mab-7 encodes a novel transmembrane protein that orchestrates sensory ray morphogenesis in C. elegans

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Received for publication 20 January 2007; revised 8 September 2007; accepted 18 September 2007
Available online 23 October 2007

Abstract

The tapered sensory rays of the male Caenorhabditis elegans are important for successful male/hermaphrodite copulation. A group of ram (ray morphology abnormal) genes encoding modifying enzymes and transmembrane protein have been reported as key regulators controlling ray morphogenesis. Here we report the characterization of another component essential for this morphogenetic process encoded by mab-7. This gene is active in the hypodermis, structural cells, the body seam and several head neurons. It encodes a novel protein with a hydrophobic region at the N-terminus, an EGF-like motif, an ShKT motif and a long C-terminal tail. All these domains are shown to be critical to MAB-7 activity except the EGF-like domain, which appears to be regulatory and dispensable. MAB-7 is shown to be a type II membrane protein, tethered on the cell surface by the N-terminal transmembrane domain with the remainder of the protein exposed to the extracellular matrix. Since ectopic mab-7 expression in any ray cell or even in touch neurons of non-ray lineage can rescue the mutant phenotype, mab-7 is probably acting non-autonomously. It may facilitate intercellular communication among ray cells to augment normal ray morphogenesis.

Keywords: Nematode; Sensory organ; Morphogenesis; Transmembrane protein

Introduction

Tissue and organ morphogenesis involve intricate cell–cell communication and genetic regulation (Bradley et al., 2001; Dale, 1998). Morphogenesis proceeds along a well-defined path to establish the normal structure and biological function of an organ. For instance, the formation of the salivary gland in Drosophila begins with the formation of an organ primordium; duct morphogenesis is initiated by localized action of a secreted growth factor among a few cells and ends with the translocation of specific transcription factors into the target cell nuclei for gene activation (Bradley et al., 2001; Myat and Andrew, 2000). Growth factors such as the hepatocyte growth factor (HGF) also stimulate branching morphogenesis in the fetal lung of the rat (Ohmichi et al., 1998). The binding of HGF to its receptor activates both the PI3K and ERK/MAP kinase pathways to induce branching in vitro (Rosario and Birchmeir, 2003), whereas the TGF-β1 antagonizes this event (Serra et al., 1994). Hence, interplay of non-autonomous signals controls cellular differentiation to shape tissues and integrate them into functional structures.

Taking advantage of the simple cellular organization of the sensory rays in the Caenorhabditis elegans male tail, the genetic requirement for organ morphogenesis was characterized in this study. The male tail is dramatically modified during late larval stages (Baird and Emmons, 1990) so that an adult male tail develops nine pairs of bilaterally organized sensory rays embedded in a cuticular fan. These sex-specific structures are essential for the contact response between the male and hermaphrodite during mating (Liu and Sternberg, 1995). Each sensory ray is composed of two neurons (RnA and RnB) held by a structural cell (Rnst) in the distal region and is enclosed by a hypodermal sheath (Sulston et al., 1980). Sensory ray morphogenesis starts at the L4 larval stage when all sensory ray components are born. Retraction of the primordial tail tip...
follows. Cells R1.p to R5.p fuse to form the tail seam (SET), while cells R6.p to R9.p fuse with ventral hyp7 (Sulston et al., 1980). During retraction, each structural cell attaches to the cuticular surface, forming a dot-and-ring-like papillus. The anterior and dorsal migration of ray cell bodies and hypodermis away from the papillus results in ray extension, including the enclosure of each ray by a thin hypodermal layer (Baird et al., 1991).

The genes required for establishing normal ray morphology include a group of ram genes (Baird and Emmons, 1990). The ram-1, ram-2, ram-4 genes encode cuticular collagen genes which generate a unique extracellular matrix in the male tail (Tam et al., unpublished data; Yu et al., unpublished data). ram-5 encodes a novel transmembrane protein residing on the structural cell surface to facilitate cellular communication (Yu et al., 2000).

Mutations in these latter three genes result in amorphous rays, possibly due to altered matrix architecture without proper post-translational modifications. Nevertheless, how and where these molecules interact during ray morphogenesis remains unclear.

_mab-7(e1599)_ is another gene with a key role in this organ morphogenesis process. Mutant _mab-7_ males have swollen sensory rays. Its hermaphrodites appear slightly dumpy but otherwise lack an obvious phenotype (Link et al., 1988; Hodgkin, 1983). Increased display of cell surface glycoproteins has been visualized by the fluorescence-labeled wheat germ agglutinin (WGA) staining of male tails in _mab-7(e1599)_ adults and in several other _ram_ mutants, suggesting the presence of a common cuticular defect among them (Ko and Chow, 2000; Link et al., 1988). In this study, _mab-7_ was shown to encode a novel protein with an EGF-like domain and a ShKT domain. Through various expression analyses, and structural and topological characterization, cellular defects in _mab-7_ mutant sensory rays were defined. A potential functional mechanism for this molecule was uncovered based on its product localization and cellular requirement during morphogenesis of the male tail.

**Materials and methods culture**

The _C. elegans_ strains were maintained through standard laboratory procedures (Brenner, 1974). All strains used in this study carried the _him-5(e1490)_ mutation, which increases the incidence of males (Hodgkin et al., 1978). Other genetic strains used in this study included CB4088: _him-5(e1490)_, KC64: _him-5_ (e1490)_; _mab-7_(e1599)_; KC69: _wsx29(pRF4+pKS+T2425phIGFPNL5-);_hin-5_(e1490)_; KC160: _wsx29(pRF4+pKS+T2425phIGFPNL5-)_; _hin-5_(e1490)_; _mab-7_(e1599)_; KC182: _him-5(e1490)_; and _mab-7_(e1599)_; _mab-7_(e1599)_; KC64: _rfj-3(p1426j);_hin-5_ (e1490)_; KC465: _hin-5(e1490)_; _mab-7_ (wsx75)_; KC466: _hin-5(e1490)_; _mab-7_(wsx74)_.

**Phenotype characterization of _mab-7_**

The body length and ray shape of the _mab-7_(e1599) were examined by Nomarski microscopy. The body length of over twenty young adult hermaphrodites and males was measured using a micrometer, and the average was calculated. The ray-swelling phenotype was assessed using a Ram index, a measurement of average Ram severity, with “0” indicating the wild type and “5” representing the most variant phenotype (Ko and Chow, 2002). Thirty males of each allele were scored and the values were averaged to obtain a semi-quantitative estimation of the phenotype severity in a mutant population. The mating efficiency of _mab-7_ was measured by scoring the cross progeny of _unc-24(e138)_ hermaphrodites sired by the _mab-7_ males against those sired by wild-type males. Five L4 males were mated with three _unc-24_ L4 hermaphrodites for 2 days. These males and hermaphrodites were removed, and the cross progeny were scored 1 day later.

Any cellular defects in the ray components were investigated using three tissue-specific _gfp_ markers: _ram-5;_ _gfp_ (structural cells) (Yu et al., 2000); _pkd-2;_ _gfp1_ (neuronal B cells) (Barr and Sternberg, 1999); and _dpy-13;_ _gfp_ (hypodermis) (Ko and Chow, 2002). A GFP marker, _gfp-lgg-1_, was used to identify autophagy in the male tails of _mab-7_ specimens (Melendez et al., 2003). The gross cellular morphology and subcellular features of male ray cells were examined under a fluorescent microscope in at least 3 transgenic lines carrying an extrachromosomal array of the reporter markers. The scores were combined and reported.

**Electron microscopy**

Fixation, thin sectioning and TEM were performed using standard procedures (Hall, 1995). Healthy adult _mab-7_ and wild-type males were washed with M9 buffer. These males were then fixed in 0.1 M HEPES buffer containing 2.5% glutaraldehyde and 1% formaldehyde for 90 min at room temperature. Their tail was then cut open with a razor blade to improve access. After buffer rinses, the samples were then re-fixed in 0.1 M HEPES with 1% osmium tetroxide and 0.5% KCN, for 60 min at room temperature, washed in buffer and stained _en bloc_ in 1% uranyl acetate with 0.1 M sodium acetate. After the fixation, the samples were mounted in 3% agarose, embedded in Scipoxy resin (Energy Beam Sciences) and heat cured. Thin sections were prepared transverse to the body axis, post-stained with uranyl acetate and lead citrate, and examined using a Philips CM10 electron microscope.

**Mutant rescue, mutation mapping and cDNA isolation**

Twenty-four overlapping cosmids spanning the region between _unc-1_ and _egl-17_ were chosen for the complementation rescue assay. These cosmids were co-injected with a _rol-6_ marker, _prF4_, in _mab-7(e1599)_ hermaphrodites as described (Mello et al., 1991). Deletion construct, _ZC13-SacIselFLL_ containing ZC13.1, ZC13.2, ZC13.3 and part of ZC13.4, was generated by _SacI_ digestion of the rescuing cosmid, followed by self-ligation. A 6.4-kb _MluI-MscI_ fragment carrying the putative _ZC13.4_ and _mab-7_ mutant rescue activity was subcloned into the EcoRV site of _pBluscript KS+_(pBS-KS+)._ to generate BS04. An EST clone, _sk70d5_ , corresponding to the incomplete _cDNA_ of _ZC13.4_ was identified. 5′ RACE analysis was performed as described in the manual of the 5′ RACE kit (version 2.0, cat. no. 18374-058, Gibco BRL, USA) to recover the 5′ end of the _cDNA_. The primers used in the 5′ RACE procedure were _KCS40; 5′-_ _GGA_ _TAT_ _AGC_ _ACA_ _GTT_ _GAC_ _G-3′_, and _KCS49; 5′-_ _GGT_ _TTG_ _AGT_ _GAG_ _AAT_ _TGT_ _GTG_ _G-3′_. Genomic DNA of _mab-7_ mutants was isolated, and _mab-7_ ORF was amplified with twelve specific primers for subsequent sequencing using a BigDye Terminal Sequencing Kit (cat. no. 4336911, Perkin Elmer) with the same primer sets to confirm the presence of molecular lesions.

**Construction of the expression clones**

The expression pattern of _mab-7_ was evaluated through a promoter- _gfp_ reporter assay. Promoters of 541 bp and 0.9 kb were used to drive the 5′ _gfp_ reporter and _mab-7_ cDNA expression in the wild-type and _mab-7_ mutant, respectively. The promoter fragments were amplified through a PCR directly from the _ZC13_ cosmid, and subcloned into a _pPD95.67NLS_ reporter plasmid. These two clones were used to examine the spatial and temporal expression of _mab-7_ in wild-type animals. Both the 5′_ rol-6_ marker _prF4_ and the _unc-119_ mutant rescuing marker _M142_ were tested in the injection experiment to ensure the expression pattern was not influenced by the markers. The efficiency of these promoters in rescuing _mab-7_ mutants was analyzed with the reporter gene replaced by _mab-7_ cDNA.
Construction of an ectopic expression transgene

The ectopic expression of *mab-7* is controlled by promoters derived from four different cell- or tissue-specific genes: *ram-4* (Yu and Chow, unpublished); *ram-5* (Yu et al., 2000); *sek-1* (K. Matsumoto, personal communication) and *mec-7* (Hamelin et al., 1992). The 1.2-kb HindIII–Apal *mab-7* cDNA fragment was subcloned into the HindIII–Apal site after the 1 kb *ram-4* promoter to generate *ram-4::mab-7*. The 3-kb SacI–SphI *ram-5* promoter was subcloned into the plasmid *mab-7*-7EcDNA, followed by *AflI*/*NfiI* self-ligation to obtain *pram-5:* *mab-7* with only 2 kb of *ram-5* promoter. The 2 kb HindIII–HindIII *sek-1* promoter treated with T4 DNA polymerase was subcloned into the Smal site of the plasmid *pma-7*-7EcDNA to generate *psek-1::mab-7*. The 1.2-kb HindIII–Acc65I *mab-7* cDNA treated with T4 DNA polymerase was subcloned into the EcoRV site of pdPD9.41 carrying the *mec-7* promoter to obtain pmec-7:*mab-7*.

Domain characterization of MAB-7 protein

All constructs used in the domain analyses harbored the 0.9-kb *mab-7* promoter, which was sufficient to drive *mab-7* cDNA to complement the mutant phenotype. Enzyme sites were generated by site-directed mutagenesis on the promoter, which was sufficient to drive *ram-5* coding sequence. Thus, *MAB-7* EcoI–Ecl136I sequence. (Hamelin et al., 1992) Ecl136I–EcoI site was introduced. Similarly, the ShKT domain in *MAB-7* was removed by site-directed mutagenesis. In parallel, the DNA sequence encoding the HR of *MAB-7* was also generated in front of the EGF-like domain coding region linked with *sek-1* promoter treated with T4 DNA polymerase was subcloned into the plasmid *pGFP*–*dIII* site of the plasmid *pPD9.41* carrying the *sek-1* promoter, followed by T4 DNA polymerase was subcloned into the EcoRV site of pdPD9.41 carrying the *mec-7* promoter to obtain pmec-7:*mab-7*.

RNA interference of *mab-7*

The *mab-7* RNAi plasmid was derived from *mab-7* cDNA (1–497 nt) subcloned into the pPD129.36 and was maintained in *Escherichia coli HT115* (DE3). Single colonies were picked and cultured in 10 ml of LB medium with 150 μg/ml ampicillin and 12.5 μg/ml tetracycline overnight at 37 °C. 100 μl of each overnight culture was then added to 10 ml LB with 150 μg/ml ampicillin and 12.5 μg/ml tetracycline and grown at 37 °C for another 8 h. IPTG was added to a final concentration of 1 mM. The bacteria were seeded onto worm plates with 150 μg/ml of ampicillin and 1 mM IPTG, dried at room temperature overnight and seeded with synchronized L1 *KC442: rfy-3*( T426); *him-5*(e1490) mutant animals. The male tail phenotype was examined 2 days later.

Results

**mab-7** is required for multiple biological functions

Three *mab-7* alleles, *e1599*, *wx74* and *wx75*, were isolated in two separate EMS screens (Hodgkin, 1983; this study). While these mutant animals shared similar phenotypes, most of the characterization was conducted with the reference allele, *e1599* (Hodgkin, 1983). The *mab-7*(e1599) mutants produce slightly dumpy hermaphrodites and adult males, and the males had swollen bursal rays (Link et al., 1988; Hodgkin, 1983). Because of the similarity between this swollen ray phenotype and *ram* mutants, this mutant was characterized in more detail. In mating assays, all three *mab-7* males consistently had an average of fewer than 5 cross progeny, a mating efficiency much lower than that of wild-type males (Table 1). The brood sizes of selfing mutant hermaphrodites were also lower (Table 1). The body lengths of both sexes in the *mab-7* mutant populations were slightly shorter than their wild-type counterparts, except for *mab-7*(wx75) (Table 1). In the male tail, the localized swollen features in the proximal part of each ray were the same among all

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<th>Phenotypic characterization of <em>mab-7</em> mutants</th>
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<td><strong>Brood size by hermaphrodite selfing (N)</strong></td>
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* *p Value<0.001; * *p Value<0.05 in comparison with results from wild-type animals.


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three mab-7 alleles (Fig. 1E) and were distinct from other ram mutants, who showed swelling evenly distributed along their entire rays. To have an objective semi-quantitative measurement of the ray swelling in mab-7 mutants, the Ram Index first introduced by Ko and Chow (2002) was adopted for comparison. In most mab-7 males, individual rays were not distinguishable at the base because of the severe swelling, and all three mutant alleles had an average Ram index of 4, indicative of having severe swelling.

Sensory ray components are defective in mab-7(e1599) males

To visualize the type-specific cell defects associated with each lumpy ray, three ray-cell-specific gfp markers were introduced into the mab-7 mutants for comparison with wild-type features. The hypodermal sheath of the sensory rays in wild-type males (marked by dpy-13∷gfp, Fig. 1B) was smooth and thin, but it was thicker and grossly irregular in shape in mab-7 males (Fig. 1F). Swelling was also noted in the mutant structural cells (marked by ram-5∷gfp, Fig. 1G) as compared to their smooth, uniform shape in wild-type animals (Fig. 1C). In addition, nodule-like structures along the neuronal processes (marked by pkd-2∷gfp, Fig. 1H) were occasionally observed in mab-7 mutants but were absent in wild-type worms (Fig. 1D).

To obtain a better resolution of the abnormality, the mutants were further analyzed in thin sections using TEM. The overall conformation of the male tail tip was observed to be variably disrupted by pronounced swelling of tissues at the base of the fan. In some animals there was a failure of retraction evident for groups of neuronal soma, which were trapped too far posteriorly (Figs. 1I and J). These trapped cell bodies displayed abnormally electron-dense cytoplasm, perhaps due to injury. More often, the base of the fan exhibited abundant acellular debris within the fan cuticle (Fig. 1I). This material probably represents an inefficient retraction of tail tip materials (hypodermis) from the primordial leptodarian tail tip during late L4 morphogenesis (Nguyen et al., 1999).

The rays of mab-7 males were virtually all malformed. Some were very short or failed to extend beyond papilla stage. They were distinctly swollen at the base, but could form a smoother ray more distally. Ray tips and ray openings were usually well formed. Ray neurons A and B had normal ciliated tips, and the most distal portion of the structural cell and hypodermis formed a normal ray opening, even in rays or papillae which were otherwise poorly established. In general, neuronal processes appeared normal at the level of resolution by TEM. On the other hand, the base of the ray was always associated with cytoplasmic swelling confined to the hypodermis and structural cells. The swollen tissue was observed to contain many of the membrane components, an autophagy reporter (GFP∷LGG-1) was adopted for visualization of the type-specific cell defects associated with each ray phenotype into the wild type. Four putative genes were predicted in ZC13 (Fig. 2). Since the deletion clone ZC13-Sa–clslf, with the first three putative genes and a part of the fourth, could not rescue mab-7 mutants, and BSG4 harboring only ZC13.4 showed a strong rescue activity, it was concluded that mab-7 is ZC13.4 (Fig. 2).

To further confirm this mab-7 identity, the full length of ZC13.4 cDNA was tested in complementation rescue and RNAi experiments. A full length of ZC13.4 cDNA was obtained by 5′ RACE analysis to recover the first exon and redefine the authentic transcription start site, which was different from the previous Wormbase annotation. Introducing this cDNA driven by the 0.9-kb mab-7 promoter into mab-7 mutant animals resulted in a wild-type phenotype in all the male progeny (100%, N=101). RNAi experiments performed with this same cDNA fragment in the RNAi-sensitive mutant strain rrf-3∷him-5 strain generated progeny with more than 5% of which having the typical Mab-7 phenotype (N>150). Both results confirmed that mab-7 is indeed ZC13.4. In addition, when mutant alleles were subjected to this same RNAi treatment, similar phenotype with no obvious enhancement of severity was observed. As a result, they were tentatively taken as loss-of-function alleles.

Temporal expression of mab-7 coincides with the onset of ray morphogenesis

To elucidate what tissues or cells were expressing mab-7, a promoter-gfp reporter was generated with 0.9 kb or 541 bp of the mab-7 promoter. Transgenic animals carrying these reporters displayed identical GFP signals in their hypodermis (Figs. 3D and H), structural cells (Fig. 3B), vulva (Fig. 3J), PQR (Fig. 3L), body seams (Fig. 3D) and several neuronal processes in the head region (Fig. 3F) at different developmental stages. The onset of mab-7∷gfp expression was first detected in the hypodermis at the two-fold stage (Figs. 3G and H). This hypodermal signal stayed on throughout the larval stages until the animals entered their adulthood. However, GFP expression in the body seam appeared only after the L4 stage and was maintained in adults. In the male tail, a GFP signal was detected at the late L4 stage in the structural cells when the ray retraction process was almost

Minute and scattered autophagosomes were observed in the hypodermis using TEM (0.2–0.4 μm in diameter; data not shown). In mab-7(e1599) males, however, GFP∷LGG-1 signals decorated the bulk of the hypodermis as large punctuate structures up to a few microns in size (Fig. 1O). These large foci were almost ten times larger than normal autophagosomes, and matched well with the membranous whorls and endosomal bodies in both their size and their localization in the TEM sections (Fig. 1I), suggesting that autophagy had been initiated. Interestingly, these autophagosome structures expanded and were not cleared even after tail retraction was complete in adults.

Cloning and confirmation of the mab-7 locus

mab-7 was mapped on chromosome X between unc-1 and egl-17. Among the cosmids tested in complementation rescue experiments, introduction of ZC13 alone could revert the mab-7 ray phenotype into the wild type. Four putative genes were predicted in ZC13 (Fig. 2). Since the deletion clone ZC13-Sa–clslf, with the first three putative genes and a part of the fourth, could not rescue mab-7 mutants, and BSG4 harboring only ZC13.4 showed a strong rescue activity, it was concluded that mab-7 is ZC13.4 (Fig. 2).

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Fig. 1. Male tail morphology of (A) _him-5(e1490)_ and (E) _mab-7(e1599)_ (ventral views). (B–F) Ray cells were marked by cell-specific markers respectively for the hypodermis (B and F, lateral views), structural cells (C and G, ventral views), and RnB neurons (D and H, ventral views). The ray hypodermis in _mab-7(e1599)_ (F) is grossly swollen along the rays (arrow) in comparison with _him-5(e1490)_ (B). Moderate local swelling (arrow) was noted in the structural cells of _mab-7(e1599)_ (G) compared to the smoothly tapered shapes in _him-5(e1490)_ (C). Localized spherical abnormalities (arrows) can also be found along the neuronal processes in _mab-7(e1599)_ (D). Scale bar=20 μm. White arrows point to the abnormal swelling in _mab-7_ mutants. (I, J and K) Electron microscopy of _mab-7_ rays. (I) Thin section through a very swollen proximal ray containing many autophagic whorls of membrane and some swollen vacuoles (**). A neuron soma, N, is retained near the base of the ray and has darkened cytoplasm, suggesting that some degeneration has begun. Despite this extreme swelling, a distal ray tip or papillus is pointing distally towards the cuticle at the extreme right of the panel, where processes of neurons and the structural cell seem to be organizing together. (P) Proximal tissue of the tail tip, from which the ray is extending. (J) Thin section showing two consecutive rays as they traverse the fan. The more distal ray (on the right) shows normal morphology; the structural cell process (str) wraps around the dendrites of two neurons, RnA and RnB. The whole ray is surrounded by hypodermal tissue (hyp) and lies within the fan cuticle. The ray on the left is more medial in the fan and looks fairly normal except for the presence of a neuron soma (N) that has failed to retract into the body. The two neuronal processes and that of the structural cell run in parallel. Small bits of cytoplasm within the fan cuticle (+) have not been retracted. (K) A more distal thin section showing a normal ray opening where the cilium of RnB extends outward to emerge at the ventral edge of the cuticular fan (arrow). This cilium is contained within a channel created by the structural cell process. Small cytoplasm can be seen in fan cuticle (+). Scale bars in panels I, J and K = 1 μm. (L) Lateral view of a wild-type male tail with a diffuse GFP: LGG-1 signal in the hypodermis (M). (N) Lateral view of a _mab-7(e1599)_ male tail with punctate GFP: LGG-1 signals (arrows) in the hypodermis (O). Scale bars=20 μm.
complete but prior to molting. This structural cell expression was also maintained in adults.

To define the specific cellular requirement, the onset of \textit{mab-7} expression in these cells was correlated with the first appearance of ray swelling in \textit{mab-7} mutant males. Ray abnormality first appeared when ray retraction started (Figs. 3N and Q), which was well before \textit{mab-7}::\textit{gfp} expression in the structural cells was detectable prior to the final molting (Fig. 3R). The hypodermal expression profile of \textit{mab-7} at the late L4 stage, however, matched perfectly with the timing of this ray morphogenesis.

\textit{mab-7} encodes an EGF-like domain containing protein

The domain organization of the MAB-7 protein was subsequently analyzed using the Simple Modular Architecture Research Tool (SMART) and the transmembrane region and orientation prediction (TMPred) program. A hydrophobic region was predicted at the N-terminus, followed by an EGF-like domain juxtaposed to an ShKT domain. However, no defined domain was noted in the C-terminal region (Fig. 4A). The genetic lesion in the \textit{mab-7(e1599)} allele was confirmed to be a single G to A mutation converting the cysteine 67 into a tyrosine in the EGF-like domain (Fig. 4A). This residue replacement could have interrupted disulphide bond formation between the cysteine pairs within this EGF-like domain, thus altering its folding and impairing \textit{mab-7} gene function. Similarly, point mutations were identified in the other two \textit{mab-7} alleles. \textit{wx74} had a G to A transition changing cysteine 108 into tyrosine in the ShKT domain (Fig. 4A). The \textit{wx75} allele had a C to T change leading to the replacement of arginine 250 by a stop codon (Fig. 4A), which suggests that a truncated MAB-7 protein would be made.

Based on the domain organization of MAB-7, another protein sharing a similar structural feature was identified in both \textit{C. elegans} and \textit{Caenorhabditis briggsae} (Fig. 5A). MAB-7 shares 48\% identity with a hypothetical protein F21A3.3 in \textit{C. elegans}. Their counterparts in \textit{C. briggsae}, CBP07831 and CBP10921, share 98\% and 87\% identity with the \textit{C. elegans} proteins, respectively (Fig. 5A). F21A3.3 was expressed only in the hypodermis according to a reporter assay. However, in the absence of any genetic mutant and RNAi phenotype (data not shown), the biological relevance of this MAB-7-like molecule in the male tail could not be ascertained.

The ShKT domain and C-terminal tail of MAB-7 are essential functional domains

To determine the functional relevance of various MAB-7 domains to ray morphogenesis, different protein truncation constructs were made to evaluate their \textit{mab-7} mutant rescue activity. The EGF-like domain, the ShKT domain or the C-terminal tail was removed individually. The deletions derived from the full-length cDNA were linked to the 0.9-kb \textit{mab-7} promoter and were transformed into \textit{mab-7} mutants. A transgene producing a wild-type MAB-7 protein could completely rescue the mutant phenotype (100\%, \(N = 101\)) (Fig. 6A). With the last three amino acids on the C-terminus of MAB-7 removed, 88\% \((N = 106)\) of the transgenic \textit{mab-7} animals displayed a wild-type phenotype. This may have been due to the induced instability of the protein. None of the transgenic animals could be rescued when regions from amino acids 124 to 287 \((N = 101)\) or from amino acids 110 to 207 \((N = 62)\) of the C-terminus were removed (Fig. 6A). Similarly, the Mab-7 phenotype could not be reverted into wild type when the entire ShKT domain \((N = 50)\) or both the EGF-like and the ShKT domains together \((N = 110)\) were
deleted. Interestingly, a substantial level of biological activity (20%, \(N = 106\)) was observed when the entire EGF-like domain was eliminated (Fig. 6B). Although not all these domain-deleted proteins have been evaluated for their stability, preliminary data from a few tagged versions suggest that they are reasonably stable. Thus, the results from these rescue assays are consistent with the previous mapping data, and the ShKT domain and the C-terminus of the protein are required for the biological activity of the MAB-7.

The ShK toxin was first defined as a potassium channel blocker originally identified in sea anemone (Lanigan et al., 2001; Pennington et al., 1999, 1996; Castaneda et al., 1995). This polypeptide toxin of 35 residues has three intramolecular disulfide bonds at the C-terminus with a specific CXXXCXXX pattern distinct from the EGF-like domain or other cysteine-rich motifs (Pennington et al., 1996). Two residues in this polypeptide, Lys22 and Tyr23, are essential for potassium channel binding (Lanigan et al., 2001; Pennington et al., 1999, 1996).
It is possible that MAB-7 regulates channel activity and it may alter ion transport across ray cells. The alignment of various ShKT-like domains, however, shows that the toxin domain of MAB-7 resembles best with that of F21A3.3, not that of the original ShK toxin, although structural similarity is preserved (Fig. 5B). Moreover, the two signature Lys22 and Tyr 23 residues are missing in the MAB-7 ShKT domain. To ascertain their functional similarity, the MAB-7 ShKT domain was replaced by the sea anemone ShKT. This hybrid construct demonstrated absolutely no mab-7 rescuing activity in mutant animals (N = 114) (Fig. 6C).

Therefore, the MAB-7 ShKT domain is obviously distinct and specific. The structurally conserved feature may simply reflect its general property as a contact interface for protein interaction.

MAB-7 is a type II transmembrane protein acting outside the expressing cells

Based on the predictions of the SMART and SignalP (protein signal prediction) programs, the hydrophobic region (HR) in MAB-7 likely constitutes a signaling peptide. The same protein segment, however, could be a type II transmembrane motif, as predicted by TMPred. A transmembrane domain at the N-terminus would suggest that MAB-7 is either a type II or type III membrane protein (Pei et al., 2000). Since the nature of this hydrophobic region has strong implications for its subcellular localization, and possibly for the mode of its biological activity, these predictions were tested experimentally. RAM-5 signal peptide and the MEC-6 type II transmembrane domain were used to replace the MAB-7 HR domain in an otherwise functional transgene (Fig. 6C) (Yu et al., 2000; Chelur et al., 2002). The chimeric constructs were able to rescue the mutant phenotype at levels of 53% (N = 190) and 59% (N = 145), respectively (Fig. 6C). These results suggest that MAB-7 can function either as a molecule secreted outside the cell guided by the RAM-5 signal peptide, or as one anchored on the cell surface as MEC-6 protein does.

Interestingly, when the cellular localization of MAB-7 was examined in transgenic animals expressing the protein products of GFP::MAB-7 (Fig. 7D) and MAB-7::TMGFP (Fig. 7B), both fusion proteins were found to be associated with the cell membrane. Since a pat-3 transmembrane domain coding region was present in this pMAB-7::TMGFP fusion protein, the GFP should be oriented inside the cell (Fig. 7K). On the other hand, the fusion protein GFP::MAB-7 without this additional transmembrane domain could still be localized to the cell membrane (Fig. 7J). A substantial level of mab-7 mutant rescuing activity was also observed (33%, N = 117). This result is consistent with the notion that MAB-7 is a type II transmembrane protein tethered on the cell surface, as suggested by the HR swapping experiments.

The topology of MAB-7 was further characterized through β-galactosidase assays sensitive to the biological environment in and outside the cell. Two fusion proteins were generated by fusing either the lacZ gene or the synthetic transmembrane domain with lacZ at the C-terminus of the MAB-7 protein (Fig. 7E). Only the MAB-7::TMGAL showed enzymatic activity, not the MAB-7::GAL (Figs. 7F and G). Since β-galactosidase is
inactive outside the cell but is catalytically active inside (Chelur et al., 2002; Hong et al., 2000) (Figs. 7H and I), this observation suggests that the functional EGF-like, ShKT and C-terminal tail domains of MAB-7 are all exposed on the cell surface.

Ectopic expression of mab-7 in a non-ray lineage is sufficient for ray development

While MAB-7 resides on the cell surface and strong mab-7 rescue activity was observed when the MAB-7 HR was replaced by RAM-5 signal peptide, MAB-7 may nevertheless be required for cellular signaling or communication. To test this idea, mab-7 was ectopically expressed in different types of mab-7 mutant male tail cells using cell-specific promoters. When mab-7 was expressed only in the hypodermis by a ram-4 promoter, all of the transgenic animals could be rescued (100%, \(N=106\)) (Fig. 8E).

A reduced but significant rescue activity could be observed when mab-7 was ectopically expressed in ray neuron A by a sek-1 promoter (37%, \(N=111\)). In this transgenic population, 25% of the transformants displayed a partial rescue phenotype with a slightly swollen basal region (Fig. 8F), and 38% of them remained mutant. Expressing mab-7 in structural cells with a ram-5 promoter resulted in 55% and 32% of the animals fully or partially rescued, respectively (\(N=22\)) (Fig. 8D). Surprisingly, the same was true when mab-7 was expressed in PLM neurons by a mec-7 promoter. Although PLM does not send any process into the rays, 18% of the transgenic animals could be partially rescued (\(N=219\)) (Fig. 8C). In summary, ectopic expression of mab-7 in cells within the male tail region was sufficient to provide mab-7 activity. Neither synthesis of its product in structural cells or the hypodermis, nor the extension of cellular protrusions by the expressing cells into the ray is an absolute requirement for wild-type ray development.

Discussion

mab-7 encodes a novel signaling molecule

Sequence analysis of the mab-7 locus revealed that it encodes a novel protein with an EGF-like domain, an ShKT domain and a C-terminal tail of 177 amino acids. Using different fusion reporters, this study has experimentally demonstrated that the product is a type II transmembrane protein residing on the surface of the expressing cell with the N-terminal hydrophobic region anchored on the membrane. As such, the bulk of the protein molecule including the various domains is exposed on the cell surface. When these domains were analyzed in functional assays, both the ShKT domain and the C-terminal
The role of the EGF-like domain, on the other hand, is more elusive. The e1599 mutant allele has a point mutation converting a cysteine into a tyrosine residue in this domain. This specific mutation may render the protein non-functional by corrupting the folding of this domain. Alternatively, it may also lead to the establishment of a disulphide bridge in this EGF-like domain using the first cysteine of the ShKT domain, which inevitably would affect the folding of the ShKT domain and abolish the MAB-7 function (Fig. 4A). The ability of an EGF-like domain for protein–protein interaction is well documented (Morimura et al., 2001; Shibata et al., 2000; Appella et al., 1988). An MAB-7 protein without an EGF-like domain may be unable to interact with its partner(s) molecule, or for maintaining intramolecular conformation. However, such interaction does not appear to be critical during ray morphogenesis, because mutant protein without this EGF-like domain still retained substantial mab-7 activity. Hence, any impact of e1599 mutation is unlikely to be due to a disrupted EGF-like domain per se. Rather, an altered ShKT domain structure or reduced protein stability is a more probable explanation for its impaired function.

On the other hand, a lesion in the wx74 allele results in substitution of a cysteine required for disulphide bridge formation in the ShKT domain. The critical role of this ShKT domain in the mab-7 gene functionality conferred by this bridge
Fig. 7. Cellular localization and topology of MAB-7. (A and B) MAB-7∷TMGFP fusion protein displayed a fluorescent signal on the surface of the structural cell. So did the GFP∷MAB-7 (C and D). (E) DNA constructs used for the examination of MAB-7 localization and topology. (F) Enzymatic activity of the β-galactosidase of the transgenic animal carrying plasmid pMAB-7∷TMGAL. A blue coloration can be observed in mab-7 expressing seam cells. (G) No enzymatic activity was detected from the transgenic animals carrying plasmid pMAB-7∷GAL. Interpretation of the topology of various fusion proteins; MAB-7∷GAL (H), MAB-7∷TMGAL (I), GFP∷MAB-7 (J) and MAB-7∷TMGFP (K) and the wild-type MAB-7 (L). Scale bar=20 μm.

Fig. 8. Non-autonomous activity of mab-7 was revealed by ectopic mab-7 expression. Male tail of (A) wild-type and (B) mab-7(e1599). The Mab-7 phenotype was rescued by turning on mab-7 cDNA with the promoters of (C) mec-7, (D) ram-5, (E) ram-4 and (F) sek-1 genes. White arrows point to the swollen region of the sensory rays. Scale bar=20 μm.
could be lost. Nonetheless, this MAB-7 ShKT domain differs from that of the ShKT toxin in the sea anemone, although they share similar structure and sequences. The two diagnostic amino acids, Lys22 and Tyr23, required for potassium channel association are absent in the MAB-7 ShKT domain. Replacing the MAB-7 ShKT domain by sea anemone ShK toxin abolished the rescuing activity of wild-type MAB-7 protein (Fig. 6B). Indeed, many other proteins containing this toxin domain, e.g., hydra metalloproteinases 1 and 2 (HMP1 and HMP2) (Yan et al., 2000, 1995) or the human matrix metalloproteinase (MMP-23) (Velasco et al., 1999), do not have these specific Lys and Tyr residues. The activity of the ShKT domain in these proteins has not been determined experimentally. The primary role of these ShKT-containing proteins as proteases does not preclude the possibility that they can block ion channels, and the ShKT domain could work simply as a protein interaction domain. Further biochemical and genetic characterization of these ShKT-like domains will be required to elucidate their biological and physical properties, which are crucial for our understanding of their natural occurrence in a variety of different proteins.

**mab-7 is required in both sexes of C. elegans during development**

Both sexes of the *mab-7(e1599)* mutant had reduced body length as compared with wild-type animals. The transcriptional reporter revealed its expression in the hypodermis and in body seam cells at both the larval and young adult stages, implying that *mab-7* acts in the hypodermis and body seam to regulate body morphology. In addition, *e1599, wx74* and *wx75* males had a reduced ability to sire cross progeny in a mating assay. This reduced mating efficiency may partly be due to the morphological defect in the rays that impedes their mechanical function during mating (Stemberg and Liu, 1995). The swelling of these rays might prevent a stable association of the two sexes, and thus productive mating. Moreover, *mab-7* is also expressed in the amphid neurons. The reduced mounting frequency and association with hermaphrodites noted among *mab-7* males could well be the consequences of an attenuated chemosensory ability of males, which had been observed in our male chemosensory assays (Tsang SW and Chow KL, unpublished data, and Chasnov et al., 2007).

**mab-7 mutations interfere with retraction during reshaping of the male tail**

Each ray cell group making up a single ray is born in close proximity to the posterior cuticle, and papilla formation precedes retraction of cell bodies from the surface cuticle (Baird et al., 1991). Elongation may require coordination between the hypodermis, a structural cell, and the two neurons, subsequent to the initial formation of ciliated endings within a papillus. TEM and LM analyses of mutants confirmed that most rays developed past the papilla stage but were often short, indicating a potential role for MAB-7 during ray elongation after papilla formation commences. Abnormal swelling could be identified in both the hypodermis and the structural cells, where swollen neuronal processes could be seen under a light microscope (Fig. 1H), probably due to stranding of neuronal cell bodies within the ray during the retraction phase.

Careful examination by TEM of the tails of three mutant adult males showed a consistent problem in morphogenesis of all rays. Ultrastructural analysis revealed that the principal defects were confined to the hypodermis and the structural cells in the rays, but in many cases one or more neuronal cell bodies were trapped too far posterior, often at the base of the rays or even along the rays. There was excess hypodermal tissue posterior to the tail, overlying the body wall muscles in addition to the swollen hypodermis at the base of most rays. Brushy material observed in the fan was probably excess debris left behind by the retracting hypodermis. Distal portions of the rays were always well formed, and likely require coordinated morphogenesis of the hypodermis and structural cell tissue. Structural cell processes also showed proximal swelling at the base of many rays. Thus, the principal defects in *mab-7* mutant could arise either in retraction or in the recycling of materials in the hypodermis and structural cells after retraction. These tissues often showed both hypertrophy and some signs of increased autophagy in the mutants, but never in wild-type specimens. Autophagy has been shown to play an important role in the reshaping (shrinkage) of seam cell tissues during the larval transition from L2 stage to dauer larva in *C. elegans* (Melendez et al., 2003). It has an important role in many of the homeostatic mechanisms in normal tissue morphogenesis (Levine and Klionsky, 2004; Cuervo, 2004). It also serves to reduce the volume of hypodermis in the process of male tail retraction, where resorption of tissue is a critical step (Nguyen et al., 1999, and Nguyen C.Q and Hall, D.H., unpublished data). While normal morphogenesis goes through this transition swiftly, with the tissue rapidly remodeled, the loss of *mab-7* function clearly disrupts this resorption event. A profusion of large autophagosomes in the distal tail, as shown by both TEM and by the GFP::LGG-1 marker, indicates that clearance of cellular debris and reduction of hypodermal volume were inhibited. Excessive tissue and large autophagosomes were left behind at the adult stage. Thus, normal *mab-7* activity might facilitate tissue remodeling by actively removing cellular debris. In mutant animals, this process is impaired. Autophagosomes are still created, and they grow large, but their content cannot be digested or cleared, leading to the persistence of these subcellular organelles in adults.

**mab-7 does not work autonomously during ray morphogenesis**

The experiments in this study have demonstrated that the expression of *mab-7* by different cells in the tail is sufficient to restore a wild-type phenotype in *mab-7* mutants. For instance, ectopic expression of MAB-7 protein by the sensory ray neurons, or even by the PLM neurons, was sufficient to rescue the morphology of mutant tissues at least two to three cell diameters away. Although a low activity of this PLM-specific promoter (*mec-7*) in other cells, such as in the hypodermis or structural cells, cannot be ruled out, the observations here and in prior studies suggest it is unlikely. Thus, *mab-7* should be
working non-autonomously at a distance during sensory ray morphogenesis. As a type II transmembrane protein acting at a distance from its expressing cell, MAB-7 probably needs to be modified to become diffusible. Growth factors acting outside the cell, including EGF, HGF, TGF-α and TGF-β, need to be cleaved before they act as mature ligands (Hinkle et al., 2003; Le Gall et al., 2003; Merlos-Suarez et al., 2001; Dubois et al., 1995; Mizuno et al., 1994). Post-translational hydrolysis and cleavage by a protease could also facilitate the release of membrane protein (Hooper et al., 1997). At this point, it is difficult to predict how or even whether this regulated cleavage of MAB-7 takes place. However, the observation that mab-7 product synthesized in any one cell type in the ray is sufficient to restore the normal morphology of all ray cells implies that the modification would not be restricted to a single cell type. It probably occurs outside the expressing cells. In an ADAMTS-like metalloprotease encoding gene, adt-1, results in a Mab-7 like phenotype with the swelling localized at the base of all the sensory rays (Kuno et al., 2002). This adt-1 product could possibly act outside the expressing cell, as most ADAMTS proteases do, to modify MAB-7 protein in the ECM. On the other hand, no matter whether MAB-7 functions as a signaling molecule or not, these results clearly demonstrate that active intercellular communication takes place among ray cells, as was first proposed for ram-5 function (Yu et al., 2000). This communication depends on the presence of an intact, tail-specific extracellular matrix of collagens encoded by ram genes. With the proper modification of the matrix by various modifying enzymes, e.g., DPY-11, a transmembrane RAM-5 protein of the structural cells can function as a receptor of intercellular signals. How this cellular crosstalk involves mab-7 remains unclear at this point. MAB-7 might be the paracrine signaling factor or a facilitating molecule. Nevertheless, this hypothetical mechanism will not be confirmed until the physical association and biochemical activities of these components have been worked out.

In summary, mab-7 has been shown to have an important role in determining body shape and sensory ray morphology in C. elegans. Both the ShKT and C-terminal domains are crucial for its protein function. Since mab-7 acts non-autonomously, this transmembrane protein may prove to be a signaling molecule acting outside the expressing cell to control cell shape. Should it function as a communication molecule, identification of mab-7’s putative receptor(s) or interacting components in the matrix will be the key to understanding the mechanism governing male tail development and ray morphogenesis. These components would then serve as a good model for dissecting cellular communication events that take place in tissues undergoing active remodeling.

Acknowledgments

We thank members of our laboratory for their comments of this manuscript. This study was supported by grants HKUST 6118/03M and HKUST 6239/04M to K.L.C. from Hong Kong’s Research Grants Council, and by grant RR12596 from the US National Institutes of Health to D.H.H. We thank Beth Levine for kindly providing the gfp: :lgg-1 marker and Y. Kohara for cDNA clones. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is supported by the NIH National Center for Research Resources.

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