Report

Current Biology

Chitin Is Endogenously Produced in Vertebrates

Highlights

- Chitin synthase gene activity was detected in zebrafish, salmon, and axolotl
- Chitin per se was detected in situ in multiple fish and axolotl tissues
- Knockdown of chitin synthase in zebrafish embryos resulted in reduction of chitin
- Polysaccharides from fish scale epithelia exhibited spectral properties of chitin

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In Brief

It has been widely held that vertebrates do not produce chitin, a glyco-polymer found abundantly in nature. However, using multiple experimental approaches, Tang et al. provide strong evidence for the endogenous production of chitin in fishes and amphibians, raising questions as to its biological role within the vertebrates.

Accession Numbers

KM203892

Tang et al., 2015, Current Biology *25*, 897–900
March 30, 2015
http://dx.doi.org/10.1016/j.cub.2015.01.058

CellPress

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Chitin Is Endogenously Produced in Vertebrates

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http://dx.doi.org/10.1016/j.cub.2015.01.058

SUMMARY

Chitin, a biopolymer of N-acetylglucosamine, is abundant in invertebrates and fungi and is an important structural molecule [1, 2]. There has been a longstanding belief that vertebrates do not produce chitin; however, we have obtained compelling evidence to the contrary. Chitin synthase genes are present in numerous fishes and amphibians, and chitin is localized in situ to the lumen of the developing zebrafish gut, in epithelial cells of fish scales, and in at least three different cell types in larval salamander appendages. Chitin synthase gene knockdowns and various histochemical experiments in zebrafish further authenticated our results. Finally, a polysaccharide was extracted from scales of salmon that exhibited all the chemical hallmarks of chitin. Our data and analyses demonstrate the existence of endogenous chitin in vertebrates and suggest that it serves multiple roles in vertebrate biology.

RESULTS AND DISCUSSION

In our surveys of vertebrate genomes and transcriptomes, numerous "unknown" fish and amphibian sequences were detected that exhibited striking homology to bona fide invertebrate chitin synthase (CHS) genes (BlastX, E value < 10^{-150}). These sequences had likely been overlooked as coding for CHSs during the ab initio annotation process. This list (Table S1) includes CHS genes of several fishes, a salamander, and two species (zebrafish and *Xenopus*) previously identified in a recent report on metazoan chitin synthase genes [3]; amniote CHS genes have not been identified. A phylogenetic tree showing the interrelationships among the identified vertebrate CHS orthologs is given in Figure S1A.

To investigate whether fish endogenously produce chitin, we employed the zebrafish (*Danio rerio*) model system. All zebrafish experiments were conducted in accordance with the Animal Care and Use Committee at the Benaroya Research Institute. The zebrafish genome assembly [4] revealed the existence of four CHS genes: three closely related and linked genes on chromosome 18 and a gene on chromosome 13 (Table S1). Published Affymetrix microarray experiments (Supplemental Information) indicated that the chromosome 13 gene (*chs1*) is preferentially expressed during embryonic and early larval development. Whole-mount in situ hybridization (ISH) showed expression of *chs1* in the larval gut (Figures 1A–1C), and subsequent transverse sections revealed expression in cells within the epithelial wall and in mesenchymal cells adjacent to the gut (Figure 1D) [5].

For detection of chitin in situ, we used affinity histochemistry with fluorescent probes containing a highly specific chitin binding domain (CBD) (Supplemental Information) [6]. When applied to a zebrafish developmental series, chitin was detected as early as 3 days post-fertilization (dpf) as multiple fibers within the intestinal bulb (Figures S1B and S1C); an endoderm population dorsal to the gut also showed chitin signals (Figure S1C) that largely disappeared by 4 dpf (cf. Figure 1E). At 4–5 dpf, the fibers coalesced in the middle intestine and were distributed throughout the digestive tract (Figures 1E–1G; Movie S1). The bulk of this chitin was extracellular and distributed throughout the intestinal lumen; however, cellular signals were detected among cells within the lumen wall and mesenchymal cells ventral to the intestinal tract (Figure 1F). The similarity in the *chs1* ISH patterns and chitin localization (Figures 1A–1F) suggest that chitin is being synthesized by cells in and around the developing intestine and sequestered within the lumen. Validation of the robustness of our chitin detection assay is shown in Figures S1D and S2A–S2C.

In order to demonstrate that chitin production is coupled with CHS expression, we knocked down the *chs1* gene. Two different splice-blocking morpholinos were designed to *chs1* and microinjected into fertilized eggs along with their mismatch controls (Supplemental Information and Figure S2E). The knockdowns resulted in marked reduction in chitin signals in the gut (Figure S2F) and are consistent with the early expression profile of *chs1*.

We also assayed for chitin in zebrafish scales since our initial RT-PCR survey indicated expression of a zebrafish chromosome 18 *CHS* gene in this tissue (Figure S2D). Fish scales are primarily comprised of a hard, partly mineralized collagenous matrix as well as partially overlying epithelium (skin). Unlike in the gut, most of the chitin signals were observed intracellularly in epithelial goblet and club cells (Figures 1H and 1I). The chitin

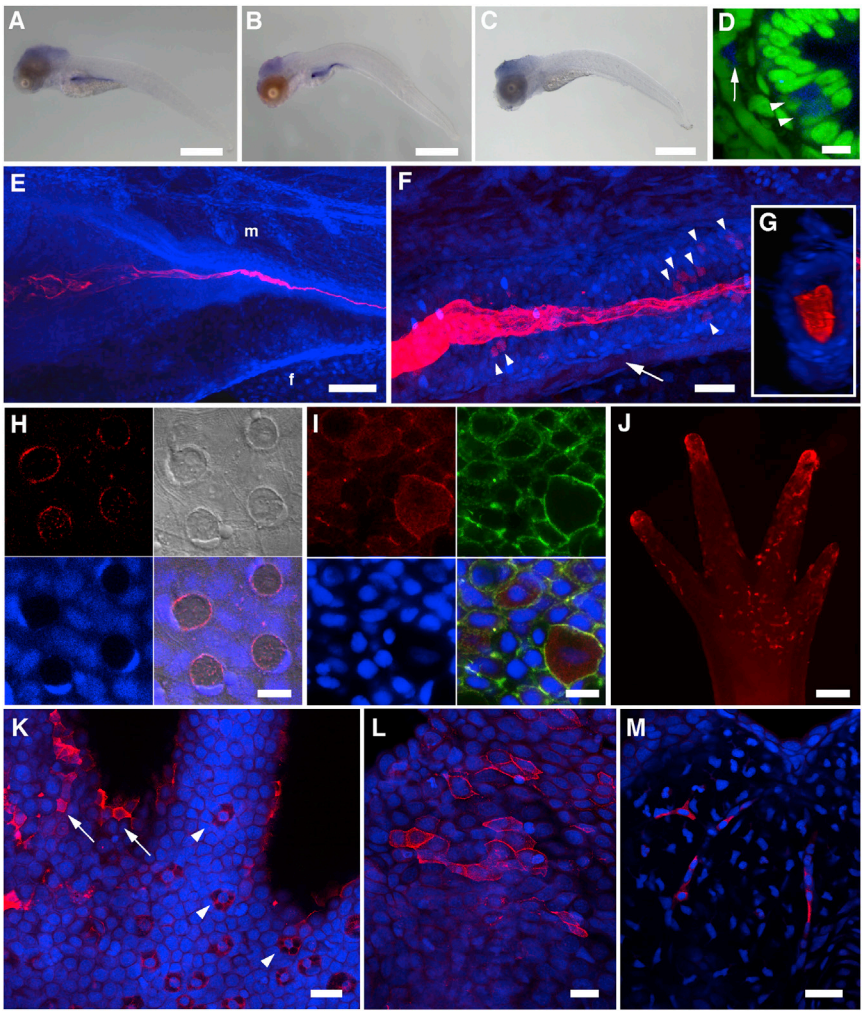

Figure 1. Detection of Chitin in Zebrafish and Axolotl

(A–D) In situ hybridization for *chs1*. NBT/BCIP signals were observed primarily in the developing digestive tract in 5 dpf (A) and 10 dpf (B) larvae, indicating that *chs1* is expressed during larval development and in the gut. A representative "sense" control larva is shown in (C). No NBT/BCIP signals were observed in the developing gut of control larvae at either 5 or 10 dpf, although background staining was observed in the cranial region. Scale bars represent 500 μm.

(D) The ventral region of the mid-intestine of a 10 dpf larva (transverse section, different specimen than B) shows *chs1* expression in select cells within the columnar intestinal epithelium (arrowheads) and in mesenchymal cells adjacent to the intestinal epithelium (arrow). NBT/BCIP deposition was imaged in the infrared range with confocal microscopy (blue), and nuclei were stained with Sytox Green (green). Scale bar represents 10 μm.

(E–M) A chitin probe (CBD-546, shown in red) was used for affinity histochemistry on fixed whole-mount specimens of zebrafish larvae (E–G) and scales (H and I) and axolotl larval forelimbs and tails (J–M). Nuclei were stained with TOTO-3 (blue), and rostral is to the left in (E) and (F).

(E) 3D-rendered projection of a 4 dpf zebrafish larva. "f" and "m" represent preanal finfold and somitic muscle, respectively; the "m" designation is above the approximate junction of the middle intestine. Chitin is observed in the developing larval gut, appearing fibrillar in the lumen of the intestinal bulb and coalescing as the lumen narrows posteriorly. Scale bar represents 100 μm.

(F) 3D-rendered projection of the mid-intestinal lumen from a 5 dpf zebrafish larva. The fibrous nature of the chitin is obvious, and several cells within the intestinal epithelial wall are observed that show strong chitin signals (arrowheads). A population of mesenchymal cells was also found to be positive for chitin signals (arrow). Scale bar represents 20 μm.

(G) Transverse section from a 3D volume-rendered reconstruction of the image in (F) showing chitin distributed throughout the lumen of the mid-intestine.

(H) Confocal image of a field of zebrafish scale epithelium. Panels show the following: CBD-546 (top left), DIC (top right), Sytox Green nuclear counterstain (pseudocolored in blue, bottom left), and merged image (bottom right). The four circular structures with red chitin signals are goblet cells that show polarized, crescent-shaped nuclei; polygonal concentric structures on the DIC overlay are squamous epithelial cells. Aggregations of small red punctate spots probably represent extracellular chitin. Scale bar represents 10 μm.

(I) Maximum intensity projection of a field of scale epithelial cells in which chitin is prominent throughout the cytoplasm of club cells. Panels show the following: CBD-546 (top left), phalloidin-FITC (top right), TOTO-3 nuclear counterstain (bottom left), and merged image (bottom right). The scale specimen was simultaneously stained for F-actin using phalloidin-FITC to help distinguish individual cells. Scale bar represents 10 μm.

(J) A forelimb (palm side up) of a larval axolotl after chitin histochemistry, imaged on a stereoscope. The tips of the developing digits and the palm area that show extensive chitin signals are primarily superficial epithelial cells. Scale bar represents 200 μm.

(K) A field from the base of a digit from a different forelimb than shown in (J), imaged by confocal microscopy. Arrowheads indicate several specialized Leydig epidermal cells, and the arrows identify a few of the superficial epithelial cells seen in (J). Scale bar represents 50 μm.

(L and M) Caudal fin of larval axolotl.

(L) Maximum intensity projection showing a subset of surface epithelial cells that exhibit strong chitin signals. Scale bar represents 50 μm.

(M) A deeper optical section showing strong chitin signals in the fibroblast-like cells of the fin mesenchyme. Scale bar represents 50 μm.

See also Figures S1 and S2.

in the goblet cells was largely restricted to the periphery of the cells, whereas it was distributed throughout the cytoplasm in the club cells. Our findings on zebrafish scales were corroborated via RT-PCR and chitin histochemistry on scales of juvenile Atlantic salmon, *Salmo salar* (Figure S2D; Movie S1).

CHS genes were also identified in two amphibian species, *Xenopus* and axolotl. Transcriptome data indicated that a CHS gene was expressed in larval axolotls (regenerating limb, Table S1); thus, appendage tissues from larvae (stage ~52–53) were obtained and used for chitin histochemistry. Chitin was found predominantly in superficial epidermal cells resembling squamous epithelia, Leydig epidermal cells, and mesenchymal (fibroblast-like) cells (Figures 1J–1M). The strong signals on the tips of the forelimb digits (Figure 1J) are due to superficial epidermal

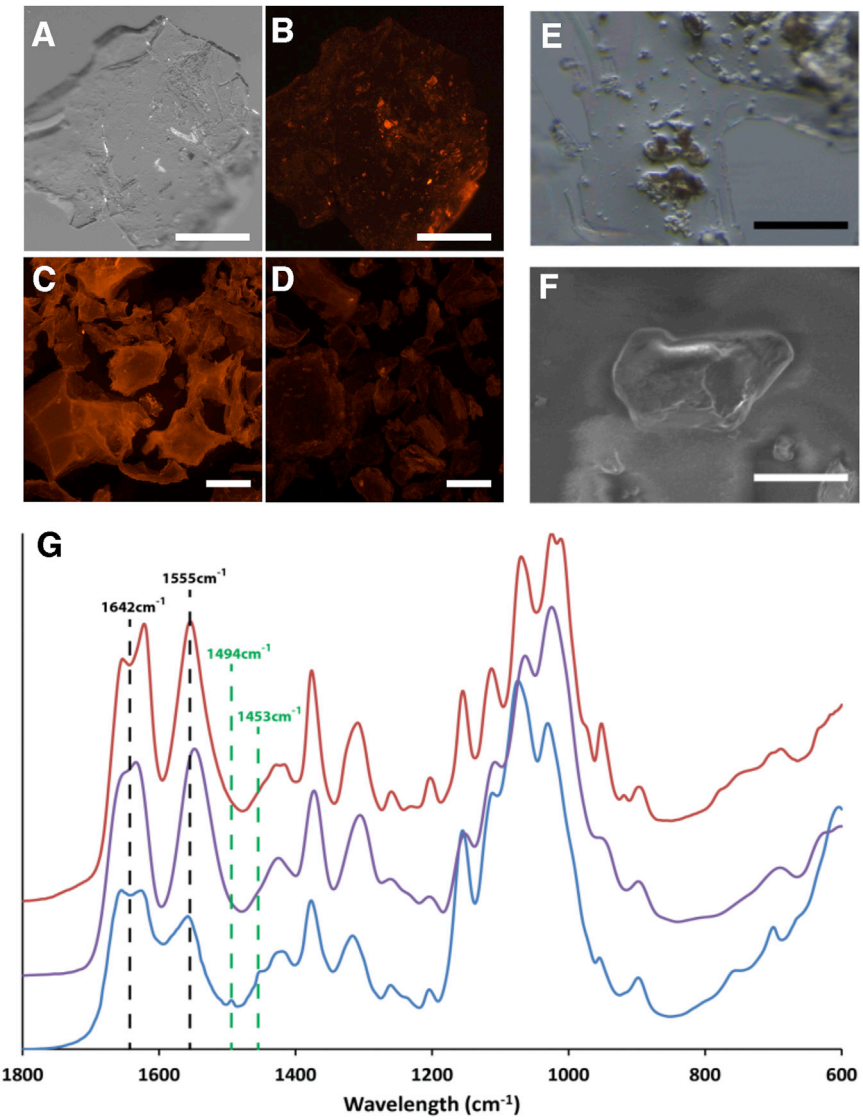

Figure 2. Extraction and Analysis of Chitin from Scale Material of Atlantic Salmon

(A–D) Skin (including scales) from a specimen of farmed Atlantic salmon was subjected to chitin extraction following procedures outlined in Supplemental Experimental Procedures. After extraction, the precipitated pellets were collected, stained for chitin with the CBD-546 probe, and imaged using an epifluorescent stereomicroscope. Scale bars represent 500 μm.

(A) A representative pellet from the chitin extraction, photographed using visible light only.

(B) CBD-546 chitin detection of the same pellet as in (A), showing strong punctate signals among a background of unstained or weakly stained material that has been shown to be comprised largely of deacetylated chitin.

(C and D) Controls included purified α-chitin from shrimp shells (C) and chitosan (75%–85% deacetylated shrimp shell chitin) (D). The controls demonstrate that our CBD-546 probe is very specific to intact chitin as it did not give appreciable signals with chitosan.

(E) Stereomicrograph of an area containing several chitinous granules within the thin, acid-soluble film. Scale bar represents 100 μm.

(F) SEM image of a chitinous granule. Scale bar represents 40 μm.

(G) FTIR analysis of the fingerprint region of the chitinous granules (blue) and its comparison with β-chitin from squid pen (purple) and α-chitin from shrimp shells (red). Two additional resonances—1,494 cm^{-1} and 1,453 cm^{-1}—stand out as main differences of our extracted chitin with respect to the controls and are diagnostic of aromatic ring breathing modes that were subsequently identified as polystyrene contamination of the salmon material (discussed in Supplemental Information).

cells. These findings in axolotl, including chitinase validation, (Figure S2C) are similar to those seen for fish scales.

Ultimate support for the existence of vertebrate chitin is isolation of chitin per se from a vertebrate. For this experiment, we used fresh scales from Atlantic salmon and employed a fastidious chemical extraction strategy. From 60 g of dried scales, we extracted a precipitate of ~1.5 mg of chitinous material for analysis. This low yield and the intractability of insoluble chitin to most characterization methods necessitated its analysis via microscopic Fourier transform infrared (FTIR) spectrometry. Micrographs of the precipitate collected from salmon scales are shown in Figures 2A, 2B, 2E, and 2F, and the FTIR spectrum of the insoluble granules confirms its identity as chitin (Figure 2G). The fingerprint region in the FTIR spectrograph revealed a split of the amine I band into two bands at 1,655 and 1,626 cm^{-1}, resulting from the two types of hydrogen bonds in the antiparallel alignment of α-chitin, though further analyses will be required to definitively rule out other isomeric forms. Our chemical results (expanded upon in the Supplemental Information) demonstrate that fish scale material contains chitin and are in keeping with molecular and histochemical data of the scale epithelia of salmon and zebrafish.

First isolated from fungi over 200 years ago, chitin has since been identified from a wide range of organisms [1, 2], including fossils [7, 8]. It is an integral structural component of invertebrate organisms, and its tractability to industrial-scale chemical extraction and bioengineering has enabled its use in many applications in biomedicine and technology [1, 9]. The difficulty of its extraction from vertebrates, however, likely has contributed to its assumed absence, and there has been but one report of the detection of a fish chitin some 30 years ago [10]. We show here the existence of endogenous chitin in fishes and amphibians, which collectively comprise over half of all extant vertebrates. Efforts can now be focused on elucidating the underlying biology and biochemistry of vertebrate chitin.

ACCESSION NUMBERS

The sequencing data reported in this paper have been deposited in the NCBI under GenBank accession number KM203892.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Discussion, Supplemental Experimental Procedures, two figures, one table, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.01.058.

AUTHOR CONTRIBUTIONS

J.J.S. and C.T.A. conceived the study. W.J.T. and C.T.A. designed and carried out the bioinformatics experiments. W.J.T. and C.T.A. designed the genomics and developmental biology experiments, which included affinity histochemistry, embryology, microscopy, and imaging. W.J.T. and C.T.A. performed the experiments. W.J.T. procured confocal images. J.G.F. and J.J.S. designed the chemical extraction experiments. J.G.F. and J.J.S. performed chemical extractions of chitin from fish scales and skin. J.G.F. developed extended procedures for chitin purification and carried out chitin characterizations, including FTIR and imaging. W.J.T., J.G.F., J.J.S., and C.T.A. analyzed the data and wrote the paper.

ACKNOWLEDGMENTS

The following individuals provided assistance, resources, and/or advice: Jesse Bengtsson, Jeramiah Smith, Igor Schneider, Michael Rego, Andrew Bornstein, Paul Freddura, Andrew Nagle, John Rawls, Gail Mueller, John Cannon, Larry Dishaw, Mark Messerli, John Dowling, and Gary Litman. Yinhua Zhang (New England Biolabs) provided the plasmid used for producing the SNAP-tag-CBD fusion protein, and Ronald Kwon helped with analysis of zebrafish microarray data. Support for this work was provided, in part, by NIH grants RR014085 and GM095471 (to C.T.A.) and discretionary funds from our respective institutions.

Received: November 11, 2014
Revised: December 19, 2014
Accepted: January 21, 2015
Published: March 12, 2015

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