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The chitin synthase genes *chs-1* and *chs-2* are essential for *C. elegans* development and responsible for chitin deposition in the eggshell and pharynx, respectively

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Abstract

It is widely accepted that chitin is present in nematodes. However, its precise role in embryogenesis is unclear and it is unknown if chitin is necessary in other nematode tissues. Here, we determined the roles of chitin and the two predicted chitin synthase genes in *Caenorhabditis elegans* by chitin localization and gene disruption. Using a novel probe, we detected chitin in the eggshell and discovered elaborate chitin localization patterns in the pharyngeal lumen walls. Chitin deposition in these two sites is likely regulated by the activities of *chs-1* (T25G3.2) and *chs-2* (F48A11.1), respectively. Reducing *chs-1* gene activity by RNAi led to eggs that were fragile and permeable to small molecules, and in the most severe case, absence of embryonic cell division. Complete loss of function in a *chs-1* deletion resulted in embryos that lacked chitin in their eggshells and failed to divide. These results showed that eggshell chitin provides both mechanical support and chemical impermeability essential to developing embryos. Knocking down *chs-2* by RNAi caused a defect in the pharynx and led to L1 larval arrest, indicating that chitin is involved in the development and function of the pharynx.

Keywords: *C. elegans*; Chitin; *chs-1*; *chs-2*; Pharynx; Grinder; Eggshell; Parasitic nematode

Introduction

Chitin is abundant in nature and found in certain lower eukaryotes and invertebrates. In the yeast *Saccharomyces cerevisiae*, chitin is present in the cell wall and a specialized cell wall structure called the septum (Cabib et al., 2001). It is also found in the cyst wall of protozoan parasites of the genus *Entamoeba* (Arroyo-Begovich et al., 1980). Arthropod chitin forms a major part of the exoskeleton and the supporting layer of the tubular trachea system (Merzendorfer and Zimoch, 2003). Additionally, it is an integral part of insect peritrophic matrices, serving to separate and protect internal tissue from the digestive process. In nematodes, chitin is found in the eggshells of many free living (Wharton, 1980) and parasitic species (Brydon et al., 1987), and in the modified eggshell or sheath that surrounds first stage larvae of filarial nematode parasites (Fuhrman and Piessens, 1985). In the model organism *Caenorhabditis elegans*, chitin is also known to be present in the eggshell (Wood, 1988); however, it is unclear if other tissues contain chitin.

Chitin synthases (EC 2.4.1.16) catalyze the polymerization of UDP-*N*-acetyl-glucosamine to produce insoluble chitin. Chitin synthases typically contain a putative catalytic domain that has the signature motif QRRRW and multiple transmembrane domains flanking the catalytic domain. Analyses of completely sequenced genomes of chitin producing organisms have revealed that two or more chitin synthase genes are present in a single species. For example, the yeast *S. cerevisiae* has three chitin synthase genes (Cabib et al., 2001), while two have been described in protozoan parasites of the genus *Entamoeba* (Campos-Gongora et al., 2004) and the insects *Drosophila melanogaster* and *Anopheles gambiae* (Merzendorfer and Zimoch, 2003). Initial molecular studies in the parasitic nematodes *Brugia malayi* (Harris et al., 2000), *Dirofilaria immitis* (Harris and Fuhrman,

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2002) and *Meloidogyne artiellia* (Veronico et al., 2001) revealed the presence of one gene. More recently, two chitin synthase genes have been identified in filarial nematodes (Foster et al., 2005). In the completely sequenced genome of the free-living nematode *C. elegans*, two putative chitin synthases (encoded by T25G3.2 and F48A11.1) have been identified (Harris et al., 2000; Veronico et al., 2001). F48A11.1 was shown to be expressed in the pharynx and its RNA transcription correlated with larval molting, while T25G3.2 transcripts were found in late larvae and adult hermaphrodites (Veronico et al., 2001). However, it is unknown what the precise roles of these two genes are in *C. elegans* development and how they contribute to chitin deposition in the worm.

In this study, we demonstrate the necessity of chitin in *C. elegans* development by revealing its distribution pattern in whole worms and studying the roles of the two predicted chitin synthases. We show definitively that chitin is an indispensable component of the eggshell and the pharynx where it is highly localized to specific regions of the lumen wall. We find that the two chitin synthases have non-overlapping functions and regulate chitin deposition in these specific tissues.

Materials and methods

Assembling C. elegans chitin synthase cDNAs

DNA sequence information for *chs-1* and *chs-2* was initially obtained from predicted genes (T25G3.2 and F48A11.1, respectively) in the *C. elegans* genome database (http://www.wormbase.org) (Harris et al., 2004), and used to design PCR primers and for sequence verification. The positions of the various RT-PCR products and cDNA clones are indicated on a representation of the corresponding genomic loci (Fig. 1).

For *chs-1*, sequence from two overlapping fragments was used to assemble a full-length cDNA. The 3′ fragment (~2.9 kb) including a 3′ UTR (135 bp) was obtained by sequencing a matching cDNA clone yk250a11 (kindly provided by Y. Kohara). The 5′ fragment (SL1 RT-cDNA, ~1.5 kb) including a 109 bp-long 5′ UTR was generated from an RT-PCR product using the trans-splicing leader SL1 (5′-GGTTTAATTACCCAAGTTTGAG) and a reverse primer (5′-ATACCACAAGAGATTGCTCG) contained in yk250a11. The PCR template used was oligo-dT primed cDNA from mixed stage worms. Products were cloned into the pCR2.1-TOPO vector (Invitrogen) and verified by sequencing.

In the case of *chs-2*, two matching cDNA clones (yk316g4, ~3.1 kb and yk98d4, ~2.6 kb, both kindly provided by Y. Kohara) were sequenced to generate the 3′ region (~3.1 kb) including a 271 bp-long 3′UTR of the cDNA. Four overlapping RT-PCR products were obtained and sequenced to assemble the 5′ region (~2.2 kb) of the cDNA. The most 5′ end of the cDNA was amplified (RT-cDNA1, ~400 bp) using a forward primer (5′-ATCT-TAAATCCCATCTCCAG) that matches the sequence at position −57 relative to the predicted translation start and a reverse primer (5′-TTGAAGACTTGCATCGTGCC) start-

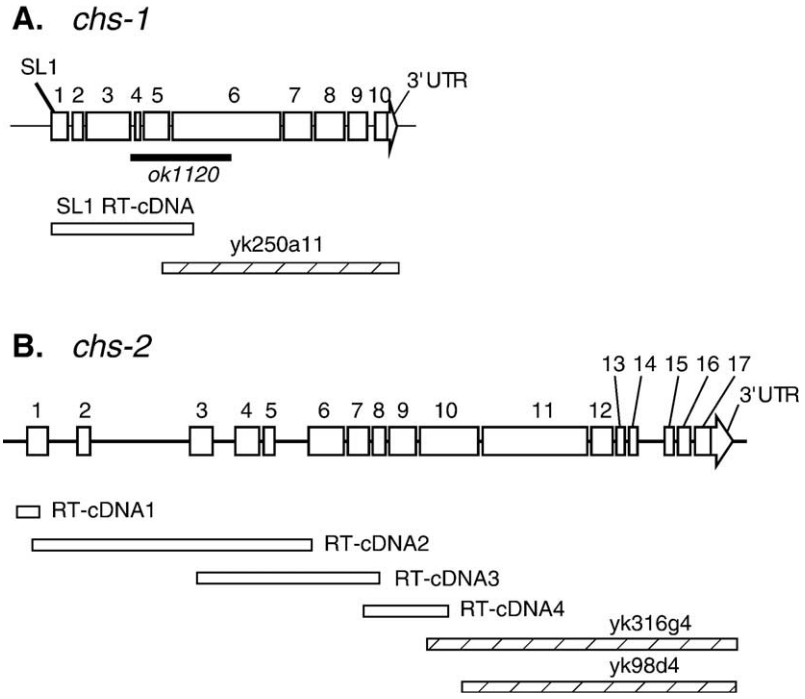

Fig. 1. Schematic representation of the gene structures of *chs-1* and *chs-2*. For each gene, the exon (boxes) and intron structure is shown. The horizontal bars represent RT-PCR and cDNA clones (hatched) that span the corresponding genomic regions. (A) *chs-1* (T25G3.2). The region deleted in *chs-1(ok1120)* is shown as a solid line under the gene structure. (B) *chs-2* (F48A11.1). Exon 13 is not predicted in Wormbase but is present in both cDNA clones.

ing from position +348. The second PCR product (RT-cDNA2, ~1.0 kb) was obtained using a forward primer (5′-GATGGAGGGGAAACCAGATG) with sequence matching at position +45 and a reverse primer (5′-AGCAGACAACG-TAGATGCAG) located at position +1090. The third PCR product (RT-cDNA3, ~1.4 kb) was obtained using a forward primer (5′-GCACGATGCAAGTCTTCAAG) located at position +330 and a reverse primer (5′-CAAAA-TGAAATCGGCAAGCGAC) at position +1734. The fourth RT-PCR product (RT-cDNA4, ~0.6 kb) was generated with a forward primer (5′-CAAATGTGCTAACTGTGGAGC) located at position +1664 and a reverse primer (5′-TTACATCGTAAGCGTCATCG) at position +2269 which is situated approximately 90 bp within the 5′end of yk316g4.

The assembled cDNA sequences for *chs-1* and *chs-2* were assigned as GenBank accession nos. AY874871 and AY874872, respectively.

RNA interference and genetic deletion

A 780 bp fragment of *chs-1* cDNA was amplified by PCR (Primers: 5′-GAAGATAGACAACAGATGGCTGA-CAAC; 5′-CGCAATCGACATGTACTTGGCATAG) from λ phage containing cDNA clone yk250a11. Similarly, a 900 bp fragment for *chs*-2 cDNA was amplified (Primers: 5′-GATGGTGCAATGGTGTGGTATCAGAAG; 5′-CTCCGTCGGCCTTGGGTCTTCGCGAG) from yk98d4. These cDNA fragments were initially cloned into pCR2.1-TOPO and then subcloned into Litmus28i (NEB) that contains a T7 promoter sequence on either side of the multiple cloning site. The cDNA inserts were then amplified using the T7 primer (5′-TAATACGACTCACTATAGGG) and the PCR product was purified and used for in vitro transcription with T7 RNA polymerase using the HiScribe™ RNAi Transcription Kit (NEB). The synthesized dsRNA was purified using an RNeasy column (Qiagen) and eluted into dH_2O. The purified dsRNA (0.5–1 mg/ml) was injected (Fire et al., 1998) into the germ line of young adult *C. elegans* worms of either the N2 wild type strain, or a mutant strain carrying *rrf-3(pk1436)* that confers higher sensitivity to RNAi for certain genes (Simmer et al., 2002). After recovering on plates overnight, each injected worm was transferred to a fresh plate every 8 or 16 h over the course of 3 days. F1 embryos and larvae were then examined for phenotype. To test the permeability of the eggshell, live eggs were incubated with small molecule dyes (Rappleye et al., 1999). Briefly, eggs were mounted on agar pads in an isotonic buffer (150 mM KCl, 5 mM HEPES, pH 7.5) containing 20 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) and 32 μM FM4-64 (Molecular Probes) and immediately examined by microscopy.

A mutant strain {RB1189: *chs-1(ok1120)/hT2[qIs48](I;III)*} carrying a deletion in *chs-1* was generated by the *C. elegans* Gene Knockout Consortium (http://www.celeganskoconsortium.omrf.org) and obtained from the Caenorhabditis Genetic Center (Minneapolis, MN). The region spanning the deletion junction was amplified using primers designed by the *C. elegans* Gene Knockout Consortium and sequenced to determine the missing DNA sequence. Homozygotes of *chs-1(ok1120)* were identified by the lack of *myo-2*::GFP that marks the chromosome translocation hT2.

chs-2 transcription reporter

For constructing a transcription GFP reporter of *chs-2* (pIP30), a 2005 bp fragment upstream of the predicted translation start was amplified from wild type genomic DNA (Primers: 5′-GCGAAGCTTTCTAGGATCTACCTG-CTATGGTATAG, 5′-CGCGGATCCGTTTGTTTAATAGA-AGCTCAGAAATTC) and cloned into the expression vector pPD95.75 for cytoplasmic GFP expression (Fire et al., personal communication). Transgenic *C. elegans* (IP113: *nbEx14[rol-6(su1006)chs-2*::*GFP]*) carrying extra-chromosomal arrays were established following microinjection of the reporter construct (~20 μg/ml) with the pRF4 plasmid (~54 μg/ml) as the transgenic marker.

Chitin detection

A construct was engineered that allowed expression of the chitin-binding domain (ChBD, 45 amino acids) of *Bacillus circulans* chitinase A1 (Watanabe et al., 1994) fused with *Escherichia coli* maltose-binding protein (MBP, 367 amino acids) (Guan et al., 1988) via a 47 amino acid-long linker. The resulting MBP-ChBD fusion protein (~48 kDa) was overexpressed in *E. coli* and affinity purified using an amylose column according to the manufacturer's instructions (NEB). The purified protein was labeled with TRITC (tetramethyl-rhodamine isothiocyanate) (Sigma) or FITC using a Fluorescein Protein Labeling Kit (Roche) and subsequently purified from free dyes by gel filtration. The purified (0.5 mg/ml) labeled probes contained approximately 2–4 molecules of dye per molecule of protein and are commercially available (NEB).

For in situ chitin detection in whole worms, mixed-stage *C. elegans* were collected from nematode growth medium (NGM) plates and then fixed and permeabilized following a protocol used for antibody staining (Bettinger et al., 1996). Bleached eggs were obtained by treating gravid worms and/or laid eggs with an alkaline hypochlorite solution (Wood, 1988). Untreated eggs were collected from NGM plates following removal of adult worms. Both bleached eggs and untreated eggs were fixed by freezing in 1× RFB buffer (160 mM KCl, 40 mM NaCl, 10 mM spermidine, 30 mM PIPES and 50% methanol) containing 1.9% formaldehyde followed by 3.5 h incubation at room temperature. Before staining, fixed eggs were washed twice for 10 min each with TTE (100 mM Tris–HCl, pH 7.4, 1 mM EDTA and 1% Triton X-100) and PTB (1× PBS, 1% BSA, 1 mM EDTA,

0.5% Triton X-100 and 0.05% sodium azide). Samples were incubated in 0.5 ml of PTB containing a 1/500 dilution of labeled probe for 4 h to overnight at room temperature on a slow rocking platform. Fluorescence was examined using a Zeiss Axiovert 200M microscope equipped with appropriate filters.

Results

Characterization of cDNAs of the predicted C. elegans chitin synthase genes

In the *C. elegans* genome, two genes are predicted to encode potential chitin synthases based on the similarity of their amino acid sequences to known chitin synthases (Harris et al., 2000; Veronico et al., 2001). These two predicted genes, T25G3.2 and F48A11.1 (previously known as Ce-*chs-2* and Ce-*chs-1*, Harris et al., 2000), are renamed *chs-1* and *chs-2* respectively to conform with the naming of chitin synthase genes of previously studied parasitic nematodes. To verify the predicted coding regions, the cDNAs were assembled using 5′ sequence derived from RT-PCR experiments and 3′ sequence obtained by sequencing existing cDNA clones (Fig. 1).

RT-PCR experiments using the trans-splicing leader SL1, but not SL2, together with an internal primer generated a specific product for *chs-1*. Sequencing this DNA revealed that SL1 is trans-spliced to the 2nd exon previously predicted in Wormbase. Interestingly, SL2 has been confirmed on the 5′ end of two downstream genes (Blumenthal et al., 2002), suggesting that *chs-1* may be the first gene in this operon. The sequence of this RT-PCR product and that obtained from cDNA yk250a11 were used to assemble a putative full-length *chs-1* cDNA (Fig. 1A). Except for the first exon mentioned above, the remaining intron/exon junctions of *chs-1* were identical to those predicted in Wormbase. The assembled *chs-1* cDNA encodes a protein of 1322 amino acids, lacking the N-terminal 58 amino acids previously predicted in Wormbase.

Similar RT-PCR experiments were performed for *chs-2* using SL1 or SL2 primers together with *chs-2* internal primers but no specific product was obtained. The sequence of the assembled *chs-2* cDNA is derived from four overlapping RT-PCR products and two overlapping cDNA clones (yk316g4 and yk98d4) (Fig. 1B). Since the assembled cDNA has an in-frame stop codon before the longest open reading frame, it likely covers the full-length of cDNA. When compared with the exons predicted in Wormbase, the assembled cDNA contains an additional 99 bp exon towards the 3′ end. Both of the sequenced *chs-2* cDNAs contained this exon. These data indicate that *chs-2* potentially encodes a protein of 1668 amino acids instead of the predicted 1635 amino acids.

Essential and non-overlapping roles of chitin synthase genes

To determine the functions of *C. elegans chs-1* and *chs-2*, their gene activities were knocked down independently by gene-specific RNAi. In addition, the role of *chs-1* was also analyzed in a strain carrying a genetic deletion.

Adult wild type N2 worms injected with *chs-1* dsRNA initially laid abnormal eggs but soon became sterile. Approximately 6 h after injection, when there was likely a partial loss of gene activity, worms laid eggs that appeared spherical, in contrast to the oval-shaped normal ones (Fig. 2A), and failed to develop further. Unlike wild type embryos, the defective embryos readily absorbed the small molecule dyes DAPI and FM4-64, indicating that their eggshells lacked the normal chemical barrier (Fig. 2A). After these permeable eggs were laid, broken and fragmented eggs often appeared in the uterus indicating that they lacked normal mechanical strength. Such eggs were sometimes extruded through the vulva (Fig. 2B). As the RNAi effect became more potent, the injected hermaphrodites had only oocyte-shaped, single-cell embryos in their uteri and ceased laying eggs. Similar phenotypes were also observed when wild type N2 worms were fed with bacteria expressing *chs-1* dsRNA, or with *rrf-3* mutant worms after dsRNA was delivered by microinjection or feeding.

To confirm the RNAi phenotype described above, worms carrying a deletion (*ok1120*) in the *chs-1* gene were also analyzed for defects. The *ok1120* deletion removed a 1378 bp genomic region (deletion starts at GTGAGCATAA and ends at AATGGTTTGG) corresponding to amino acids 285–698 (from VSAVY to GIMVW) deduced from the assembled *chs-1* cDNA. This region includes most of the predicted catalytic domain, and therefore likely removes the *chs-1* gene activity completely. Homozygotes hatched normally and grew into sterile adults. They produced embryos that resembled unfertilized mature oocytes since these embryos lacked the normal oval shape (Fig. 2C), and they showed no cell division when examined by DNA staining using DAPI (Y. Zhang et al., unpublished data, 2005). When incubated with the chitin-specific probe, these eggs did not fluoresce, while eggs containing single-cell embryos within the heterozygote mother readily bound the probe (Fig. 2C). This result is in agreement with the complete removal of chitin synthase activity in the *ok1120* deletion mutant.

When *chs-2* dsRNA was injected into RNAi sensitized worms carrying the *rrf-3* mutation, eggs hatched normally. However, at 3.5 days post-hatching, most of the progeny remained as L1 (57/89 progeny scored from 5 injected hermaphrodites), whereas the progeny of untreated worms had reached adulthood (Figs. 3A–C). These developmentally arrested larvae had typical L1 alae on the lateral hypodermis (Fig. 3D) and showed normal motility. When

examined using DIC microscopy, they were consistently found to have a disorganized and larger grinder compared to that of wild type L1 worms (Figs. 3E–F). Careful monitoring of 22 of the arrested L1 worms showed that all except one failed to develop further after an additional week. Other progeny showed delayed larval development (16/89 had body sizes of L2/L3 larvae and 13/89 were the size of L4 larvae at 3.5 days post-hatching), while a few grew normally and reached adulthood (3/89). No defect was observed when *rrf-3* worms were fed with bacteria expressing *chs-2* dsRNA, or when N2 worms were subjected to RNAi by injection or bacterial feeding.

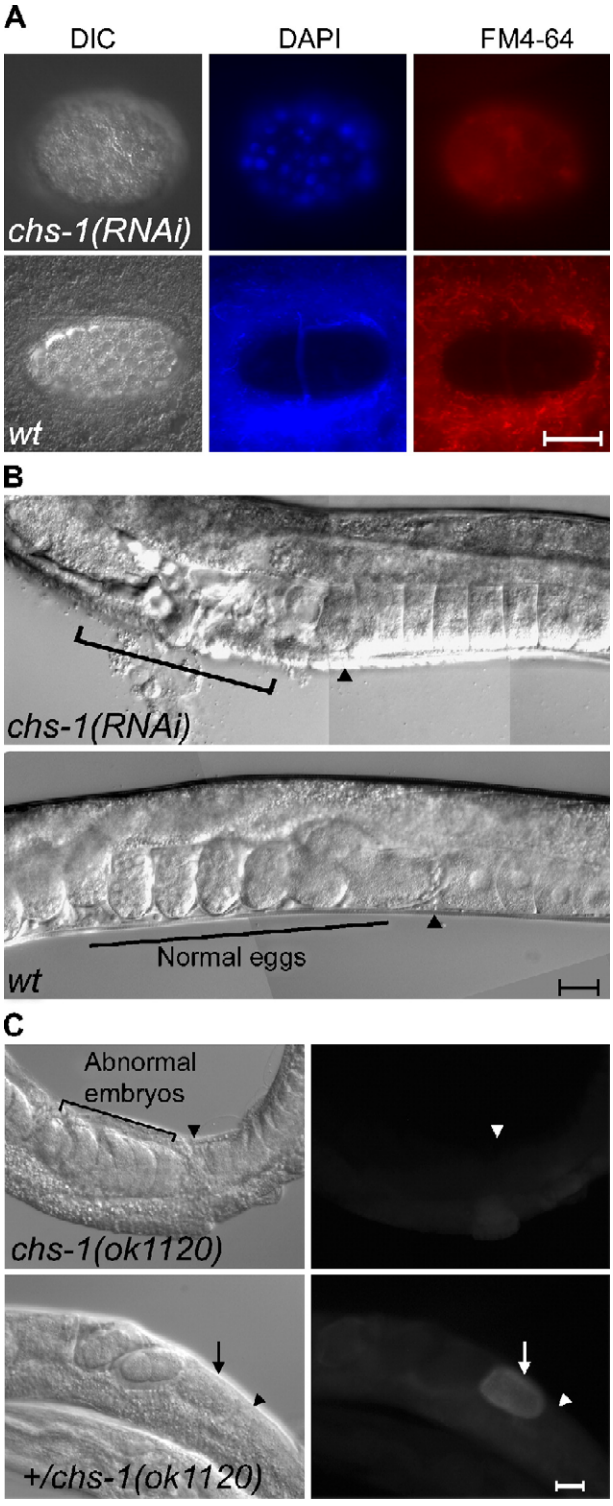

Fig. 2. Characterization of *chs-1* function by RNAi and deletion mutant analysis. (A) *chs-1(RNAi)* embryos show abnormal morphology and permeability to small molecule dyes DAPI and FM4-64. Differential Interference Contrast (DIC) images (left panels) show the defective eggs (upper row) produced by *chs-1(RNAi)* worms compared with those from wild type (wt) worms (lower row). The nuclei of *chs-1(RNAi)* eggs were stained with DAPI (middle panel) and the cytoplasm was stained using FM4-64 (right panel). (B) DIC images of a *chs-1(RNAi)* hermaphrodite (upper panel) in comparison to a wt hermaphrodite (lower panel). The uteri of *chs-1(RNAi)* worms contain abnormal and broken eggs (indicated) in contrast to normally developing embryos (indicated). Arrowheads indicate the position of the spermatheca. (C) The phenotype of the *chs-1(ok1120)* mutation observed in DIC images (left) and the fluorescent images (right) obtained following chitin detection using a rhodamine-labeled probe. The uteri of *chs-1(ok1120)* homozygotes (upper panels) contain oocyte-like abnormal eggs in contrast to normal developing eggs present in heterozygotes [+/*chs-1(ok1120)*] (lower panels). Chitin staining was absent in the abnormal eggs but detected in the egg (arrow) adjacent to the spermatheca (arrowhead) of the heterozygote. Scale bar, 20 μm.

Detection of chitin in the eggshell and pharynx

A fluorescent probe incorporating the chitin-binding domain (ChBD) from chitinase A1 of *B. circulans* (Watanabe et al., 1994) was developed for specific detection of chitin in situ. This domain has been shown to bind chitin with high affinity while having only negligible affinity to other polysaccharides in biochemical assays (Hashimoto et al., 2000) and has been used to detect chitin in fungal cell walls (Hardt and Laine, 2004). In this study, a fusion protein of ChBD with maltose binding protein (MBP), which serves as a carrier protein, was produced and then labeled with different fluorescent dyes. The fusion with MBP did not alter the specificity of the ChBD since identical patterns of fluorescence in *C. elegans* were observed when ChBD was fused to green fluorescent protein (GFP) (Y. Zhang et al., unpublished data, 2005). The specificity of the probe was further confirmed by its lack of binding to eggs from mutant worms homozygous for a putative null mutation *chs-1(ok1120)* (Fig. 2C).

Using the probe, we detected chitin in wild type *C. elegans* and eggs laid by these worms. In intact hermaphrodites, fluorescence was detected in the eggshell of single-cell embryos located closest to the spermatheca, while other eggs in the uterus had a faint signal or none at all (Fig. 4A). However, after laid eggs were treated with bleach, they stained equally well (Fig. 4B), indicating that all eggs contain chitin.

Intact wild type worms were also found to contain chitin in the pharynx with similar patterns of fluorescence observed in larval and adult stages. Fluorescence was observed in the pharynx of *chs-1(ok1120)* mutant worms,

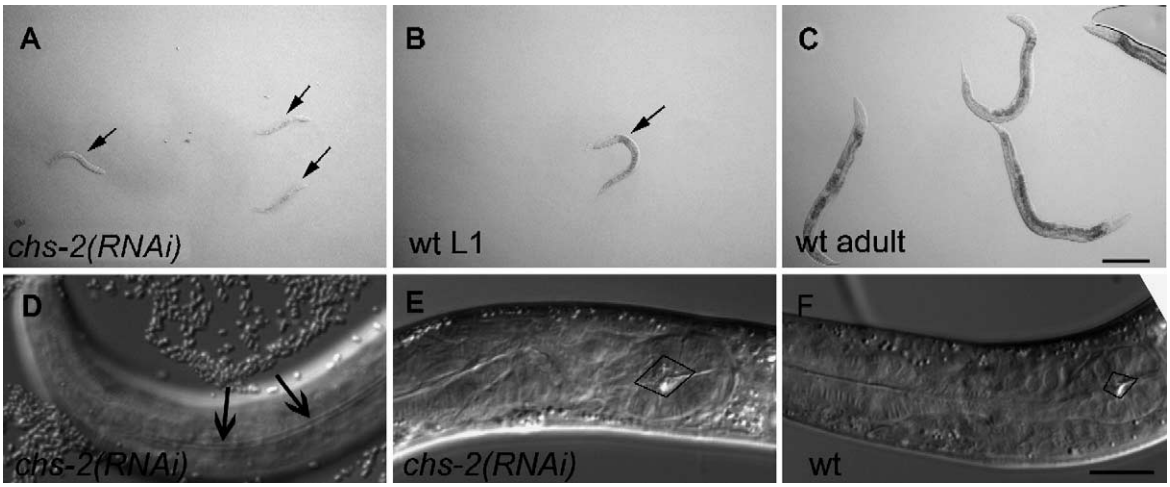

Fig. 3. Characterization of *chs-2* function by RNAi. DIC images of *chs-2(RNAi)* and wt worms. (A) 3.5 day old *chs-2(RNAi)* worms (arrows) arrested as L1 larvae. (B) Normal wt L1 larva (arrow). (C) 3.5 day old adult wt worms. (D) *chs-2(RNAi)* arrested worms have typical L1 alae (arrows). (E) *chs-2(RNAi)* arrested worms have a larger grinder (indicated) with disorganized and irregular morphology. (F) Normal grinder (indicated) of a wt L1. Upper panel scale bar, 100 μm; lower panel scale bar, 10 μm.

indicating that pharyngeal chitin is not the product of the *chs-1* gene (data not shown). To determine the location of chitin more precisely, the fluorescent structures in the pharynx were analyzed through serial microscopic Z sections. Fluorescence was observed in the lumen walls of the pharynx with greater intensity in particular regions as seen in two representative sections (Figs. 5A–B). In the buccal capsule, an intense signal was found along the lumen walls and was brightest in the mesostom region

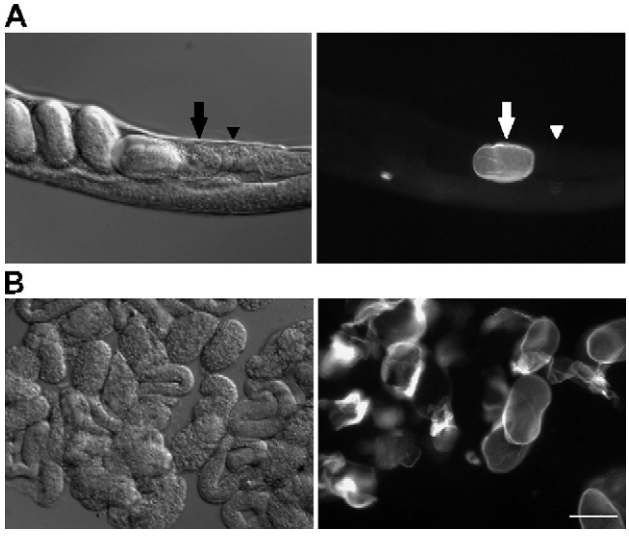

Fig. 4. Detection of chitin in the *C. elegans* eggshell. DIC (left) and fluorescent images (right) obtained following chitin detection using a rhodamine-labeled probe. (A) Visualization of chitin in eggs in whole worms. The fluorescent signal is present on the eggshell surrounding a single-cell stage embryo (block arrow) adjacent to the spermatheca (arrowhead), but not on the eggshells of more advanced embryos. (B) Visualization of chitin in isolated eggs pre-treated with bleach. All eggs with an eggshell showed a fluorescent signal. A few embryos lost their eggshells following the treatment and therefore showed no staining. Scale bar, 20 μm.

(Figs. 5A and C). At the junction between the buccal capsule and the procorpus, two fluorescent spots of about 1 μm in diameter were observed (Figs. 5A and C), likely representing a cross sectional view through a collar-like structure. From the procorpus to the isthmus, three identical chitinous longitudinal bundles were visible in serial sections (Figs. 5A–B), likely corresponding to the walls of the 3-fold symmetrical pharynx lumen (Albertson and Thomson, 1976). Each fluorescent bundle was wider in the anterior procorpus and became narrower towards the posterior procorpus (Fig. 5B). Upon closer examination, each bundle in the procorpus region consisted of two lateral narrow stripes running along the A–P axis (Fig. 5E), consistent with chitin being present on both sides of the lumen wall in each lobe. Measurements were obtained for the spacing between stripes within a bundle and between the bundles themselves (Fig. 5E) and they were also found to be in agreement with the dimensions of the triangular lumen of the pharynx (Albertson and Thomson, 1976). The fluorescent signal was relatively bright in the procorpus, faint in the metacorpus, stronger in the anterior isthmus and gradually tapered off towards the posterior isthmus (Figs. 5B and D). In the terminal bulb, there was a strong signal forming a ragged band corresponding to the grinder (Fig. 5F). The localization of chitin in cross sections spanning various regions of the pharynx is proposed in Fig. 6.

Expression of chs-2∴GFP reporter in the pharynx

To further support the role of chitin in the pharynx, the expression of *chs-2* was studied using a transcription GFP reporter. GFP was expressed throughout the entire pharynx (Fig. 7), at a low level in the region surrounding the buccal cavity and abundantly in the procorpus, metacorpus and terminal bulb. No GFP expression was

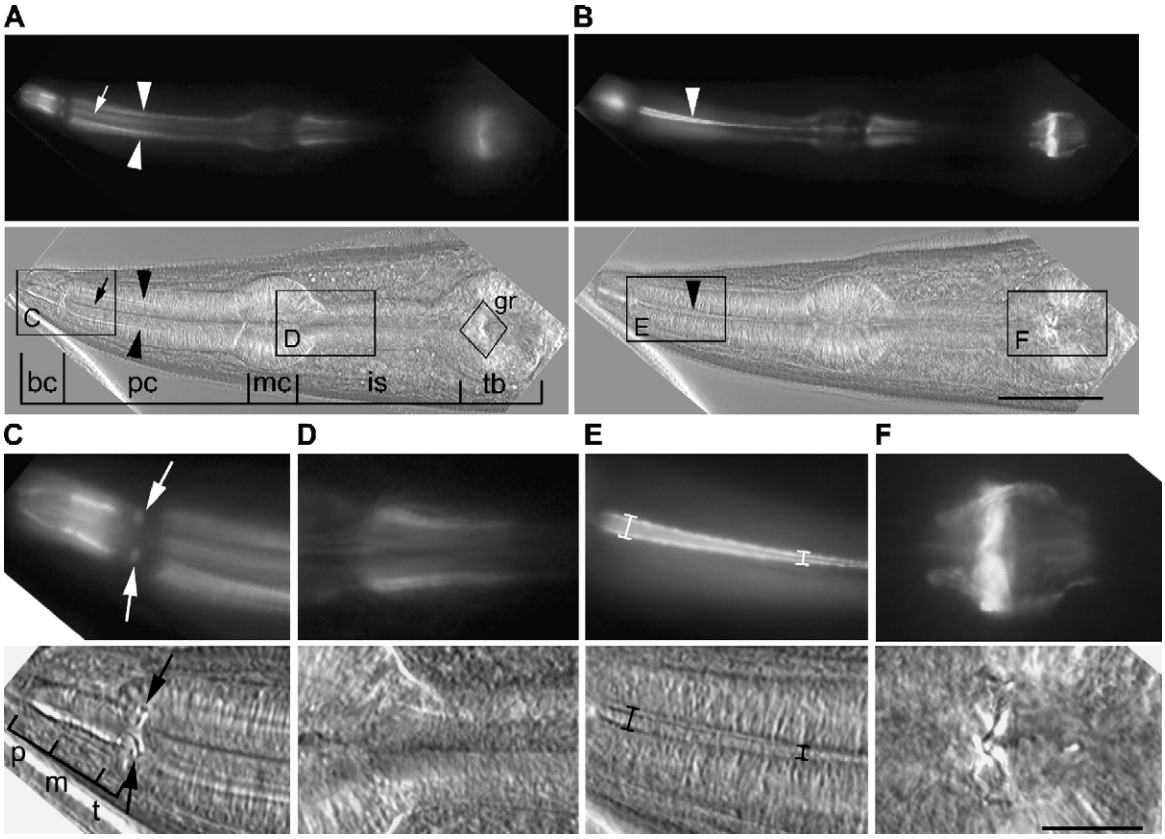

Fig. 5. Detection of chitin in the *C. elegans* pharynx. DIC (lower panels) and fluorescent images (upper panels) obtained following chitin detection using a rhodamine-labeled probe. Two representative Z-sections (~3.15 μm apart) are shown enabling visualization of the chitinous structures in the pharynx with enlargements (indicated) of selected regions. (A) Section showing two of the three chitinous bundles (arrowheads) and a third bundle present on a different focal plane partially in view (arrow). The various regions of the pharynx are indicated in the DIC image. Abbreviations are: bc, buccal capsule; pc, procorpus; mc, metacorpus; is, isthmus; tb, terminal bulb; gr, grinder. (B) Single chitinous bundle in focus (arrowhead). (C) Enlarged view of the buccal capsule to the anterior procorpus. Two fluorescent spots (arrows) are observed at the junction between the buccal capsule and the procorpus. The various regions of the buccal capsule are indicated in the DIC image. Abbreviations are: p, prostom; m, mesostom; t, telostom. (D) Enlarged view of the posterior metacorpus to the anterior isthmus. (E) Enlarged view of the anterior procorpus. The distance between the two chitinous stripes is indicated. (F) Enlarged view of the grinder region. Upper panel scale bar, 30 μm; lower panel scale bar, 10 μm.

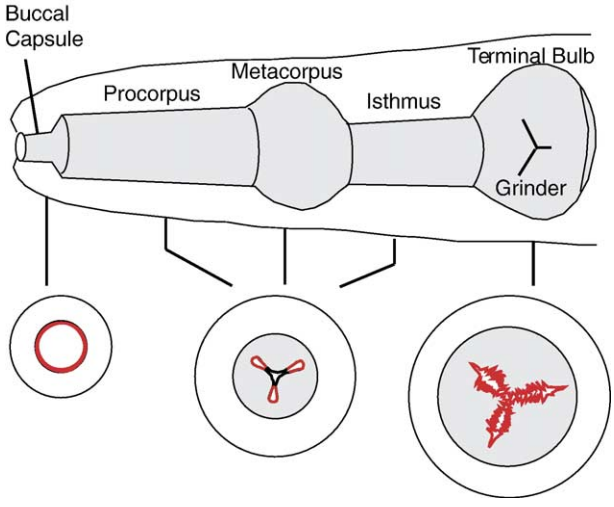

Fig. 6. A schematic representation of the proposed localization of chitin (red) in transverse sections of the pharynx (shaded gray).

observed with a GFP transcription reporter for *chs-1* (data not shown).

Discussion

In this report, we describe a detailed study of the roles of chitin and chitin synthase genes in *C. elegans*. We verified the open reading frames of the two predicted *C. elegans* chitin synthase genes by cDNA analysis and showed that each gene is essential for development. *chs-1* is critical for eggshell production while *chs-2* has an indispensable role in the pharynx. This conclusion is supported by our analysis of individual gene activities, and is consistent with our specific detection of chitin in the eggshell and the pharynx.

Gene function studies of *chs-1* and *chs-2* provided the most convincing evidence for their separate roles in development. RNAi and gene deletion analysis demonstrated the importance of *chs-1* in production of eggshell chitin. Eggshell defects of escalating severity were observed over time in the eggs produced by *chs-1(RNAi)* worms, likely

Fig. 7. Expression of a *chs-2*::GFP transcription reporter. GFP expression (upper panel), DIC (middle panel) and merged image (lower panel) of a late L3 larva are shown. The insert in the GFP panel shows the GFP expression in the buccal cavity region (bracket) with a 2-fold enlargement. Regions of the pharynx are indicated in the merged image using the abbreviations described in Fig. 5A. Scale bar, 20 μm.

reflecting decreasing gene activity associated with increasing RNAi potency as reported for other genes (Fire et al., 1998). The eggshell defects in their most severe form were also observed in worms carrying the *chs-1(ok1120)* null mutation. These phenotypes are consistent with the finding that *chs-1* transcripts are abundant in the ovary as detected by RNA in situ hybridization (Y. Kohara, personal communication, 2004), and the fact that the gene activity appears to be maternal since the phenotype produced by *chs-1(RNAi)* could not be rescued by fertilization with wild type sperm (Y Zhang et al., unpublished data, 2005) (Hanazawa et al., 2001). These data agree with the sterility observed in previous large-scale RNAi screens (Hanazawa et al., 2001; Kamath et al., 2003). In contrast, knockdown of *chs-2* by RNAi resulted in eggs that hatched normally but the L1 larvae failed to develop further. Closer examination of these worms revealed visible defects in the grinder. These phenotypic data correlate with our GFP transcription reporter studies and those of a previous report (Veronico et al., 2001) showing that *chs-2* is only expressed in the pharynx. Therefore, it is clear that the two chitin synthase genes in *C. elegans* have distinct functions. Non-overlapping roles have also been described for chitin synthases in yeast (Cabib et al., 2001) and suggested in insects (Arakane et al., 2004; Ibrahim et al., 2000; Ostrowski et al., 2002; Tellam et al., 2000; Zhu et al., 2002; Zimoch and Merzendorfer, 2002).

Further insights into the role of chitin synthases in *C. elegans* were obtained by our unequivocal detection of chitin in situ using a ChBD-based probe. This probe enabled us to circumvent the limitations of traditional indicators such as wheat germ agglutinin and calcofluor (Brydon et al., 1987; Fuhrman and Piessens, 1985; Neuhaus et al., 1997), both of which bind other polysaccharides (Neuhaus et al., 1997). We formally demonstrated that chitin is present in the *C. elegans* eggshell, which is consistent with studies on eggs from other nematode species (Wharton, 1980) and the observation that treatment of *C. elegans* eggs with chitinase together with chymotrypsin is able to weaken eggshells (Edgar, 1995). The chitin layer of nematode eggs has been proposed to provide structural strength, with the underlying lipid layer conferring impermeability to toxic chemicals (Bird and Bird, 1991; Rappleye et al., 1999). However, in our experiments, when the gene responsible for eggshell chitin, *chs-1*, was knocked down, eggs became not only fragile and abnormal in shape, but also permeable to small molecules. When chitin is absent from the eggshell as in the case of the *chs-1(ok1120)* null mutant, fertilized eggs failed to divide, indicating that a chitinous eggshell is needed for embryonic division. Furthermore, our study revealed that *C. elegans* eggs contain a bleach-sensitive layer overlying the chitin layer, since chitin was detectable only in intra-uterine eggs containing single-cell embryos but in all eggs irrespective of stage after bleach treatment. The formation of a similar outer layer has been described in the eggs of several other nematodes (Bird and Bird, 1991; Wharton, 1980).

We also discovered that chitin is present in the lumen walls of specific regions of the pharynx. The *C. elegans* pharynx consists of three regions: the buccal capsule that opens to the outside, a middle cylinder including the corpus and the isthmus with a 3-fold symmetrical lumen, and a terminal bulb containing a grinder (Albertson and Thomson, 1976). These parts coordinate to function as a feeding organ that collects, transports and grinds bacterial food (Avery and Shtonda, 2003; Avery et al., 1987). Chitin was mainly detected in the buccal cavity and grinder, and in narrow regions along the triangular lumen of the corpus and anterior isthmus. The expression of cytoplasmic *chs-2*::GFP reporter in these regions of the pharynx is consistent with the presence of chitin. This elaborate layout suggests that chitin may have critical roles in the functions of the pharynx, such as providing rigidity to the pumping pharynx and/or strength for the mechanical breakdown of food in the grinder. It also implies that, during development of the pharynx, sophisticated controls, such as spatial transcription regulation and protein targeting, are necessary for such precise chitin deposition. The proposed roles are consistent

with the phenotypes observed when the pharyngeal chitin is reduced by knocking down *chs-2* gene activity. The *chs-2(RNAi)* worms had a deformed grinder, and arrested as L1 larvae probably as a result of starvation. A similar starvation-induced larval arrest is known to occur when wild type L1 are deprived food upon hatching (Hope, 1999). Interestingly, when *chs-2(RNAi)* arrested larvae were fed on fluorescent latex beads, fluorescence was found in the intestine indicating that the lumen of the pharynx was not blocked (data not shown). However, further quantitative studies are needed to determine if the pharynx is less efficient at pumping and crushing.

The presence of chitin in the pharynx may help explain interesting aspects of *C. elegans* development. It has been recognized that the pharynx contains a specialized cuticle that is shed and regenerated at each larval molt (Wright and Thomson, 1981). Recently, it was shown that the growth of the buccal cavity is non-continuous and occurs only at molting (Knight et al., 2002). This may in part be due to the presence of chitin in the lumen wall, which could limit growth in a similar way to that described for the chitin-containing exoskeleton in insects (Merzendorfer and Zimoch, 2003). Therefore, when *C. elegans* larvae grow, the chitin layer would have to be removed and re-synthesized to accommodate a larger size. This may also explain the L1 arrest phenotype observed in *chs-2(RNAi)*. Consistent with a possible role in molting, the *chs-2* gene was shown to be transcribed immediately prior to each larval molt (Veronico et al., 2001).

The distinct functions of the two chitin synthase genes demonstrated here in *C. elegans* may apply to other nematodes. Although both are typical chitin synthases, the encoded amino acid sequences of these two genes show some differences and can be distinguished from each other in sequence alignment analyses. CHS-1 is more similar to CHS-1 from the parasitic nematodes *B. malayi* (Harris et al., 2000), *D. immitis* (Harris and Fuhrman, 2002) and *Onchocerca volvulus* (Foster et al., 2005) and they are considered orthologs. In the case of *B. malayi* and *D. immitis*, the *chs-1* ortholog was found to be expressed in the germ line (Harris and Fuhrman, 2002; Harris et al., 2000). This supports the notion that these nematode CHS-1 orthologs are also responsible for the production of eggshell chitin. On the other hand, CHS-2 is more related to the insect chitin synthase genes than to CHS-1 (Harris et al., 2000; Zhu et al., 2002). Orthologs of CHS-2 have also recently been identified in *B. malayi* and *O. volvulus* (Foster et al., 2005). It is likely that these CHS-2 orthologs may also produce pharyngeal chitin, although this remains to be determined. Interestingly, lectin and calcofluor binding studies have suggested the presence of chitin in the pharynx of the porcine parasite *Oesophagostomum dentatum* (Neuhaus et al., 1997), implying that this nematode may possess a *chs-2* ortholog.

These studies have shown that the two chitin synthase genes in *C. elegans* have essential and non-overlapping roles in development. The likely presence of orthologs of these two genes in other nematodes and their absence from vertebrates and plants indicate that these enzymes warrant continued investigation as drug targets in parasitic species.

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