenic lines were generated by the standard procedure. For rescue experiments, each two of the independent transgenes of hs-DMBS-L or hs-DMBS-S on the third chromosome were used, and the transgenes were combined with either DMBSP1 or DMBSP2 by recombination. Matings were performed between w; DMBS/TM3, Sb and w; DMBS hs-DMBS-L/TM3, Sb or w; DMBS hs-DMBS-S/TM3, Sb, and the cultures were either kept at 25°C or treated at 37°C for 1 hour twice a day throughout development. The full-length DRhk cDNA was cloned into pUAST (Brand and Perrimon, 1993) to generate UAS-DRhk transgenic lines.

**Immunohistochemistry and confocal microscopy**

Phalloidin staining

Embryos were fixed for 20 minutes in 7.2% formaldehyde/PBS: heptane, 1:1, and were devitellinized by hand. Embryos were incubated in rhodamine-conjugated phalloidin (Molecular Probes, Inc.) for 20 minutes, and were then washed with 0.2% Triton X-100/PBS three times. The DMBS mutant embryos were distinguished from the paternally rescued embryos by using the blue balancer, TM3, P[fr+]7=H22.7;DB2.

Immunostaining

Fixation and staining were carried out by the standard procedures. The primary antibodies and dilutions used are as follows. A mouse monoclonal anti-phosphotyrosine antibody (UB1) at a dilution of 1:1000; the rabbit polyclonal anti-myosin heavy chain antibody (Jordan and Karess, 1997), a gift from R. Karess, at a dilution of 1:1000; and the anti-phospho-MRLC antibody (Matsumura et al., 1998) at a dilution of 1/20. The secondary antibodies used were a rhodamine-conjugated anti-mouse IgG antibody (TAGO) or a FITC-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). The DMBS mutant embryos were distinguished as described above.

For the observation of MRLC distribution, the GFP-Sqh transgenic line (Sisson et al., 2000) provided by R. Karess was used. Embryos were fixed for 20 minutes in 4% paraformaldehyde/PBS: heptane, 1:1, and were devitellinized by vigorous shaking for 10 seconds before substitution of the aqueous layer with methanol. Then, embryos were rehydrated twice with 0.2% saponin/PBS for 10 minutes before mounting.

All images were collected on a Zeiss 512 laser scanning confocal microscope and processed with Adobe Photoshop software.

**Expression and purification of DMBS recombinant proteins**

For the production of GST-fused DMBS-L, the coding sequence of DMBS-L was amplified by PCR using the primer set, 5'-TGAACTTTCTACCTGTCTGCGACGACGCAAC-3' and 5'-GGTGACGTTGCTCTTTACCTAACATGTTGCTCTTAC-3', to create the EcoRI restriction sites immediately before the first codon. The amplified DNA fragment was cloned into pGEX-3T-1 (Pharmacia) to fuse with GST at the N terminus. The construct was verified by DNA sequencing. Mutagenesis at the putative phosphorylation site in DMBS was done by PCR using the following primers: 5'-AGGGAGACTCGACGTCCTGCCCAGGTGTGC-3' and 5'-CCAGG-
TGACACCTGAGCCAGACCGT-3'. Purification of the GST-fusion proteins was carried out essentially as described previously (Frangioni and Neel, 1993).

**Immunoprecipitation, immunoblotting and in vitro kinase assay**

Immunoprecipitation and immunoblotting of the HA-tagged DRho-kinase were performed as described previously (Mizuno et al., 1999). The immunoprecipitated DRho-kinase was incubated with 0.6 μg of GST-fused DMBS in the kinase buffer and was assayed as described previously (Mizuno et al., 1999).

**RESULTS**

**Identification of the Drosophila homolog of MBS**

To identify the *Drosophila* homolog of MBS, we searched the *Drosophila* Genome Database for sequences similar to vertebrate MBSs. The vertebrate MBSs share three conserved domains or motifs: amino-terminal ankyrin-repeats, a centrally located major phosphorylation site, and a carboxy-terminal leucine-zipper motif (Fig. 1A). A BLAST search for the putative open reading frame encoding a sequence similar to the phosphorylation site revealed a genomic fragment flanked by sequences encoding ankyrin repeats and a leucine zipper motif.

Analysis of the corresponding EST clones generated by the Berkeley *Drosophila* Genome Project revealed that none of them contained all three conserved domains. So, we predicted the 5' and 3'-UTR sequences from the EST and genomic sequences, and amplified the cDNA fragments containing the complete coding sequence by PCR using cDNA libraries for templates (see Materials and Methods). Two types of cDNAs were thus obtained from different cDNA libraries, each encoding a polypeptide of 927 and 797 amino acid residues with the predicted relative molecular mass of 101,409 and 87,509, respectively. The two sequences are identical except for an insertion of a 129 amino acid sequence upstream of the putative phosphorylation site in the longer polypeptide (Fig. 1A).

They contained the three conserved motifs, and the amino acid identities to their corresponding regions of human MYPT2 (Fujioka et al., 1998) are 57.8%, 55.6% and 77.8%, respectively, from the amino terminus (Fig. 1A). The similarity to vertebrate MBSs is restricted to these regions. Vertebrate MBSs and the homolog of *Caenorhabditis elegans*, MEL-11, contain seven ankyrin repeats (Fujioka et al., 1998; Wissmann et al., 1997), but the sequences in the fourth and seventh repeats diverged in *Drosophila*. In the *Drosophila* Genome Database, no other sequence similar to MBS was found, and we consider them as *Drosophila* homologs of MBS. We refer to the longer and shorter forms as *Drosophila* MBS-long (DMBS-L) and *Drosophila* MBS-short (DMBS-S), respectively (Fig. 1A).

The two cDNAs should derive from a single gene by alternative splicing (Fig. 3A). The DMBS-L-specific, 7th exon encodes a sequence of 129 amino acid residues. Furthermore, two in-frame consecutive splicing acceptor sites are present 5’ of the 4th exon (data not shown), and splicing variations at this site added one more amino acid residue in DMBS-L as compared to DMBS-S. We also obtained several partial cDNA fragments different from DMBS-L and DMBS-S (data not shown). Thus, the DMBS gene encodes multiple forms of DMBS through differential splicing. Splicing variants have been reported also for the vertebrate MBSs and MEL-11 of *C. elegans* (Hartshorne et al., 1998; Wissmann et al., 1999).

**Phosphorylation of DMBS by DRho-kinase**

Previous studies revealed that DRho-kinase physically interacts with DRho1 in the GTP form and phosphorylates the
vertebrate MBS in vitro (Mizuno et al., 1999; Winter et al., 2001). The sequence at the putative phosphorylation site of MBS is well conserved in DMBS (Fig. 1B), and we tested whether DRho-kinase phosphorylates DMBS in vitro. The GST-fused DMBS-L was expressed and purified from Escherichia coli, and was found to be phosphorylated by wild-type DRho-kinase but not by kinase-dead DRho-kinaseK116A (Fig. 1C, lanes 2 and 3).

Thr594 may correspond to the major phosphorylation site in vertebrate MBS (Fig. 1B). The threonine residue was replaced with an alanine, and this recombinant DMBS was used as a substrate. As shown in Fig. 1C, the level of phosphorylation was significantly reduced, indicating that Thr594 is the major site phosphorylated by DRho-kinase. It has been reported that mammalian MBS is phosphorylated at several sites by Rho-kinase (Kawano et al., 1999), and there presumably are other phosphorylation sites in DMBS as well.

**Expression of DMBS during development**

The pattern of the expression of DMBS during development was analyzed by in situ hybridization using DMBS-L as a probe (Fig. 2). A significant amount of DMBS mRNA was uniformly detected in blastoderm stage embryos (Fig. 2A), and it would be mostly of maternal origin. DMBS is expressed ubiquitously throughout embryogenesis (Fig. 2B,C). In the imaginal discs from third instar larvae, the DMBS transcript was uniformly detected (data not shown). Tissue- and stage-specificity of the expression for each isoform remain to be analyzed.

**Identification of DMBS mutants**

From the Drosophila Genome Database, DMBS was located at the 72D region on the left arm of the third chromosome. The intron/exon structure of DMBS was deduced from a comparison between the genomic and cDNA sequences (Fig. 3A). In this region, two P-element insertions, l(3)72Db03802 and Ep(3)3727, have been registered in FlyBase (Fig. 3A). They and an EMS-induced mutation, l(3)72Dd3, failed to complement to each other. l(3)72DbD03802 and l(3)72Dd3 are lethal during early larval stages, while Ep(3)3727 results in development to adults with a mild wing defect (data not shown). Excision of the P insertions reversed the lethal and wing phenotypes of l(3)72DbD03802 and Ep(3)3727, respectively, indicating that the P insertions caused the mutations. Imprecise excision of the P insertion from Ep(3)3727 produced a new mutation, P2r31, whose lethal phase spans from the third instar larva to early pupal stages.

A polyclonal antibody against a synthetic polypeptide corresponding to the carboxy-terminal region of DMBS was developed, producing a major band of about 95 kDa and several minor bands on the immunoblot (Fig. 3B). Arrows indicate the positions of DMBS bands. (C) Increase of phospho-MRLC in DMBS mutant larvae. The extracts were prepared as described (Winter et al., 2001) from the larvae as in B, except for lane 5 for which sqhAX2; GFP-Sqh larvae were used. Protein blots were probed with an anti-phospho-MRLC antibody (upper panel) or an anti-Sqh antibody (lower panel). The band corresponding to the GFP-Sqh fusion protein is not included. (D) Reduction in the amounts of DMBS proteins in the DMBS mutant females. Protein blots were probed with either an anti-DMBS-C1 antibody (upper panel) or an anti-Pnut antibody (lower panel). The bands corresponding to the GFP-Sqh fusion protein are not included. (D) Reduction in the amounts of DMBS proteins in the DMBS mutant females. The bands corresponding to the GFP-Sqh fusion protein are not included.

**Fig. 2. Expression of DMBS during development.** Antisense (A-C) or sense RNA (D) probes were used to detect the transcripts. (A,D) Blastoderm embryos, (B) germband-elongated embryo, and (C) germband-retracted embryo. Anterior is to the left and dorsal to the top.

**Fig. 3. Identification of the DMBS mutations.** (A) The genomic structure at the DMBS locus. The exon/intron structure is shown with columns and lines under the genomic map. The coding and untranslated regions are indicated as red and blue columns, respectively. The DMBS-L-specific exon is represented by a yellow column. The sites of the P-element insertions are indicated above the map. (B) Reduction in the amounts of the DMBS proteins in DMBS mutants. Protein blots were probed with either an anti-DMBS-C1 antibody (upper panel) or an anti-Pnut antibody (lower panel). Extracts were prepared from late third instar larvae of the following genotypes: wild type (lane 1), Df[3L]yrk117DMBSP2 (lane 2), DMBSP1/DMBSP2271 (lane 3), and DMBSP1/DMBSP2271 (lane 4). Arrows indicate the positions of DMBS bands. (C) Increase of phospho-MRLC in DMBS mutant larvae. The extracts were prepared as described (Winter et al., 2001) from the larvae as in B, except for lane 5 for which sqhAX2; GFP-Sqh larvae were used. Protein blots were probed with an anti-phospho-MRLC antibody (upper panel) or an anti-Sqh antibody (lower panel). The band corresponding to the GFP-Sqh fusion protein is not included. (D) Reduction in the amounts of DMBS proteins in the DMBS mutant females. Protein blots were probed with either an anti-DMBS-C1 antibody (upper panel) or an anti-Pnut antibody (lower panel). The bands corresponding to the GFP-Sqh fusion protein are not included.
Table 1. Rescue of DMBS mutations by transgenic DMBS-L or DMBS-S

<table>
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<tr>
<th>Genotype</th>
<th>– Heat shock</th>
<th>+ Heat shock</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBSP2/DMBSF1</td>
<td>0/167 (0)*</td>
<td>0/110 (0)*</td>
</tr>
<tr>
<td>DMBSF1/DMBSF1 hs-DMBS-L</td>
<td>0/404 (0)</td>
<td>1368/164 (83)</td>
</tr>
<tr>
<td>DMBSF1/DMBSF1 hs-DMBS-S</td>
<td>0/377 (0)</td>
<td>117/195 (60)</td>
</tr>
<tr>
<td>DMBSP2/DMBSP2</td>
<td>0/262 (0)</td>
<td>0/138 (0)</td>
</tr>
<tr>
<td>DMBSF1/DMBSF1 hs-DMBS-L</td>
<td>0/491 (0)</td>
<td>210/313 (67)</td>
</tr>
<tr>
<td>DMBSF1/DMBSF1 hs-DMBS-S</td>
<td>0/466 (0)</td>
<td>176/245 (72)</td>
</tr>
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*The numerator and denominator represent the number of animals surviving to adulthood and the theoretically expected number of flies with indicated genotype, respectively. The numbers in parentheses represent the respective percentages of surviving flies.

From these results, we conclude that they are the mutations in DMBS, and we refer to l(3)72Dd03802, EP(3)3727, l(3)72Dd and P2r31 as DMBSP1, DMBSP2, DMBSF1 and DMBSF2r31, respectively. The strength of their phenotype can be ordered as DMBSF1 = DMBSP1 > DMBSP2r31 > DMBSF2. Since DMBSF1 and DMBSF2 gave identical results, only the results obtained with DMBSF1 are presented in the following sections unless otherwise mentioned.

**Defects in dorsal closure in embryos lacking DMBS**

The animals homozygous for or transheterozygous between the strong DMBS alleles are larval lethal and embryonic development seems to proceed normally (Fig. 7A). This would be because of the maternal contribution of DMBS+ activity, a notion consistent with the observation that a significant amount of maternal mRNA is present in early-stage embryos (Fig. 2A). To analyze the function of DMBS during embryogenesis, we tried to reduce the maternal contribution.

The mutants transheterozygous between the weak allele, DMBSP2, and the strong alleles or Df(3L)th117, survived to adults. Examination of such female flies by immunoblotting revealed that the amount of DMBS proteins is greatly reduced (Fig. 3D). When the females transheterozygous between DMBSP2 and Df(3L)th117 were mated with wild-type males, embryonic development proceeded normally in most of the embryos. However, about 25% of the embryos failed to hatch in matings involving males heterozygous for the strong alleles (Fig. 7A). Similar results were obtained with the females of the genotypes, DMBSF1/DMBSF1 or DMBSF1/DMBSF2 (data not shown). Furthermore, embryonic defects were suppressed by paternal expression of the DMBS transgenes (data not shown). These results indicate that embryos fail to develop when DMBS is defective both maternally and zygotically, and that the maternal defect is rescued by the paternal expression of a wild-type gene.

About 80% of the dead embryos in the above experiments demonstrated the “dorsal open” or “dorsal hole” phenotype, which can be typically seen in embryos defective in the dorsal closure (Fig. 4B). In the remaining lethal embryos, the pattern of dorsal hairs was disturbed along the dorsal midline (Fig. 4E). These phenotypic variations would be due to the residual activity of maternal DMBS derived from the weak allele, DMBSP2. The results indicate that DMBS is required in the process of dorsal closure.

**Activation and inactivation of nonmuscle myosin II during dorsal closure**

To elucidate the roles of DMBS during dorsal closure, the distribution of nonmuscle myosin II and its activation were analyzed during the course of dorsal closure. The process can be divided into three phases. It starts with elongation of the dorsal-most, leading edge cells of the lateral epidermis along the dorsoventral axis. This is followed by elongation of the other lateral epidermal cells with the amnioserosa becoming covered by the elongating epidermis. Finally, the two lateral edges of the epidermis meet at the dorsal midline and fuse to close the epidermis.

Before the onset of the lateral epidermis elongation, the heavy chain of nonmuscle myosin II and MRLC outline the inner surface of the plasma membrane of the lateral epidermal cells at low levels, and they are concentrated at moderate levels along the leading edge of the lateral epidermis (Fig. 5A,B). The phosphorylated form of MRLC is localized similarly (Fig. 5C). During the course of extensive elongation, the heavy chain and MRLC accumulate at high levels along the leading edge (Fig. 5D,E). Phosphorylated MRLC is also detected at a high level along the leading edge (Fig. 5F). After the meeting of the two lateral epidermal sheets, both the heavy chain and MRLC at the leading edge become diffuse (Fig. 5G,H), and the phosphorylated form of MRLC disappears at the site of fusion (Fig. 5I). These observations indicate that the distribution and phosphorylation of nonmuscle myosin II are dynamically regulated during dorsal closure.

We next examined the distribution and activation of nonmuscle myosin II and F-actin in DMBS mutant embryos. Examination of the cell shape with anti-phosphotyrosine antibody revealed that the leading edge cells fail to fully elongate, and some of them remain polygonal (Fig. 6B). In contrast, the epidermal cells located more ventrally elongate nearly normally. A significant amount of phosphorylated MRLC is detected along the dorsal side of the boundaries.
between the leading edge cells in the mutant embryos (Fig. 6J, arrowheads), while the distribution of the heavy chain of nonmuscle myosin II is essentially not affected (Fig. 6H). F-actin is highly accumulated along the leading edge of the lateral epidermis (Fig. 6D). In the mutant embryos, an aberrant accumulation of F-actin within the leading edge cells is observed, while its distribution along the leading edge is essentially unaffected (Fig. 6E). This may correspond to the morphological aberrations of the leading edge cells.

**Genetic interactions of DMBS with mutations for the heavy chain of nonmuscle myosin and the Rho signaling pathway**

DRho-kinase is thought to be responsible for the inactivation of myosin phosphatase through phosphorylation of DMBS. If this inactivation turns out to have a considerable effect on the levels of phosphorylated MRLC, it can be expected that the phenotypes in the DMBS mutant embryos and in the embryos overexpressing DRho-kinase would be similar. When DRhk+ was expressed with the arm-GAL4 driver, about 80% of the embryos failed to hatch (Fig. 7B). A similar result was obtained with the 69B-GAL4 driver that induces the target gene in the ectoderm (Staehling-Hampton et al., 1994) (data not shown). Most of the lethal embryos showed a dorsal open or dorsal hole phenotype (Fig. 4C), and the pattern of dorsal hairs was disturbed along the dorsal midline in the remaining embryos (Fig. 4F) as observed in the DMBS mutant embryos. Examination of the cell shape (Fig. 6C) and the F-actin distribution (Fig. 6F) revealed the same aberrations as those in the DMBS mutant embryos.

To examine whether defects in the dorsal closure in the embryos lacking DMBS or overexpressing DRhk+ are due to an aberrant activation of nonmuscle myosin II, the genetic interactions with zipper (zip), which encodes the heavy chain of nonmuscle myosin II, were analyzed. As already described, about 25% of the progeny from crossing the females transheterozygous with DMBS<sup>P2</sup> and Df(3L)th117 to the males heterozygous for DMBS<sup>E1</sup> are embryonically lethal (Fig. 7A). We expected that a reduction in the gene dosage of zip+ would suppress the defects in the DMBS mutant or DRhk+ -expressing embryos. When DMBS<sup>P2</sup>/Df(3L)th117 females are mated with males heterozygous for both DMBS<sup>E1</sup> and zip<sup>Ebr</sup>, half of the embryos defective for both maternal and zygotic DMBS should

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**Fig. 5.** Distribution of nonmuscle myosin II during dorsal closure. (A-C) Lateral views of embryos before elongation of the lateral epidermis, (D-F) Lateral views of embryos during elongation of the lateral epidermis, and (G-I) Dorsal views of embryos during fusion of the lateral epidermis. Embryos were stained with an anti-myosin heavy chain antibody (A,D,G) or an anti-phospho-MRLC antibody (C,F,I). MRLC was visualized by expression of the GFP-Sqh transgene (B,E,H). Arrows indicate the leading edge of the lateral epidermis in A-F, and the dorsal midline in G-I.

**Fig. 6.** Cellular aberrations of dorsal closure in DMBS mutant embryos and in embryos overexpressing DRhk+. The wild-type embryos (A,D,G,I), DMBS mutant embryos (B,E,H,J) and embryos overexpressing DRhk+ (C,F) were stained with an anti-phospho-tyrosine antibody (A-C), phalloidin (D-F), an anti-myosin heavy-chain antibody (G,H), and an anti-phospho-MRLC antibody (I,J). The DMBS mutant embryos were obtained from a mating between DMBS<sup>P2</sup>/Df(3L)th117 females and DMBS<sup>E1</sup>/TM3, P[ry<sup>t7.2=HZ2.7</sup>]DB2 males, and the mutant embryos were identified by use of the blue balancer. DRhk+ was driven by 69B-GAL4. The aberrantly distributed phospho-MRLC is indicated with arrowheads in I. Polygonally shaped leading edge cells (B,C) and the leading edge cells with aberrant accumulation of F-actin (E,F) are boxed.
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be heterozygous for zipEbr. As expected, the embryonic lethality was reduced to nearly half that of the corresponding cross (Fig. 7A). Similarly, the heterozygosity for zipEbr considerably suppressed lethality due to ectopic DRhk+ expression (Fig. 7B). These results strongly suggest that either loss of DMBS+ or overexpression of DRhk+ causes hyperactivation of nonmuscle myosin II through increasing the levels of phosphorylation of MRLC.

zipEbr is a point mutation reported to be highly sensitive to genetic backgrounds (Halsell and Kiehart, 1998; Halsell et al., 2000). About 70% of the flies transheterozygous between zipEbr and zip02957 have malformed wings with varying degrees of severity (Fig. 8B-D). Although zipEbr is recessive, a considerable percentage of the flies heterozygous for both zipEbr and the mutations in the components of the Rho signaling pathway such as D RH o1 and D RhoGEF2 produced similar defects (Fig. 8D) (Halsell et al., 2000). A half reduction of Drok, which encodes DRho-kinase (Winter et al., 2001), also dominantly enhanced zipEbr. This indicates the involvement of the Rho signaling pathway and its effector, DRho-kinase, in the myosin function of adult wing morphogenesis (Halsell et al., 2000). When the flies are also heterozygous for DMBS(+)E1, wing malformation is significantly suppressed (Fig. 8D), suggesting that DMBS functions antagonistically to the Rho signaling pathway (Fig. 8E) and is also involved in adult morphogenesis.

DISCUSSION

Involvement of DMBS in morphogenetic processes

The force-generating activity of nonmuscle myosin II is regulated mainly by phosphorylation and dephosphorylation of its regulatory light chain, MRLC. Myosin phosphatase is responsible for the negative regulation of nonmuscle myosin II through dephosphorylation of MRLC, and its regulatory myosin-binding subunit, MBS, is crucial in regulating the activity of myosin phosphatase and determining its substrate-specificity. Here we identified the mutants for the Drosophila gene encoding a homolog of MBS, DMBS. Genetic analyses of DMBS revealed its roles in dorsal closure during embryonic development and in adult wing morphogenesis. Extensive cell shape changes take place during both morphogenetic processes, and the genetic interactions with zip indicate the importance of DMBS in the regulation of nonmuscle myosin II in these processes. DMBS is expressed ubiquitously throughout development, and the mutants are lethal at early larval stages, suggesting yet other functions for DMBS during development.

Interactions with the Rho signaling pathway

The phenotype produced by overexpression of DRhk+ resembles that of DMBS mutant embryos, and both phenotypes can be suppressed by reducing the gene dosage of zip+. The results suggest that these phenotypes are the result of the hyperactivation of nonmuscle myosin II, and that myosin phosphatase and DRho-kinase function antagonistically toward each other in regulating nonmuscle myosin II (Fig. 8E). A similar observation has been reported for let-502 and mel-11 of C. elegans, which encode the homolog of Rho-kinase and MBS, respectively. The genes have been demonstrated to function in the hypodermal cell-shape change associated with the elongation of the embryo in C. elegans (Wissmann et al., 1997), thus indicating the conservation of the regulatory mechanisms of nonmuscle myosin II in morphogenesis. The genetic link between zip and the mutations in the Rho signaling pathway has been demonstrated (Halsell et al., 2000) (Fig. 8A-D), and this indicates the regulation of nonmuscle myosin II by the Rho signaling pathway. The suppression of both phenotypes generated by double heterozygosity for zip and the
mutations in the components of the Rho-signaling pathway by DMBS demonstrates that DMBS functions antagonistically toward the Rho-signaling pathway in the regulation of nonmuscle myosin II (Fig. 8E).

Roles of DMBS in dorsal closure

During dorsal closure, an extensive cell shape change in the lateral epidermis takes place. One of the major forces underlying the morphogenetic process is provided by the constriction of a supacellular purse-string revealed by the high level accumulation of F-actin and the heavy chain of nonmuscle myosin II along the leading edge of the lateral epidermis (Young et al., 1993) (Fig. 6D,G). We found that MRLC is also highly accumulated along the leading edge (Fig. 5D,E; Fig. 6G). Small quantities of these components of actomyosin are also detected along the inner surface of the plasma membrane. Phosphorylated MRLC was detected in significant amounts along the leading edge of the lateral epidermis (Fig. 5F and Fig. 6I), indicating that nonmuscle myosin II is persistently activated along the leading edge during extensive epidermal spreading.

In DMBS mutant embryos, the defects in dorsal closure seem to be confined to the leading edge cells, and these cells fail to fully elongate. In contrast, the lateral epidermal cells located more ventrally elongate more or less normally. It has been reported that dorsal closure is driven by multiple forces and that it can proceed in the absence of an intact contractile purse-string at the leading edge (Young et al., 1993; Kiehart et al., 2000). These lateral epidermal cells are under tension during dorsal closure (Kiehart et al., 2000), and they themselves may produce the forces to elongate.

It should be noted that the accumulation of nonmuscle myosin II in the leading edge cells was essentially not affected. Activation of nonmuscle myosin II takes place along the leading edge as in normal embryos, since the phosphorylated MRLC was detected there in the mutant embryos (Fig. 6H). In addition to the distribution along the leading edge, a significant accumulation of phosphorylated MRLC was detected also on the dorsal side of the boundaries between the leading edge cells in the mutant embryos (Fig. 6J). This may indicate the role of myosin phosphatase in inactivating nonmuscle myosin II in this subcellular location to coordinate elongation of the leading edge cells.

The results suggest the localized activation of myosin phosphatase during the normal course of dorsal closure. One possible explanation for this localized activation is that there is a subcellular distribution of myosin phosphatase itself in this region. However, a suitable antibody would have to be raised against DMBS to determine if this was so. Another possible explanation is the localization of an activator of myosin phosphatase in this region. Thus, there must be a subcellular localization of mechanisms for the regulation of nonmuscle myosin II. It has been demonstrated that the cellular polarity of the leading edge cells is altered from basolateral to apical in the leading edge during elongation (Ring and Martinez-Arias, 1993; Woods and Bryant, 1993; Fehon et al., 1994). It would be of interest to learn how cellular polarity affects the pattern of regulation of the nonmuscle myosin II in the leading edge cells. The results obtained in this study demonstrate the importance of both positive and negative regulation of nonmuscle myosin II in morphogenesis.

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