Masquerade: a novel secreted serine protease-like molecule is required for somatic muscle attachment in the *Drosophila* embryo

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Diverse developmental processes, such as neuronal growth cone migration and cell shape changes, are mediated by the interactions of cells with the extracellular matrix. We describe here a secreted molecule encoded by the *Drosophila masquerade* (*mas*) gene. Total loss of *mas* function causes defective muscle attachment. This mutant phenotype suggests that *mas* normally acts to stabilize cell–matrix interaction and represents a novel functional and limiting component in the adhesion process. *mas* encodes a 1047-amino-acid preproprotein that is further processed by proteolytic cleavage to generate two polypeptides. The carboxy-terminal polypeptide is highly similar to serine proteases and has an extracellular localization; however, it is unlikely to possess proteolytic activity, because the catalytic site serine has been substituted by a glycine residue. During embryonic development, the *mas* amino- and carboxy-terminal polypeptides are differentially localized. The *mas* carboxy-terminal polypeptide accumulates at all somatic muscle attachment sites, which corresponds well with the morphological defect seen in the *mas* mutants. Our findings demonstrate the involvement of an extracellular component in somatic muscle attachment. We propose that *mas* acts via its modified serine protease motif, either as a novel adhesion molecule and/or as a competitive antagonist of serine proteases, to stabilize muscle attachment.

[Key Words: Muscle attachment; serine protease; *Drosophila*; extracellular molecule; masquerade]

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The development of the *Drosophila* somatic musculature is a multistage process. After germ-band retraction, myoblasts fuse to form muscles and the differentiating structures make attachments at specific sites on the epidermis (Williams and Caveney 1980). By 13 hr after egg laying, the formation of muscle pattern is complete and the muscles have established or are establishing muscle insertion points at characteristic locations on the epidermis (Bate 1990). The assembly of developing muscle insertions in insects involves the formation of adhesive junctions between the interdigitating processes of the epidermis and the muscle fibers (Lai-Fook 1967; Poodry 1980).

Members of the *Drosophila* integrin family (Leptin et al. 1987), named PS integrins, were first identified as antigens expressed in a position-specific manner in the wing imaginal disc (Wilcox et al. 1981; Brower et al. 1984). PS1 ($\alpha_{ps1}\beta_{ps}$) and PS2 ($\alpha_{ps2}\beta_{ps}$) integrins are both heterodimers with identical β -subunits but different α -subunits. The α_{ps2} -subunit is encoded by the *inflated* (*if*) gene (Bogaert et al. 1987; Brower and Jaffe 1989; Brown et al. 1989; Wilcox et al. 1989; Brown 1994),

whereas the common β_{ps} -subunit is encoded by *l(1)mysospheroid* (*mys*) gene (MacKrell et al. 1988; Leptin et al. 1989). Defects seen in *mys* embryos (for reviews, see Brown 1993; Bunch and Brower 1993), which fail to synthesize both PS1 and PS2, include the formation of mysospheroid bodies caused when somatic muscles detach from the body wall and round up. Mutant *if* embryos show defects in embryonic somatic muscle attachment that are similar to (Brabant and Brower 1993) but less severe than those seen in *mys* embryos (Brown 1993, 1994). Ultrastructural studies indicate that there are matrix components in the junction between the apposing epidermal and muscle cells (Newman and Wright 1981). The differential localization of PS1 and PS2 on complementary sides of the epidermal–muscle junction and the differences in the severity of the phenotypes of the mutants lacking these molecules support the idea that they both bind to protein components of the extracellular matrix (ECM) to effect muscle attachment (Brown 1993, 1994).

In the developing embryo, the ECM affects cell differentiation and proliferation, provides an environment for cell migration, and serves as a substratum for guidance and adhesion (Bard 1990). Genes encoding several major

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classes of extracellular matrix (substrate) adhesion molecules [for review, see Fessler and Fessler 1989], as well as cell adhesion molecules (see Hortsch and Goodman 1991; Goodman and Doe 1993), have been identified and characterized in *Drosophila*. These molecules show striking structural similarities to their mammalian counterparts, suggesting a conservation in function [Hynes 1992; Kreis and Vale 1993]. However, with the notable exception of the integrins, the functional roles of most of the *Drosophila* ECM/cell adhesion molecules have not been defined precisely for want of an informative mutant phenotype. For example, null alleles of the gene encoding the substrate adhesion molecule laminin A [Garrison et al. 1991; Kusche-Gullberg 1992; Henchcliffe et al. 1993] exhibit no obvious embryonic morphological abnormalities. However, an array of defective adult structures was reported [Henchcliffe et al. 1993]. It would seem, at least in the embryo, that the loss of function of substrate adhesion molecules like laminin A, as well as many of the cell adhesion molecules, can be compensated in part by other components of the ECM or cell surface. Hence, it may be necessary to remove multiple components of the system to observe a phenotype.

Proteases can modulate the interactions of the cell surface with the extracellular environment either directly, for example, by localized and directional degradation of the ECM [e.g., Pollanen et al. 1988; Werb et al. 1989], or indirectly by activating signal transduction pathways [Vu et al. 1991] to effect, for example, the reorganization of cytoskeleton [Jalink and Moolenaar 1992] or neurite retraction [Suidan et al. 1992]. The enzymatic activities of most proteases are regulated, being synthesized as inactive zymogens that only become enzymatically active following processing. Once activated, the domain of enzymatic activity can be further modulated by enzymatic inhibitors (see Hecht and Anderson 1992). The effects of the interplay of proteases [e.g., Gurwitz and Cunningham 1988; Dihanich et al. 1991] and their inhibitors [Gloor et al. 1986; Reinhard et al. 1988] have been proposed to play an important role in growth cone motility and in neurite outgrowth [Monard 1988; Suidan et al. 1992; Sumi et al. 1992]. Mutations in the *Drosophila* *Stubble-stubloid* (*Sb-sbd*) gene, which encodes an apparent transmembrane protein with an extracellular serine protease domain, cause defects in cell shape and changes in the organization of microfilament bundles required for normal imaginal disc and bristle morphogenesis [Appel et al. 1993]. Although the molecular substrates for the protease encoded by *Sb-sbd* have not been

elucidated, it seems likely that the molecular interactions (e.g., between receptors like integrins and protein components of the ECM) that have effects on cell shape and movement are regulated, at least in part, by the interplay between proteases and their inhibitors.

Here, we describe the characterization of the gene *masquerade* (*mas*) that encodes a secreted molecule with striking similarities to serine proteases; however, it is unlikely to possess proteolytic activity, because the essential catalytic serine residue has not been conserved in *mas*. Our results indicate that *mas* is processed to generate two polypeptides that are differentially localized. The carboxy-terminal "modified serine protease domain" accumulates at all embryonic somatic muscle attachment sites coinciding with the defects seen in animals lacking *mas* function. Total loss of function of *mas* is embryonic lethal and results in the formation of myospheroid bodies and detached somatic muscles. This phenotype points to a role for *mas* as an extracellular component required for the stabilization of somatic muscle attachment. We discuss possible mechanisms by which *mas* might act in this process.

Results

Identification, cloning, and analysis of the mas gene and three mutant alleles

mas was identified originally from a screen of homozygous viable single P-element insertion lines that show recessive defects in chemosensory behavior [B. Murugasu-Oei, R. Balakrishnan, W. Chia, and V. Rodrigues, in prep.]. Results from reversion experiments have shown that this P-element insertion is responsible for defective chemosensory perception in the third-instar larvae and in adults (data not shown). Using standard methods (see Materials and methods), ~20 kb of genomic DNA flanking the *mas* P-element insertion was isolated. Within the cloned region, a single 4-kb transcript was detected that was located ~500 bp upstream of the P-element insertion. Using the genomic DNAs as probes, cDNA clones were obtained that encompassed the entire coding region of this transcript. The organization of the transcription unit was determined by comparing the cDNA sequence with the genomic sequence. A schematic summary of the organization of the *mas* transcription unit relative to the genomic DNA is shown (Fig. 1a,c).

The expression of the *mas* transcription unit was assessed by Northern hybridization using poly(A)⁺ RNA prepared from various developmental stages. The results

Figure 1. Schematic representation of the *mas* gene, its transcript, deduced protein, and the lesions associated with three P-element-mediated deletion mutations. (a) A restriction map of the genomic region encompassing the *mas* transcription unit. (b) The structure, deduced from Southern mapping experiments, of three P-element-induced deletion mutants affecting the *mas*-coding region are schematically represented (see text). (c) The intron/exon organization of *mas* derived from a comparison of cDNA and genomic sequences. The sizes of the introns are indicated; the 1.6-kb intron in the 5'-transcribed nontranslated region has not been sequenced in its entirety. Horizontal arrow and solid square indicate the putative positions of transcriptional start and stop sites based on a full-length cDNA clone; transcribed but nontranslated regions are indicated by open boxes; protein-coding regions are indicated by solid boxes. The deduced *mas* protein and structures of deletion mutants are also represented. Absent sequences are shown as a solid bar, with the broken bar indicating the unknown endpoint of the deletion. The restriction sites shown are *Bam*HI (B), *Eco*RI (R), *Hind*III (H), *Sac*I (Sc), *Xmn*I (X), *Pst*I (P), and *Sal*I (S).

Figure 1. (See facing page for legend.)

(see Fig. 2) indicate that the 4-kb *mas* mRNA is detected primarily during embryonic, first-instar larval and pupal development.

The original P-element insertion, located at cytological position 64B, clearly did not abolish *mas* function, because it lies just 3' to the *mas* transcription unit and did not cause gross alterations in the *mas* transcript distribution (not shown). To generate total loss-of-function alleles of *mas*, we mobilized the original P element and attempted to isolate new insertions into the *mas*-coding region as well as imprecise excisions that removed the *mas*-coding region (see Materials and methods). The mutations obtained were analyzed (see Materials and methods), and the structural defects associated with three of the alleles are schematically summarized in Figure 1, b and c. We have analyzed in detail the embryonic phenotype associated with these three alleles that remove either all or part of the *mas*-coding region. *mas*^{xs76} and *mas*^{x124} are protein minus null alleles (see below) in which the whole of the *mas* protein-coding region has been removed; in *mas*¹⁵⁴, only part of the protease-like portion of the carboxy-terminal region of *mas* is deleted (Fig. 1c). These mutants are recessive embryonic lethal mutations that exhibit indistinguishable phenotypes either as homozygotes or as transheterozygotes. In addition, we have observed that animals homozygous for mutational lesions farther 3' to *mas*, which do not affect the *mas* transcribed region, are phenotypically normal (not shown).

Figure 2. Developmental Northern blot of *mas*. A Northern filter containing 2 μ g of polyadenylated RNA from each of the stages indicated was hybridized to a *mas* cDNA probe (*top*). The ages of the embryonic samples are given in hours; the ages of the larval and pupal RNA preparations are given in days. The filter was rehybridized to an actin probe as a control for loading (*bottom*).

Loss of *mas* function results in defective somatic muscle attachment

To examine the musculature in mutant embryos, we constructed strains bearing the *Mhc-lacZ* transgene in the mutant background. The *Mhc* promoter directs expression of *Escherichia coli* β -galactosidase in all somatic muscles, thus serving as a convenient marker (Hess et al. 1989; Broadie and Bate 1993). Muscle development and patterning are normal in mutants up to stage 15. The defects only become apparent during stage 16. We often observed muscles that appear collapsed [e.g., in Fig. 3b', lateral transverse muscles 1–3 (LT1–LT3); in Fig. 3b, solid arrowheads] in comparison to their counterparts in normal animals (Fig. 3a, solid arrowheads). The most frequent defects are ones in which the muscles appear rounded up in myospheroid-like bodies [e.g., in Fig. 3c', ventral acute 2 (VA2); in Fig. 3c, solid arrow] or have detached from one side [e.g., in Fig. 3d', ventral lateral 1 (VL1); in Fig. 3d, open arrows]. In some instances, muscles [e.g., in Fig. 3a', lateral oblique 1 (LO1); in Fig. 3a, open arrowhead] appear absent, presumably because they had detached from both ends (absence of LO1 in Fig. 3b, b'). The overall muscle pattern was, however, not altered significantly, and the structure of muscle fibers appeared intact when examined by polarized light or by activity staining of the *Mhc-lacZ*; *mas*^{xs76} animals. The phenotype of all three alleles is reminiscent of *l(1)mys* although not as severe.

To determine whether *mas* loss of function has an effect on somatic muscles in general or whether the effect is specific to subsets of muscles, a quantitative analysis of the phenotype was performed for two of the *mas* mutant alleles (*mas*¹⁵⁴ and *mas*^{xs76}). In these experiments, we scored individual muscles in whole mounts of homozygous mutant animals and control embryos stained with anti-MHC (Kiehart and Feghali 1986); for ease of scoring, we examined primarily the ventral and lateral somatic muscles (although similar results are seen with the dorsal muscles from a smaller data set). Our results (Table 1) indicate that animals homozygous for the *mas* loss-of-function alleles (*mas*¹⁵⁴ and *mas*^{xs76}) show a frequency of muscle attachment defects one and one-half orders of magnitude higher than those seen in wild-type animals and in animals homozygous for a precise excision of the original *mas* insertion line. Moreover, the defects seen in the mutants appear to affect all the somatic muscles at similar frequencies. These observations clearly indicate a general role for *mas* in somatic muscle attachment and are consistent with the view that *mas* acts to promote and/or stabilize cell-matrix interactions.

mas encodes a putative secreted molecule sharing many of the structural features of serine protease zymogens but is unlikely to possess protease activity

DNAs covering the whole of the *mas* protein-coding region were isolated and sequenced. The largest open reading frame starting with a methionine is 1047 amino ac-

Figure 3. Null mutations of *mas* cause defective muscle attachment. Muscle nomenclature is according to Bate (1993). The somatic musculatures of the abdominal segments of dissected embryonic preparations of wild-type (a) and mutant (b–d) animals were examined. Both the wild-type and mutant animals carry an insertion (*Mhc-lacZ*), in which the *E. coli lacZ* expression is driven by a myosin heavy-chain promoter, which allows the somatic muscles to be visualized by staining with anti- β -galactosidase. Schematic drawings showing the relevant muscles are given immediately above each panel (a'–d'). Anterior is to the left and dorsal is toward the top. (a) A wild-type hemisegment from an *Mhc-lacZ* embryo; muscles LT1–LT3 (solid arrowheads), muscle LO1 (open arrowhead), muscle VL1 (open arrow), and muscle VA2 (solid arrow), which lies just below the plane of focus, are indicated. (b) A mutant hemisegment (from an *Mhc-lacZ; mas^{xs76}* embryo) in which muscles LT1–LT3 have detached and collapsed (solid arrowheads; horizontal stripes in b') and muscle LO1 is absent. (c) A mutant hemisegment in which muscle VA2 has rounded up into a ball-like structure (solid arrow; hatched circle in c'). (d) A mutant segment in which muscle VL1 appears to have detached from one of its attachment sites (open arrows; vertical stripes in d'). Similar results are seen with the other *mas* alleles described in Fig. 1d (also see Table 1). Bars (a,b), 25 μ m.

ids, although the sequence context of the second methionine (at codon 8) has a better agreement with the consensus sequence for translational initiation (Cavener 1987). Translational termination codons are found in all three reading frames upstream of the putative initiation codon. The *mas* nucleotide and amino acid sequences (Fig. 4), as well as a schematic representation (Fig. 5a) showing the major structural features of the deduced protein, are shown. A hydrophobic sequence that immediately follows the initiation methionine is probably a signal peptide (von Heijne 1985); inspection of the amino-terminal residues suggests that the first 30 amino acids comprise a presequence and that signal sequence cleavage occurs between residues 30 and 31 (von Heijne 1986).

The protein contains five repeats of a disulfide knotted motif that has been found in other arthropod serine proteases (Gay and Keith 1992), including the *Drosophila Sb-sbd* (Appel et al. 1993), *snake* (DeLotto and Spierer 1986), and *easter* (Chasan and Anderson 1989) gene products and the *Limulus* proclotting protein (Muta et al.

1990). For the *Limulus* proclotting protein, it has been demonstrated that intramolecular disulfide bridges formed between the 6 cysteine residues (found in each of these motifs) result in the formation of a knot-like structure that is thought to act as a receptor for the protein that cleaves at the zymogen activation site. Like other protease zymogens, there are stretches where single amino acids of G, E, P, and T are repeated. Interspersed among the disulfide knots and the amino acid repeats are six putative N-glycosylation sites.

The carboxy-terminal domain possesses many of the features associated with serine proteases (see Fig. 5b); however, the position normally occupied by the serine residue within the invariant catalytic triad essential for catalysis has been replaced by a glycine. The authenticity of this glycine has been confirmed by sequencing of both the genomic and two independent cDNA clones. Hence, *mas* is unlikely to possess protease activity. The residues lining the substrate binding pocket have also been conserved (see Fig. 5b), and the substrate binding pocket most resembles those of the trypsin-like serine

Table 1. Quantitative analysis of the muscle detachment phenotype in *mas* mutants

Ventral muscles (number of defective muscles/total number of muscles)														
Muscle	VA1	VA2	VA3	VO1	VO2	VO3	VO4	VO5	VO6	VL1	VL2	VL3	VL4	VT1
Genotype														
WT	0/137	0/137	0/137	0/137	0/137	0/137	0/137	0/137	0/117	0/137	0/137	0/137	0/137	1/117
Rev	0/136	0/136	0/136	1/136	1/136	0/136	0/136	0/136	0/122	0/136	0/136	0/136	1/136	0/122
<i>mas</i> ¹⁵⁴	30/138	33/138	39/138	31/138	31/138	29/138	29/138	31/138	28/118	19/138	21/138	18/138	18/138	29/118
<i>mas</i> ^{<i>xs76</i>}	37/140	35/140	40/140	32/140	32/140	33/140	31/140	36/140	32/120	24/140	29/140	30/140	31/140	27/120
Lateral muscles (number of defective muscles/total number of muscles)														
Muscle	SBM		LO1		LL1		LT1		LT2		LT3		LT4	
Genotype														
WT		1/137		0/137		0/137		1/137		0/137		3/137		5/137
Rev		0/136		2/136		2/136		1/136		1/136		3/136		3/136
<i>mas</i> ¹⁵⁴		19/138		35/138		22/138		21/138		22/138		28/138		33/138
<i>mas</i> ^{<i>xs76</i>}		28/140		37/140		41/140		28/140		28/140		32/140		38/140
							WT		Revertant			<i>mas</i> ¹⁵⁴		<i>mas</i> ^{<i>xs76</i>}
Number of defective hemisegments/total number of hemisegments							6 137		9 136		127 138		130 140	
Defective hemisegments (%)							4.4		6.6		92.0		92.9	

Nomenclature of the muscles is according to Bate (1993). Defective muscles are defined by the phenotypes described in Fig. 3, b–d. Muscles phenotypes of abdominal segments 1–7 were assessed at embryonic stage 16 using anti-MHC antibodies (Keihart and Feghali 1986). (WT) Wild type; (Rev and Revertant) precise excision of the original *mas* P-element insertion, as determined by Southern blot analysis; (*mas*¹⁵⁴ and *mas*^{xs76}) *mas* mutant alleles (see Fig. 1d).

proteases that cleave after basic amino acid residues. The 6 cysteines that form disulfide bonds within the protease domain of other serine proteases are also found at conserved positions in the carboxy-terminal domain of *mas*.

mas is specifically processed to generate two polypeptides

The structural features of the predicted *mas* protein show striking similarities to serine proteases, many of which are activated by processing (zymogen activation). To examine the possibility that *mas* is also processed, we produced affinity-purified antibodies against two antigens; a synthetic peptide (residues 164–188) corresponding to a region of *mas* lying near the amino terminus and a fusion protein that includes the *mas* carboxy-terminal modified serine protease domain (see Fig. 5a; Materials and methods). That these antibodies specifically recognize *mas* is supported by the observation that they fail to stain animals homozygous for a mutation that deletes the *mas* coding region (see Fig. 8e,f,m–o, below). With wild-type embryos, the in situ patterns of staining seen with the two antibodies differ (see Fig. 8a–d,g–l); these observations are consistent with the notion that *mas* is processed to generate distinct polypeptides that are differentially recognized by the two purified sera.

In Western experiments using 0- to 22-hr embryonic lysates, the purified serum raised against the amino-terminal synthetic peptide specifically recognizes a 35-kD band (Fig. 6a, lane 3), whereas the purified serum raised

against the carboxy-terminal fusion protein specifically recognizes a 77-kD band (Fig. 6a, lane 1). A 112-kD band seen in some but not all preparations is consistent with the existence of a transient proprotein that is processed into an amino-terminal and a larger carboxy-terminal polypeptide. The 35- and 77-kD bands were not seen when the experiments were done with the preimmune sera (Fig. 6a, lanes 2,4). To further ascertain the authenticity of these bands, an additional control was done, using a strain (*hs-mas*) homozygous for a transposon insertion carrying a transgene in which the full-length *mas* cDNA is placed under the control of the *hsp70* promoter. *hs-mas* embryos were collected during a developmental interval when there is normally no detectable *mas* protein expression (2–5 hr) and subjected to a 1-hr heat shock at 37°C (see Materials and methods). Two hours following heat shock, accumulation of both the 77-kD (Fig. 6b, lane HS) and the 35-kD (Fig. 6c, lane HS) polypeptides is readily detected in Western blots using the appropriate purified serum. In the corresponding control embryos that were not subjected to heat shock, little if any of the 77-kD (Fig. 6b, lane C) and 35-kD (Fig. 6c, lane C) polypeptides can be detected, because *mas* protein expression does not normally occur until late in embryogenesis (see below). These data indicate clearly that the *mas* protein is processed into two polypeptides of 35 kD (amino-terminal) and 77 kD (carboxy-terminal). The processing mechanism therefore appears to be ubiquitous and is present during stages preceding the normal appearance of the *mas* protein.

1	AGTCGGCTTT TGGCTTTCCG AAGGTGCACT CGCGTCGTGA TCCCATCTCT CAGATACATAT ATATACATAT ATATATATATA TACACACAAA AGAGATACCG	
101	AGGATCGCTC TAATTTAAAC AGAAACCTTT ATTCTTGGTA AGAATGAAAC ACUTGCTGAC CATTTTGGAC CAACGAATT	
195	AGC ACA ATG AGC CGC CTT GTT CTG CCG CTG ATC TTC AGC ATT TTG CTG GTC AGT AAG CCA TCG CCA TCA CAG GCT CAG GAT	32
276	GAA TCC CTG GCT GGC AGT TTC CTA TCA GAT CTT TTG GAC ACC ATA ACG AGC ACC GCA GAC TCC AAG GAT TGC CTT GGA GTA	59
357	TGT GTC CAC ACT TTG GCA ACT TTA ATT TGC TAC GAA GTT CTA GAC GAT GTG GCT TGT CTT TCG CCC AGC ATG AAA TGC TGC	86
438	ATA GAA AAT GCT CCA GGC GGC AAG AAT GGC ACT GCT GTG AGA GCA ACA ACA ACG CTT AAA ACC ACA ACC ACT GCT AGC ACC	113
519	ACT ACC ACC CAG ACA ACC ACC ACC AGT GTG GGC ACT ACT AGC ACC ACA AAG AGA ACC ACC ACA AGC AGC ACC ACT CCA AAA	140
600	CCA AAA CCA AAG CCA CAG ACC AAG CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA	167
681	ACC ACC AAG AAA GTG GCT ACG GGC AAA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA	194
762	GGA GTT TGC GTG GGC GAT GGC ATA GCG GAG TAC TGT GAG GGC TAT TTG ACC AGC GAT GGA CTG TGC AAG GAA GGC ACC AAG	221
843	TGC TGC GTC TCC GAT GAT GAT TAT TCC AAT GGC AAG GAT ATC TAT ATT CCA GGC AAA CAT ATG AGC AAG TTG	248
924	AAG CCC AAC CAG AGC CTG TCC GAA AAA TCC GGT CCC GGC AAG AGT TCC AGT ACG TCC ACG AGC TCC ACT ACT ACC ACC ACC	275
1005	AGC ACT ACC ACA ACA CCC GAA CCG GCT AGA ATA AAC AAC AGT TCT CCG AAG CCC ACC AAA CAC AAG AAG CAT CCG ACC ACC	302
1086	ACA ACG ACG GAG GCA GGC GAG GAA GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG	329
1167	AAG CTG AAG TCC GCA CAA GGT CAG GGT CAG GTG TTG AAG GAA TGC GAG GGC GAG TGC AAT AAT GGA ATA TTT GCG ATC TTC	356
1248	TGT GAT GAC ATC GAT TCC GAT GCT TTC TGT CCG GAA GGA AGC TGT TGT GTG ACT GGA GAT GGC TCG GAG GCA ACA CCC	383
1329	TCC TCC AAG GCT CCA CCC ACC AAG CCG GGC ATC AAG CAT GCT CCC AAG CCA GCA GGC AAG CCG GCT CCG CCC GCG TCT CCA	410
1410	CCG CCA GCA CCA CCA TCT TCG ACC TCT GGA GGC GGA GGA GGT GGT GAT TTC CTC TCT CAA ATC ATA TCC TTT GCG GAG AGC	437
1491	ACT CTG AAC TCG CCA TCT CCA CCA CCG CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA CCA	464
1572	AAC ATT ATG GGC GGC TTT GCG AGA CCA CCG CTG GTT TCC ACA CCG ACA CCG TGT GGC AAG GGT TCG GTA TGC TGC	491
1653	GAC TCC TCA AGA GCG GCT CAG CCA CCG AAG TTG CCA CCA CCG AGC CCG TCG CCG GCG GCG TCG CCC ACT GCT CCA CCA	518
1734	TAT GTC CTG CCC AAT CCG AGT CCG GAT CCG GGA GAA GAA TCC CCA GGA TCC TGT ATC GTC AGC CTG CTC AGC TTC ACT	545
1815	TGC TTC AAA AAC CCG GAG ATG ACT GAT TTT CCG GAG CCG AAG GGT TCC GGC CAG ATT TGC TGT CCG CCC AAG AGC AAG ATC	572
1896	CTT GAG AAG CAG CAG TTT CAG ACC CCG AAC GAC ACC GGC TAC TAC CCG GCA CCG CCG CCA CCG CCC ATT GGT CCA CCA CAG	599
1977	GCA TAT CCC CCA CAG ACA CCA CCC TAC TCC TAC ATG AAC AAT CCA CCG CCA CAG GGA CCA CCA CCC CAG ATG CCG CCA CCA	626
2058	CAC CCC AAT CCC TAC CAG CCA CCG CCA CCG GCG CCC AAC TAC GCA GAT TAC TAT CCA GGT TCC GCA CCG GGA CTA CCA CCA	653
2139	CAA CCT CAG CCA CCG ATG ACC ACC CCG CCG CCG ACA ACT ACA ACC ACC ACC ACT CCA CCG CCA CAT GTC TAC TCC AAG TAT	680
2220	GTG TGT GGT GTG AAG GCT CTG GCA AGC AGA TCC CAG GCT CTA TCT TTC GTC TCC TAC GCG GGT GGC AAC TAC GGA	707
2301	GTA CAG AGA ACA GCT CCG CAG ATG ACC TCC GCG GCG GGA TAC TCT CCC AAC TTC AAC AAA TCC AAT GAG AGA TTG GTC CTA	734
2382	GGA TCC GGC ATT GTG CCC CAG CAG ATT CAC AAC CAG CCA CCG GGT GAC CTA GTG GAG TCC AAC AGC AGT CTA CAG AGC AAC CAA	761
2463	TTA COT TCG TAC CAC AAT CAC CAA GCT CAG GCA GAT CAG CCG GAT CTG GTC TAT CCG GAA TAC TAC CAA CAG AGG TCT CTT	788
2544	TAC GGA CTC CAG TCG AAC TTC AGC GGA AGA CCA AGG GCA CCA GTG GTG GGA GAG GAT GGT GAG AAT GGA GAG TCG TCC	815
2625	TGG CAG GTG GCT TTG ACT TAT TTT TTG AAT CAA TAT CTA TGT GGA GCT GGT TTA ATC GCA ACC CAG TGG GTT CTT GCA CCA	842
2706	GTC CAT TGT GTT ACC AAC ATT GTT GGT TCC GGA GAT GCT ACT TAC GTG CCG GTT GGT GAT TAC GAT CTA ACC CCG AAA TAC	869
2787	GGC AGT CCA GGA ACA CAG ACT TTG CCG GTG GCG ACC ACC TAC ATT CAT CAC AAC CAC AAT AGC CAG ACT CTG GAC AAC GAT	896
2868	ATT GCT CTC CTG AAG CTC CAT GGT CAA CCG GAG CTT CCA GAT GGA GTG TCG TTG GTT CTT CTT CCA GGA GAG GTC AGC	923
2949	CAT GCG GCG GCG AAG CCA TGC ACG GTT ACC GGA TAC GAT ATG GGT GAA GCT GGT CCC ATT CCC CTG CCG GTG CCG GAG	950
3030	GCG GAA ATT CCG ATC GTG AGC GAT ACG GAG TGC ATC CCG AAG GTG AAC GCG GTG ACG GAG AAG ATC TTC ATC CTG CCC GCG	977
3111	TCC AGT TTC TGT GCG GCG GGA GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG GAG	1004
3192	GCG TTC TAC GAG CTG GCG GCG CTC GTC TCT TGG GGA TTC GCG TCG GCG CAG GAT GTG CCC GCG GTC TAT GTG AAG ACG	1031
3273	TCC TCG TTC ATC GCG TGG ATC AAC CAG ATC ATC AGC GTG AAT AAC TTA TAGTGAGGAA TCTGCAAG GACACACTGA CACCATGTGC	1047
3361	GGGAGGAACC CGGCTCTTGT GCGGATTTCC AGGACTGAGC ACTAGCCTGG TGTAGCTTAC GATTTTTC AGGGTTTCCC GCTCTTGAC TGTACGGA	
3461	ACTCCATCCT AGTGTATCTT ATTACTACTT AAACCTAACT CTAATGACTC CAAATCCAGA AAGCAACTAT ATTAACTATT ATGATTATCG GTAGCGTGA	
3561	CGGCAATCTT TAAAGTATT CCCCTATTGT ATACGCGCCT ATGGTAAAT GTTTTGTAT ATACAAAATA AATACAAAT CTTCAGTTAT AT	

Figure 4. The nucleotide and deduced amino acid sequence of *mas*. The nucleotide sequence of the full-length *mas* cDNA, excluding the run of A's at the 3' end, is shown along with the amino acid sequence of the longest deduced open reading frame starting with a methionine. The locations of the putative signal peptide (bold italics), signal peptidase cleavage site (arrowhead), N-glycosylation sites (broken lines), disulfide knotted domains (underline), introns (open arrowheads), and polyadenylation site (double underline) are shown. The amino acids occupying positions that in serine proteases correspond to the triad of residues responsible for catalysis (see text) are in bold and indicated with asterisks (*). There are several potential protein cleavage sites between residues 330 and 357.

The *mas* RNA expression pattern

The *mas* RNA expression pattern was assessed using the whole-mount in situ method (Tautz and Pfeifle 1989). The earliest expression of *mas* RNA is seen at stage 11 in small cells along the midline of the central nervous system (CNS) that persists until the end of stage 13 (Fig. 7b,c,e,f, fine arrow); we have not been able to identify

these cells. *mas* RNA expression is seen in the epidermis starting at stage 12 as lateral patches of cells (Fig. 7a–c, solid arrowhead); during stage 13, the number of expressing cells increases (Fig. 7d,f, solid arrowhead); from stage 14 onwards (Fig. 7g,j), the great majority of epidermal cells express *mas* RNA, although a few nonexpressing cells can be seen; the level of staining is highest at the anterior border of each segment (Fig. 7g,i, open arrow-

Figure 5. The main structural features of the mas protein. (a) The deduced mas protein is schematically represented; the approximate locations of the consensus signal peptidase cleavage site and the putative protein cleavage site (arrows), the five disulfide knotted domains (knotted symbol), and six putative N-glycosylation sites (Y symbol) are indicated. The region bearing homology to serine proteases is shown. The locations of the peptide sequence (amino acids 164–188) and the fusion protein (which includes amino acids 736–1047) used to generate antibodies against the mas protein are indicated. (b) The carboxy-terminal region of the predicted mas protein (amino acids 801–1047) aligned against four members of the trypsin serine protease superfamily. (CHY) Chymotrypsinogen (Smillie and Hartley 1966); (TRY) trypsin (Hermodson et al. 1973); (PLA) macaque plasminogen precursor (Tomlinson et al. 1989); (SNK) snake, a putative *Drosophila* serine protease (DeLotto and Spierer 1986). Generally conserved residues within the serine protease catalytic region are in boldface type. (*) Positions corresponding to the residues of the catalytic triad (note the substitution of glycine for the conserved serine in mas). (▽) Residues in the substrate binding pocket important in determining substrate specificity. The three pairs of cysteines forming disulfide bonds within the serine protease domain are joined.

b

MAS	ARV VGG EDGENGEWCWQVALINSLNQYL.....CGAALIGTQWVLTA AHC VTNIVRSGD
CHY	ARI VNG EDAVPGSWPWQVSLQDSTGFHF.....CGGSLISED VV TA AHC GVTTSDVVV
TRY	DKI VGG YTCAANSIPYQVSL.NSGSHF.....CGGSLINSQW VV SA AHCYKS
PLA	GRV VGG CVAYPHSWPWQISLRTRLGMHF.....CGGT LIS PE V LT AHC LEKSSRPS.
SNK	PLI VGG TPTRHGLFPMAALGWTQGGSGKDQDIKWCGGALVSELY V LT AHC ATSGANHRT

MAS	AIYVRVGDYDL..TR...KYGS...PGAQTLRVATTYIHHNHSQTLDND IALLKL HGQAE
CHY	AGEFDQGLETE.DTQVLKIGKVFKNPKFSIL.....TVRND I T LLKL ATPAQ
TRY	RIQVRLGEHNI.D..VLEGNE.....QFINAAKIIITHPNFNGNTLDND IMLIK LSSPAT
PLA	.FYKVLGAHR..EVHLE.....PHVQETEVSKMF.....SEPAR ADIAL L KL SSPAI
SNK	WFAWRPQLNETSATQQDIKILIIVLHPKYR.....SSAYYH DIAL L KL TRRVK

MAS	LRDGVCLVCLPARGVSHAAGKRCVT..GYGYMGE.AGPIPLRVREAE P IVSDTEC..IR
CHY	FSETVSAVCLPSADEDFPAGMLCATT...GWGKTYNALKTPDKLQATL P IVSNTDC..RK
TRY	LNSRVATVSLP.RSCA.AAGTECLIS...GWGNTKSSGSSYPSSLQCLKAPVLSDSSC...
PLA	ITDKV P ACLPSPNYVVDARTECFIT...GWGETQ..GTYGAGLLKEARL P VIENKVC..NR
SNK	FSEQVR P ACLVQCG.APHT...TVVAA G WGRTEFL.GAKSNALRQVLDLVSPQMTCKQI.

MAS	KVNAVTEKIFIL..PASS F CAGGEEGH.DAC QGD GGGP...LVCQDDGFYELAGL V SWGFG.
CHY	YWGSRTVDVMI.....CAGAS.GV.SSCMGDSGGP...LVCQKNGAWTLAG I VSWGSST
TRY	KSSY.PGQI...TGNMICVGFLEGGKDS CQGD SGGP...VVCNQ...LQGI V SWGYG.
PLA	..YEFNLGT..V..KTTEL C AGHLAGGTDSC CQGD SGGP...LVCPEKDKYIL Q GVTSWGLG.
SNK	YRKERRLPRIIE.GQ..FCAGYLQAQ.GH CQGD SGGP I HALLPEYNCVAFVVG I TSFGKF.

MAS	CGRQDV PGV YVKTSSFIGWINQIISVNNL
CHY	C.STST P AVYARVTALMPWQETLAAN
TRY	CAQKN K PGVYTKVCNYYNWIQQTIAAN
PLA	CARP NK PGVYVRVSRFVTWIEGVMRNN
SNK	CAAP N AP PGV YTRLYSYLDWIEKIAFKQH

head). *mas* RNA is also detected in the cells of the tracheal system from stage 14–17 (Fig. 7k,l, thick arrow).

mas protein expression

We probed embryos with antibodies that recognize the amino-terminal and carboxy-terminal regions of the mas protein (see Fig. 5a; Materials and methods). The expression patterns reveal interesting aspects of the differential localization of the two processed polypeptides. The amino-terminal antibody labels epidermal cells and the membrane of cells of the tracheal system. Staining in the epidermal cell membrane is first detected at stage 14 and increases in intensity until stage 17 (a stage 16 embryo is shown in Fig. 8a); in the older embryos, the strongest

staining is associated with the denticles (Fig. 8b, arrow-head). There is an apparent lag between the times at which the *mas* RNA (stage 12) and protein expression (stage 14) are first detected in the epidermis. Staining in the tracheal system can be seen from stage 14–17 (Fig. 8c,d); the staining appears to be primarily in the cells lining the tracheal lumen (see Fig. 8d, arrow).

The pattern of expression seen with the anti-fusion protein antibody (see Fig. 5a; Materials and methods), which recognizes the carboxy-terminal 77-kD polypeptide on Western blots, is shown in Figure 8, g–l. The most intense staining is seen in the attachment sites of somatic embryonic muscles late in embryogenesis, starting in early stage 16 (Fig. 8h, fine arrow; Fig. 8l, long arrow) and persisting until the beginning of pupation

Figure 6. The mas protein is processed to give two cleavage products. Western blot analyses. (a) 12- to 24-hr wild-type embryonic extracts with mas carboxy-terminal fusion antibody (lane 1), preimmune serum prior to the production of carboxy-terminal antibody (lane 2), amino-terminal peptide antibody (lane 3), and preimmune serum prior to the production of the amino-terminal antibody (lane 4). (b,c) A transgenic line was established in which the *mas* cDNA was placed under the control of the *hsp70* promoter (*hs-mas*). Embryos (2–5 hr) were given a 1-hr heat pulse and allowed to recover for 2 hr (lanes HS); the embryonic extracts were compared with the extracts of 5- to 8-hr uninduced *hs-mas* (lanes C) by probing with anti-carboxy-terminal antibody (b) and anti-amino-terminal antibody (c).

(data not shown). Whole-mount in situ hybridization does not detect *mas* RNA in muscle (see previous section), suggesting that the carboxy-terminal moiety recognized by the fusion antibody is secreted into the intercellular region of the attachment site by the epidermal cells. Further evidence for the secretion of the carboxy-terminal polypeptide is provided by its localization in the tracheal lumen (Fig. 8g,j, thick arrow), which is clearly extracellular. In the CNS, *mas* RNA is found in some unidentified cells in the midline (see previous section), whereas the carboxy-terminal antibody not only stains these cells (Fig. 8i, open arrowhead) but also the axons of the longitudinal connectives (Fig. 8g,h,k, solid arrowhead), again consistent with the carboxy-terminal polypeptide being secreted.

Discussion

We argue that the muscle phenotype observed in animals null for *mas* function is attributable to a decrease in cell–matrix adhesion. Sequence analysis and biochemical data suggest that mas is produced as a preproprotein that is processed to release an extracellular carboxy-terminal polypeptide. The correlation of its distribution and the loss-of-function phenotype suggests that the carboxy-terminal polypeptide is the functional portion of mas. The mas protein, as a whole, contains many structural features found in serine proteases; however, the catalytic serine residue is replaced by a glycine, making it unlikely to possess enzymatic activity. We suggest two mutually nonexclusive models for how mas might act to stabilize cell–matrix interactions. In the first model, we propose that mas acts as a novel substrate adhesion molecule and directly promotes cell–substrate adhesion. In this scenario, the conserved serine protease motif, which is normally employed to facilitate protease–substrate interactions, has been adapted in mas for a similar role in mediating protein–protein interactions. An alternative model would be that mas acts to counteract the effects of serine proteases (which can affect

cellular morphology either directly, by cleavage of proteins involved with the anchoring of cell surface to the substratum, or indirectly through the activation of cell surface receptors). In this scenario, we propose that mas competes for serine protease substrates and behaves, effectively, as a competitive serine protease antagonist to indirectly stabilize cell–substrate interactions.

Loss-of-function muscle phenotype suggests a role for mas in the maintenance of cell–matrix interactions

The observation that the formation of the somatic muscle attachments occurs prior to the accumulation of the carboxy-terminal polypeptide of mas suggests that this molecule plays a role in the maintenance of muscle–epidermal adherence. The phenotype of the null alleles further substantiates this view because the mutant animals show detachment of the somatic muscles (stage 16 and later) around the time of the first muscular contractions. Ultrastructural studies of somatic muscle attachment sites indicate that there are matrix components lying between the adhering epidermal and mesodermal structures. It is thought that the binding of the PS integrins, found on the apposed cells, to sites on the matrix would facilitate the formation of the attachment site. The defects in muscle attachments seen in the *mas* mutants suggest a role for *mas* in stabilizing this type of cell–matrix binding. As would be expected for this proposed role, the effect of the absence of mas function is general to all somatic muscles; moreover, the incomplete expressivity of the phenotype can be explained by the presence of other ECM molecules that can partially compensate for the absence of mas.

The defect caused by mas loss of function coincides with the localization of the carboxy-terminal modified protease domain of mas at muscle attachment sites

The carboxy-terminal polypeptide of mas appears to be the functional portion of the mas protein; its localization

Figure 7. (See facing page for legend.)

to all somatic muscle attachment sites coincides with the defects observed in the *mas* mutant animals. Consistent with this notion, the mutant allele *mas*¹⁵⁴, which removes only the carboxy-terminal portion of *mas*, is phenotypically a null allele. Several lines of evidence indicate that the carboxy-terminal polypeptide has an extracellular location. First, the primary sequence of the *mas* protein as well as *in vitro* translation results (see next section) suggest that the entire protein is secreted. Second, the localization of the carboxy-terminal protein in the tracheal lumen is clearly extracellular. Third, its localization is not what might have been expected from the *mas* RNA expression pattern, implying that there must be some mechanism by which the carboxy-terminal polypeptide is able to reach its functional sites. It is possible that the localization of the carboxy-terminal polypeptide is receptor mediated. In contrast to the carboxy-terminal polypeptide, there is no indication that the amino-terminal polypeptide plays any functional role other than to promote the processing and trafficking of the carboxy-terminal modified serine protease domain. Epidermal cells (which accumulate the amino-terminal polypeptide) do not appear to be affected in mutants that remove the entire *mas* coding region.

Implications of the structural features of *mas*

As one might expect from the *mas* protein localization data, the structural features of the deduced protein suggest that *mas* is synthesized as a preproprotein; the presence of the amino-terminal signal peptide and the absence of a hydrophobic stretch of sufficient length to traverse the membrane suggest that *mas* is secreted. Consistent with this scenario, preliminary data indicate that *mas* is translocated into the membrane fraction and protected from protease cleavage when a T7 RNA polymerase transcribed full-length *mas* mRNA is translated *in vitro* in the presence of canine pancreatic microsomes (A. Kornezos, pers. comm.).

Both the processing of the *mas* protein and its primary structure share several common features with serine proteases. It is processed; however, our Western blot data show that *mas* is cleaved, not at the expected zymogen activation site between residues 802 and 803, but nearer the amino terminus (see Fig. 5a and legend) to generate two polypeptides of 35 kD (amino-terminal) and 77 kD (carboxy-terminal). Like other arthropod serine protease proproteins, for example, *Drosophila* easter and snake proteins and the proclotting enzyme of *Limulus*, *mas* also contains disulfide knotted domains. These are thought to mediate complex formation as a prerequisite

to zymogen activation (Smith et al. 1994). It is possible that one of these knots in *mas* serves in this capacity.

By analogy with serine proteases, the cleavage of *mas* proprotein facilitates the folding of the *mas* carboxy-terminal-modified protease domain into its final functional conformation. This domain is located extracellularly and, presumably, can be localized to its site of action by binding to specific receptors. Because the catalytic serine has been replaced by a glycine in *mas*, it probably cannot act as a protease; however, the high degree of overall conservation with the protease domains of other serine proteases, that is, the cysteine residues involved in forming disulfide bridges and the residues lining the substrate binding pocket, raises the possibility that the *mas* carboxy-terminal domain can adopt a similar conformation to that of serine proteases.

Possible mechanisms of action of *mas*

How does the extracellular carboxy-terminal-modified protease domain of *mas* act to promote or stabilize cell-matrix interactions? One possibility is that it acts as a novel substrate adhesion molecule/ligand to directly promote or to signal cell-substrate adhesion mechanisms. In this scenario, we propose that the conserved serine protease domain that is normally used to facilitate enzyme-substrate interactions has been adapted (in an enzymatically inactive form) in *mas* for a similar role in mediating protein-protein interaction.

This biological strategy appears to have been used in several growth factors and adhesion molecules. The human hepatocyte growth factor (HGF), the *Drosophila* adhesion molecules glutactin and neurotactin, as well as vertebrate thyroglobulin, are some examples of molecules that show homology to enzymes except that they lack critical catalytic groups. Like *mas*, HGF is structurally similar to serine proteases but has alterations in 2 of the 3 residues in the catalytic triad (Nakamura et al. 1989). It has been suggested that the modified protease domain acts as a ligand to activate *c-met* tyrosine kinase activity (Bottaro et al. 1991). The adhesion molecules glutactin and neurotactin both belong to the family of serine esterases (Barthalay et al. 1990; de la Escalera et al. 1990; Hortsch et al. 1990; Olson et al. 1990). In these proteins the catalytic serine is not conserved, and it is unlikely that the molecules possess esterase activity. The conserved regions may be responsible for the adhesive properties of neurotactin and glutactin. Vertebrate thyroglobulin also contains the defective cholinesterase domain lacking the catalytic serine and may be involved

Figure 7. The *mas* embryonic RNA expression pattern. Whole-mount embryos were hybridized to digoxigenin-labeled *mas* cDNA (see Materials and methods). Lateral (L) and ventral (V) views of stage 12 (a–c), stage 13 (d–f), stage 14 (g–i), and stage 15 (j–l) embryos are shown, with anterior at left. a, d, g, and j focus on the progression of epidermal expression of *mas*; note the stronger expression in cells near the segmental boundaries (g and i, open arrowhead). b, e, and h focus on the progression of *mas* expression in a few cells along the midline of the CNS. *mas* is expressed in these midline cells during stages 12 and 13 (b and e, fine arrow), but midline expression ends by stage 14 (h). Higher magnifications of b and e are shown in c and f, respectively. k and l focus on the expression seen in the tracheal system (thick arrow); higher magnification of an older stage 15 embryo is shown in l. Bars (k,l), 25 μ m.

in the dimerization of cholinesterases and thyroglobulin (Mercken et al. 1985).

An alternative mechanism, which is not necessarily

excluded and which may work in conjunction with the model proposed previously, is that mas stabilizes adhesive interactions between the cell surface and extracel-

Figure 8. (See facing page for legend.)

lular components by acting as a protease antagonist. Given the predicted structural similarities between mas and serine proteases, it is certainly conceivable that the carboxy-terminal domain of mas can compete for the substrates of trypsin-like serine proteases. In this scenario, mas would act to antagonize the activity of serine protease (e.g., molecules like the membrane-associated serine protease encoded by *Sb-sbd*), and its absence would result in an excess of protease activity at a specific locale; an imbalance in favor of protease activity can potentially destabilize cell–substrate interaction in at least two different ways. First, destabilization can be caused by the excessive local proteolytic degradation of components of the ECM as well as receptors for matrix molecules on the cell surface (e.g., Monard 1988). A second possible mechanism for destabilization of cell–substrate interaction could be effected by protease-mediated activation of a signal transduction pathway; for example, the activity of the protease thrombin causes the activation of cell surface receptors (Vu et al. 1991), this being the apparent reason why thrombin causes neurite retraction in culture (Suidan et al. 1992).

The issue of mechanism aside, it is interesting to note that the defective muscle attachment seen in *mas* mutants occurs with incomplete expressivity. We believe that this is a reflection of the fact that many components are involved in effecting muscle attachment; the function of the components overlap, and the loss of some of the components can be compensated by those that remain. There are examples of mutations that abolish substrate and cell adhesion molecule function that do not cause obvious phenotypes as a result of the high degree of redundancy. In this respect, the embryonic defects observed for the *mas* null alleles are unusual and implicate mas as a functional and limiting extracellular component in the process of muscle attachment.

Materials and methods

All standard molecular biology procedures were performed according to Sambrook et al. (1989).

Strains

The $P[w^+ ry^+]$ 64B insertion strain was kindly provided by Bob Levis (Levis et al. 1985). The original strain contained an unlinked lethal that was recombined out by constructing a homozygous $w; P[w^+ ry^+]$ 64B *h th cu sr e* strain. This strain was used in all subsequent experiments. The *Mhc-lacZ* transformant line was a gift from S. Bernstein (Hess et al. 1989). Details of balancer and marker strains are listed in Lindsley and Zimm (1992).

Isolation of *mas* genomic DNAs and cDNAs

An EMBL4 genomic library of *SalI* fragments was prepared from the *mas* P-element insertion line. Clones containing DNA flanking the P element were isolated using the 5' end of the P element as a probe. The flanking DNA was used to screen a wild-type genomic EMBL3 library (Tamkun et al. 1992), and ~20 kb of DNA encompassing the insertion site was isolated and restriction mapped. The wild-type genomic clones were used as probes for Northern blots and to screen embryonic cDNA libraries (Poole et al. 1985; Brown and Kafatos 1988).

Northern blot analysis

Poly(A)⁺ RNA was prepared using standard methods. Two micrograms of poly(A)⁺ RNA from each developmental stage were separated on formaldehyde/agarose gels, transferred to nylon membranes, hybridized, and exposed as described in Sambrook et al. (1989).

DNA sequencing

The cDNA was subcloned into Bluescript(+) (Stratagene), and nested deletions were prepared by the exonuclease III Erase-a-base system (Promega). Both strands of the cDNA were sequenced by the dideoxy chain-termination method with Sequenase (U.S. Biochemical). Genomic DNA was sequenced using oligonucleotide primers based on the cDNA sequence. Protein homology searches were carried out using the data bases at the National Center of Biotechnology Information, National Institutes of Health (NIH) with the suite of BLAST programs.

Whole-mount embryo RNA in situ hybridizations

Staging of the embryos was according to Campos-Ortega and Hartenstein (1985). Localization of RNA in whole-mount em-

Figure 8. The differential localizations of the carboxy- and amino-terminal portions of the mas protein. (a–d) Stage 16 embryo whole mounts stained with an antibody directed against a peptide corresponding to an amino-terminal region of mas (see Fig. 5a), viewed at different focal planes; anterior is at left and dorsal is on top. (a) Staining is seen in the epidermis in a pattern that corresponds with the denticle belts (for higher magnification, see b, arrowhead). (c) Staining can be seen in the tracheal system primarily along the luminal surface (for higher magnification, see d, short arrow). (e,f) Stage 16 *mas* null embryo whole mounts double-stained with antibody against the amino-terminal mas peptide and anti-β-galactosidase antibody. Epidermal (e, arrowhead) and tracheal (f, short arrow) staining are absent in the *mas* null embryos. (g–l) Filleted embryos stained with antifusion protein antibody directed against the carboxy-terminal modified serine protease domain of mas (see Fig. 5a). (g) Flattened preparation of a stage 15 embryo opened along the dorsal midline; accumulation of the carboxy-terminal portion of the mas protein can be seen in the tracheal lumen (thick arrow) [higher magnification of the tracheal protein localization is shown in j (thick arrow)] and along the axons (solid arrowhead) of the longitudinal connectives [higher magnification shown in k (solid arrowhead)]. (h) A stage 16 embryo opened along the dorsal midline; note the high levels of staining seen at all somatic muscle attachment sites (fine arrow) that were not present at stage 15 [higher magnification of the lateral and ventral muscle attachment site staining is shown in l, long arrow]; the axons of the longitudinal connectives (arrowhead) and the tracheal lumen (below the plane of focus) are stained. (i) Dorsal view of a dissected stage 13 embryonic CNS; staining can be seen in some unidentified midline cells in the CNS (open arrowhead). (m–o) Stage 16 *mas* null embryo whole mounts double-stained with anti-fusion protein antibody and anti-β-galactosidase antibody. (m) High magnification of the tracheal lumen (thick arrow) shows no staining with anti-fusion protein antibody. (n) The axons of the longitudinal connective of the CNS (arrowhead) showing the absence of the carboxy-terminal portion of mas. (o) The attachment sites of the lateral and ventral muscles show no accumulation of mas carboxy-terminal protein (long arrow). Bars (c,d,g,h,i), 25 μm.

bryos was performed essentially as described by Tautz and Pfeifle (1989) with a modification (Yang et al. 1991), using random-primed, digoxigenin-labeled cDNA probes (Boehringer Mannheim). Antidigoxigenin–HRP (horse radish peroxidase) antibody was used to detect the hybridized probe. Development was with 0.25 mg/ml of diaminobenzidine, 2 mg/ml of D-glucose and 4 ng/ml of glucose oxidase as described (Hsu et al. 1988).

Generation of *mas* alleles by P-element mobilization

To generate mutations in *mas*, the P-element inserted 3' of *mas* was mobilized, as described previously (Robertson et al. 1988), to try and generate transpositions and imprecise excisions that affect the *mas*-coding region. Females homozygous for the P-element insertions were crossed to males carrying the *Sb* P[Δ2-3][99B] element. Progeny jump-starter males containing the P element over *Sb* P[Δ2-3][99B] were mated to *w/w*; *TM3 Ser/TM6 Tb* females. From each of 250 independent crosses set up using single jump-starter males, individual balanced lines were established from one *Sb*⁺ white-eyed male (representing an excision chromosome) and two *Sb*⁺ red-eyed males (representing unselected chromosomes that still retain *w*⁺). Altogether, lines representing 250 independent excision chromosomes and 500 unselected *w*⁺ chromosomes were established. These lines were analyzed by Southern blotting and PCR. *mas*^{*x*76} was one of the excision chromosomes; *mas*^{*15a*} represents one of the unselected chromosomes.

Our analysis indicated that two of the lines carrying unselected *w*⁺ chromosomes appear to have resulted from duplicative transposition events with P elements flanking the *mas* transcription unit. For one of these double P-element insertion lines, we looked for the simultaneous excision of both elements using the strategy described by Cooley et al. (1990). *mas*^{*x*124} represents one of the chromosomes resulting from such a double excision event.

Generation of antibodies

For expression of the carboxy-terminal *mas* fusion protein, the 1.27-kb *Bam*HI–*Eco*RI fragment from the cDNA clone that includes amino acid residues 736–1047 was cloned into a modified pGEX vector (Guan and Dixon 1991). Using standard protocols, fusion proteins electroeluted from SDS-PAGE gel slices were used to immunize rabbits. The peptide CKKPSTTK-KVATAKPKDKEEATKADD, which corresponds to residues 164–188 of *mas*, was coupled to bovine serum albumin (BSA) via the amino-terminal cysteine, not present in *mas*, using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Sigma) (Harlow and Lane 1988). Two-hundred milligrams of the fusion protein and 100 mg of the conjugated peptide were emulsified 1:1 with Freund's complete adjuvant (BRL) and injected intradermally into female rabbits. The rabbits were boosted every 3 weeks and bled 10 days later. The peptide antibody was affinity purified using Pierce's Sulfolink coupling gel. The fusion protein antibody was purified using the low-pH method described in Sambrook et al. (1989).

Whole-mount staining of embryos with antibodies

The whole-mount staining protocol of Mitchison and Sedat (1983) was used with modifications by Gould et al. (1990) and Rob White (pers. comm.). Briefly, embryos were fixed for 40 min in heptane/paraformaldehyde, devitellinized, and washed extensively in PBS containing 0.1% Triton X-100 (PTX). They were blocked with 0.1% BSA in PTX (PBTX) and incubated overnight with primary antibody. Secondary antibodies conju-

gated to biotin and the avidin–biotin complex (ABC Kit, Vector Lab) were used, and the staining was developed as described by Hsu et al. (1988). Unless stated otherwise, mutant alleles were balanced over “blue balancer” chromosomes that carried an insertion containing *Ubx-lacZ* to facilitate the identification of homozygous mutant embryos. Antibodies directed against bacterial β-galactosidase were obtained from Sigma, and the anti-MHC antibody was a kind gift from Dan Kiehart (Kiehart and Feghali 1986). Activity staining was performed according to Ashburner (1989). Embryos were observed using Nomarski optics and staged according to Campos-Ortega and Hartenstein (1985).

Western blot analysis

Homogenates were prepared in 2× SDS gel-loading buffer (Sambrook et al. 1989), with 1 mM PMSF, and electrophoresed on a 10% SDS–polyacrylamide gel. The transfer to a nitrocellulose filter (Amersham's Hybond-C Extra) was performed using transfer buffer (pH 8.3) (Sambrook et al. 1989). The filter was preincubated for 1 hr at room temperature with PBS containing 1% BSA and 0.1% Tween 20. Primary antibody was added to the appropriate dilution followed by an overnight incubation at 4°C. The filter was washed, incubated with peroxidase-conjugated secondary antibody for 1 hr, and, after washing, developed using the BM Chemiluminescence Western blotting reagents (Boehringer Mannheim).

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Note added in proof

The nucleic acid sequence of *mas* has been deposited to the GenBank data library under accession number U18130.

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