In many organisms, sophisticated mechanisms facilitate release of peptides in response to extracellular stimuli. In the ciliate *Tetrahymena thermophila*, efficient peptide secretion depends on specialized vesicles called mucocysts that contain dense crystalline cores that expand rapidly during exocytosis. Core assembly depends of endoproteolytic cleavage of mucocyst proproteins by an aspartyl protease, cathepsin 3 (*CTH3*). Here, we show that a second enzyme identified by expression profiling, Cth4p, is also required for processing of proGrl proteins and for assembly of functional mucocysts. Cth4p is a cysteine cathepsin that localizes partially to endolysosomal structures and appears to act downstream of, and may be activated by, Cth3p. Disruption of *CTH4* results in cells (∆*cth4*) that show aberrant trimming of Grl proproteins, as well as grossly aberrant mucocyst exocytosis. Surprisingly, ∆*cth4* cells succeed in assembling crystalline mucocyst cores. However, those cores do not undergo normal directional expansion during exocytosis, and they thus fail to efficiently extrude from the cells. We could phenocopy the ∆*cth4* defects by mutating conserved catalytic residues, indicating that the in vivo function of Cth4p is enzymatic. Our results indicate that as for canonical proteins packaged in animal secretory granules, the maturation of mucocyst proproteins involves sequential processing steps. The ∆*cth4* defects uncouple, in an unanticipated way, the assembly of mucocyst cores and their subsequent expansion and thereby reveal a previously unsuspected aspect of polypeptide secretion in ciliates.

**Regulated secretion of peptides from intracellular stores plays many key roles in intercellular communication and tissue coordination in animals** (1). A highly specialized organelle in neuroendocrine cells, the secretory granule, is required for peptide generation, storage, and release (2). The bioactive peptides are generated within secretory granule formation by the action of proteolytic enzymes on polypeptide precursors (3). Proteolytic processing occurs in a post-trans-Golgi network (TGN) compartment, the immature granule, to which propeptides are sorted away from bulk protein traffic, and is a multistep process involving several classes of enzymes (4–6). The best studied among these are a family of serine proteases called prohormone convertases (7). Related to bacterial subtilisins, prohormone convertases (PCs) catalyze endoproteolytic cleavage at defined motifs in their substrates. The products of that cleavage can then undergo trimming, for which one important factor is carboxypeptidase E (CPE) (8). Defects in both PCs and CPE are linked with disease (9, 10).

Signaling via peptide secretion appears to be widespread in eukaryotes and has also been well documented for fungi, amoebae, and plants (11–13). In the oligohymenophorean ciliates *Tetrahymena thermophila* and *Paramecium tetraurelia*, regulated peptide secretion occurs from organelles that share striking similarities with secretory granules in animals (14, 15), although they may be more closely related to lysosomes (16). Called mucocysts in *Tetrahymena* and trichocysts in *Paramecium*, these organelles serve to concentrate a subset of newly synthesized proteins in the secretory pathway and to store them in the form of elaborately structured units (17). Such stimuli provoke mobilization of extracellular calcium, which promotes exocytic membrane fusion (18, 19). During exocytosis, the secretory proteins are rapidly extruded, due to the fact that the crystalline lattices expand rapidly and directionally. Thus, the function of secretory organelles in ciliates depends upon the assembly of protein crystals that can undergo spring-like elongation (20, 21).

The most abundant proteins stored in and released from *Tetrahymena* mucocysts belong to two families, called GRT (for granule tip) and GRL (for granule lattice) (22, 23). Studies of the Grl proteins, and the related *P. tetraurelia* tmp proteins, have demonstrated that extensive proteolytic processing occurs during mucocyst and trichocyst synthesis (20, 24–26). Processing is essential to generate the crystal-ordered luminal core, whose formation can therefore be thought of as the product of a morphogenetic program (26). The enzymes involved, however, and the precise roles of processing in core assembly have in general only been inferred. Recently, we reported that an aspartyl cathepsin, Cth3p, plays an essential role, since disruption of the *CTH3* gene resulted in cells (∆*cth3*) that completely failed to process Grl proproteins and, consequently, to assemble crystalline mucocyst cores (27). *CTH3*

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This article is dedicated to our late colleague, friend, and mentor, Don Steiner, a pioneer in the field of proprotein processing.

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was originally identified as a candidate based on expression profiling, which is a powerful approach in this organism thanks to rich databases for gene expression (28–32).

Here, we show that a second enzyme identified by expression profiling, Cth4p, is also required for processing of proGrl proteins and for assembly of functional mucocysts. Cth4p is a cysteine cathepsin that localizes to endolysosomal structures and appears to act downstream of Cth3p. Disruption of CTH4 results in cells (Δcth4) that show aberrant processing of Grl proteins during mucocyst biogenesis. Surprisingly, Δcth4 cells succeed in assembling crystalline mucocyst cores. However, those cores do not undergo normal directional expansion and thus fail to efficiently extrude from the cells, which therefore demonstrate grossly aberrant regulated exocytosis.

MATERIALS AND METHODS

Cell culture. Wild-type T. thermophila strains CU428.1 and B2086 were cultured in SPP medium (1% proteose peptide 0.2% dextrose, 0.1% yeast extract, 0.003% ferric EDTA). Experimental cultures were grown at 30°C with shaking at 100 rpm to 2 × 10^5 to 4 × 10^5 cells/ml unless otherwise indicated. Culture densities were determined using a Z1 Coulter Counter (Beckman Coulter Inc., Indianapolis, IN). All reagents were from Sigma-Aldrich Chemical Co. unless otherwise indicated. Details of T. thermophila strains are in Table 1. Strains described as wild type in the text refer to CU428.1, which is wild type with respect to mucocyst exocytosis.

Gene expression profiling and in silico analyses. Expression profiles were derived from the Tetrahymena Functional Genomics Database (http://tfgd.lblb.ac.cn/); for graphing, each profile was normalized to that gene’s maximum expression level. Alignment of protein sequences was done using ClustalX (1.8) with default parameters.

Phylogenetic tree construction. Using protein BLAST (blastp), the T. thermophila CTH4 and CTH3 genes were used to identify potential homologs in ciliates, apicomplexans, Arabidopsis, and Homo sapiens, listed in Table S2 in the supplemental material. For tree building, the top hits were selected from each lineage, assembled, and aligned with ClustalX (1.8), and maximum-likelihood trees were constructed with MEGA5 (Molecular Evolutionary Genetics Analysis [http://www.megasoftware.net/]). Gapped regions were excluded in a complete manner, and percentage bootstrap values were derived from 1,000 replicates.

Expression of cathepsin 4 (CTH4) gene fusions. The Gateway (Invitrogen, Grand Island, NY) system was used to engineer cyan fluorescent protein (CFP)-tagged CTH4 (THERM_00445920). The cloning strategy and expression conditions were identical to those used for CTH3 (27).

Generation of cathepsin 4 knockout strains. The CTH4 upstream region was amplified (1,688 bp), together with a portion of the open reading frame (ORF) plus downstream flank (1,793 bp total), which were subsequently subcloned into the SacI and XhoI sites of the neo4 cassette, respectively, using in-fusion cloning (Clontech, Mountain View, CA). The construct resulted in deletion of CTH4 genic region from ~50 to 925. The sequences of the primers are listed in Table S1 in the supplemental material. To assess the gene disruption, total RNA was isolated using an RNasy mini kit as per the manufacturer’s instructions (Qiagen, Valencia, CA). Forward and reverse primers used for CTH4 are given in Table S1 in the supplemental material. The presence of the CTH4 transcripts was assayed by one-step reverse transcription-PCR (RT-PCR) (Qiagen, Valencia, CA).

Biologic transformations. The knockout vector was linearized by digestion with KpnI and SapI and transformed into CU428.1 cells by biolistic transformation (33).

Expression of Cth4p-green fluorescent protein (Cth4p-GFP) at the endogenous locus. CTH4 (THERM_00445920) and 817 bp of CTH4 downstream genomic sequence were amplified and cloned into the BamHI and HindIII sites of pmeGFP-neo4, respectively, by in-fusion cloning. To generate enzymatically disabled Cth4p, we used pCTH4mEGFP-neo4 vector as the substrate. We generated Cys552→Ala and His562→Ala mutations, in which selected TGC (Cys) and CAC (His) codons in CTH4 were replaced by GCA (Ala) and GCT (Ala), respectively, using the GeneArt site-directed mutagenesis PLUS kit (Invitrogen, Grand Island, NY). Final constructs were confirmed by DNA sequencing.

Expression of Cth4p-6×His. CTH4, without the stop codon, was amplified using a forward primer containing a Pmel restriction site and a reverse primer containing the coding sequence for 6×His, followed by a stop codon and an Apal restriction enzyme site. The amplified product

### Table 1: Description of *Tetrahymena* strains

| Strain name | Phenotype | Details of relevant genetic modification | Source |
|-------------|-----------|------------------------------------------|--------|
| B2086       | Wild type for exocytosis | None | J. Gaertig (University of Georgia, Athens, GA) |
| CU428       | Wild type for exocytosis | None | P. Bruns (Cornell University, Ithaca, NY) |
| UC810       | Δcth4; no detectable CTH4 expression | Replaces nucleotides ~50 to 925 of macronuclear CTH4 ORF with NEO4 cassette | This study |
| Unstable cell line | High-level inducible expression of CFP-tagged Cth4p (cth4-1) | C-terminal fusion of Cth4p and CFP, expressed under the control of the MTT1 promoter, on the minichromosome | This study |
| UC811       | Endogenous-level expression of GFP-tagged Cth4p (cth4-2) | C-terminal fusion of Cth4p and GFP, integrated at the macronuclear CTH4 locus | This study |
| UC812       | Endogenous-level expression of enzymatically disabled, GFP-tagged Cth4p (cth4-3) | Mutated variant of Cth4p (Cys552→Ala; His562→Ala), C-terminally fused to GFP, integrated at the macronuclear CTH4 locus | This study |
| UC813       | Inducible expression of His-tagged Cth4p (cth4p-6×His) | C-terminal fusion of Cth4p and 6His, expressed under the control of the MTT1 promoter, at the macronuclear MTT1 locus of CU428 | This study |
| UC814       | Inducible expression of His-tagged Cth4p (cth4p-6×His) | C-terminal fusion of Cth4p and 6His, expressed under the control of the MTT1 promoter, at the macronuclear MTT1 locus of Δcth3 (UC803) | This study |

* rDNA, ribosomal DNA.
was cloned into the pNCVB vector as described previously (27). The con-
struct was linearized by digestion with SfiI and transformed into CU428.1
and Δcth3 cells by biolistic transformation, and transformants selected
using 60 µg/ml of blasticidin and 1 µg/ml of CdCl₂. To induce transgene
expression in growth or starvation media, cells were incubated for 2 h in
1 µg/ml of CdCl₂ (for SPP medium) or 0.1 µg/ml of CdCl₂ (for 10 mM Tris
[pH 7.4]).

Live-cell microscopy. Live-cell imaging of cells expressing GFP-
tagged fusion proteins was performed as described recently (27). Cth4p-
GFP cultures were analyzed at ×3 × 10⁵ cells/ml unless otherwise indicated. Simultaneous imaging of Cth3-GFP with Lysotracker (Invitrogen) or
FM4-64 (Life Technologies, CA) was performed as recently described
(27).

Electron microscopy. Cells were grown overnight to stationary phase
(10⁶/ml), washed, fixed in 2% glutaraldehyde, 1% sucrose, and 1% os-
mium at 25°C in 0.1 M sodium cacodylate buffer, and section stained with
uranyl acetate and lead citrate after embedding. Thin sections were viewed
in a Tecnai G2 F30 Super Twin microscope (FEI).

Dibucaine and alcian blue stimulation. Dibucaine and alcian blue
stimulation of exocytosis was performed as described previously (27, 34).
Importantly, cells were washed and suspended in fresh medium immedi-
ately prior to stimulation. To assess secretion following dibucaine stimula-
tion, 2 ml of the cell-free supernatants following low-speed centrifuga-
tion were carefully withdrawn and precipitated with 10% trichloroacetic
acid (TCA). To remove flocculent, the remaining supernatant was as-
pirated, and the tube was cut with a razor just above the flocculent layer.
Flocculent was then removed using a wide-mouth 200-µl pipette tip.

Flow cytometry. Detergent-permeabilized and antibody-stained cells
were analyzed by FACS (BD FACSCalibur), as described previously (35).  

Immunofluorescence. To visualize mucocyst or mucocyst interme-
diates, cells were fixed, permeabilized with detergent, immunolabeled
with monoclonal antibody (MAb) 5E9 or MAb 4D11, and analyzed as
described previously (27). For simultaneous imaging of Cth4p-CFP/GFP
and mucocyst core proteins, cells were double stained with mouse MAb
5E9 and rabbit anti-GFP (27). Cells were imaged using a Leica SP5 II
confocal microscope, and image data were analyzed as previously de-
scribed (27).

SDS-PAGE and Western blotting. Whole-cell lysates and Western
blots were prepared as described previously (27). GFP-tagged fusion pro-
teins were immunoprecipitated from detergent lysates using polyclonal
rabbit anti-GFP antiserum as described previously (16).

Isolation of secreted mucocyst contents for mass spectrometry. Af-
after dibucaine stimulation, 45 ml of cells at 10⁶/ml was pelleted (15 ml/ 
tube) for 2 min at top speed (2,000 × g) in a clinical centrifuge. All sub-
sequent steps were at 4°C. After the supernatant was aspirated, the tubes
were cut with a razor just about the flocculent layer, and the top of the
flocculent layers were collected using a wide-mouthed transfer pipette and
transferred to 1.5-ml conical tubes. Flocculent was then diluted 10-fold
with 10 mM Tris (pH 7.4) and pelleted at 5,000 × g for 5 min, to extract
small pellets of cells that were trapped in the flocculent. The flocculent
samples were checked by light microscopy for cellular contamination, and
dilution and repelleting was repeated 5 or 6 times until no cells were visible.
The final samples were dissolved in 2 × SDS-PAGE sample buffer, incubated
for 15 min at 90°C, and then centrifuged (18,000 × g, 20 min, 4°C). The
small solid pellets were discarded and the supernatants resolved by 4 to 20% SDS-
PAGE, following by staining with Coomassie brilliant blue.

Mass spectrometry: tryptic digestion. The gel sections were chopped into
∼1-mm² pieces, washed in distilled water (dH₂O), and desiccated
using 100 mM NH₄HCO₃ (pH 7.5) in 50% acetonitrile. Reduction was
performed by addition of 100 µl of 50 mM NH₄HCO₃ (pH 7.5) and 10 µl
of 200 mM tris(2-carboxyethyl)phosphine HCl at 37°C for 30 min. The
proteins were alkylated by addition of 100 µl of 50 mM iodoacetamide
prepared fresh in 50 mM NH₄HCO₃ (pH 7.5) and allowed to react in the
dark at 20°C for 30 min. Gel sections were washed in water and then
acetonitrile and vacuum dried. Tryptic digestion was carried out over-
night at 37°C with a 1:50 to 1:100 enzyme-protein ratio of sequencing
grade-modified trypsin (Promega) in 50 mM NH₄HCO₃ (pH 7.5) and 20
mM CaCl₂. Peptides were extracted with 5% formic acid and vacuum
dried.

HPLC for mass spectrometry. Samples were resuspended in high-
performance liquid chromatography (HPLC)-grade water containing
0.2% formic acid (Fluka), 0.1% trifluoroacetic acid (TFA; Pierce), and
0.002% Zwittergent 3-16 (Calbiochem), a sulfobetaine detergent. The
peptide samples were loaded onto a 0.25-µl μC OptiPak trapping cartridge
custom-packaged with Microm Magic (Optimize Technologies) C8,
was washed, then switched in-line with a 0-µm C18 packed spray
tip nanocolumn packed with Microm Magic C18AQ, for a 2-step grad-
ient. Mobile phase A was water-acetonitrile-formic acid (98%/2%/0.2%),
and mobile phase B was acetonitrile-isopropanol-water-formic acid (80%/10%/10%)/0.2%). Using a flow rate of 350 nl/min, a 90-min, 2-step
LC gradient was run from 5% mobile phase B to 50% mobile phase B in 60
min, followed by 50% to 95% mobile phase B over the next 10 min, a hold
for 10 min at 95% mobile phase B, and back to starting conditions, fol-
lowed by reequilibration.

LC-MS/MS analysis. Samples were analyzed via electrospray tandem
mass spectrometry (LC-MS/MS) on a Thermo Q-Exactive Orbitrap mass
spectrometer, using a 70,000 RP survey scan in profile mode, m/z 350 to
2,000 Da, with lock masses, followed by 20 MS/MS/higher-energy collis-
sional dissociation (HCD) fragmentation scans at 17,500 resolution on
doubly and triply charged precursors. Single charged ions were excluded,
and ions selected for MS/MS were placed on an exclusion list for 60 s. An
inclusion list of expected 2+ and 3+ m/z tryptic ions was used for a subset
of targeted proteins (in-house software). These candidate ions were se-
lected for MS/MS if detected in the MS scan, regardless of their abun-
dance.

Database searching. Tandem mass spectra were extracted by Protein
Wizard version 3.0.6447. All MS/MS samples were analyzed using Mascot
(Matrix Science, London, United Kingdom; version 2.3.02) and X! Tan-
dem (The GPM [http://thegpm.org]; version CYCLONE [2010.12.01.1]).
Mascot was set up to search the 140401_SPROT_Tetrahymana_thermo-
phila database (unknown version; 24,698 entries) assuming the digestion
enzyme trypsin. X! Tandem was set up to search the 140401_SPROT_Tetra-
hymana_thermophila database. Mascot and X! Tandem were searched with
a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of
20 ppm. Carbamidomethyl of cysteine was specified in Mascot and X!
Tandem as a fixed modification. Glu→pyro-Glu of the N terminus, am-
monia loss of the N terminus, Gln→pyro-Glu of the N terminus, oxida-
tion of methionine, formyl of the N terminus, and carbamidomethyl of
cysteine were specified in X! Tandem as variable modifications. Oxidation
of methionine and formyl of the N terminus were specified in Mascot as
variable modifications.

Criteria for protein identification. Scaffold (version Scaffold_4.2.1;
Proteome Software Inc., Portland, OR) was used to validate MS/MS-
based peptide and protein identifications. Peptide identifications were
accepted if they could be established at greater than 91.0% probability to
achieve a false-discovery rate (FDR) of less than 1.0%. Peptide probabil-
ities from X! Tandem were assigned by the Peptide Prophet algorithm (36)
with Scaffold delta-mass correction. Peptide probabilities from Mascot
(Ion Score Only) were assigned by the Scaffold Local FDR algorithm.
Protein identifications were accepted if they could be established at
greater than 99.0% probability to achieve an FDR less than 1.0% and
contained at least 2 identified peptides. Protein probabilities were as-
signed by the Protein Prophet algorithm (37). Proteins that contained
similar peptides and could not be differentiated based on MS/MS analysis
alone were grouped to satisfy the principles of parsimony.

RESULTS  
Cth4p, a cysteine cathepsin, is a strong candidate for a mucocyst
processing enzyme based on expression profiling. In prior work,
we mined an expression database of T. thermophila transcripts to
discover that a set of genes encoding mucocyst cargo proteins, a VPS10 family receptor involved in cargo sorting, and several putative proteases were all transcriptionally coregulated (16, 27, 34). Among those proteases is a gene for cathepsin 4 (CTH4), whose expression pattern was strikingly similar to that of several other mucocyst-associated genes (Fig. 1A). A phylogram including CTH4 and related genes in other organisms, which were identified as top BLAST hits, shows that CTH4 falls within the genes for the family of cysteine cathepsins, including cathepsin C in H. sapiens (labeled Hs8-9) (Fig. 1B). CTH4 is only distantly related to CTH3, the gene for an aspartyl cathepsin previously shown to be involved in mucocyst proprotein processing (see Fig. S1 in the supplemental material). In addition, the genes most closely related to CTH4 in the T. thermophila genome show a different expression pattern, suggesting that CTH4 is unlikely to have a functionally redundant paralog (Fig. 1A).

**Cth4p-CFP, expressed from an inducible promoter, localizes to cytoplasmic puncta and docked mucocysts.** We expressed a copy of Cth4p, with cyan fluorescent protein (CFP) appended to the C terminus, under the transcriptional control of the inducible MTTI promoter, in order to visualize both initial and potentially processed products (38). Western blotting of cell lysates at 30, 60, and 120 min of induction, using an anti-GFP antibody, showed a protein of the expected size, as well as a polypeptide of the size expected for monomeric CFP (Fig. 2A). This pattern is similar to cleavage in Tetrahymena of a GFP-tagged variant of Cth3p, releasing monomeric GFP (27).

To determine whether Cth4p localizes to mucocyst intermediates and/or mature organelles, we fixed and permeabilized cells at time points shown in Fig. 2A and then dually labeled them with antibodies against GFP and a mucocyst cargo protein, Grl3p (Fig. 2B). Cth4p-CFP colocalized extensively with Grl3p in docked mucocysts after 120 min but less clearly at the earlier time points when Cth4p-CFP was found primarily in cytoplasmic puncta. An ambiguous feature of these results, explored further below, was that CFP signal in mucocysts at 120 min might reflect the localization of monomeric CFP that is proteolytically cleaved from the chimeric protein and which may be different from that of Cth4p-CFP.

**Cth4p-GFP, expressed at the endogenous locus, shows partial overlap with endolysosomal markers.** The cysteine cathepsin family contains numerous members in other eukaryotes that function in endolysosomal compartments, which may be represented by the Cth4p-positive cytoplasmic puncta noted above (39). To examine this possibility, we first expressed Cth4p-GFP at the endogenous CTH4 locus, under its native promoter, to avoid potential overexpression artifacts. Western blotting of cell lysates, using an anti-GFP antibody, showed both full-length protein and monomeric GFP (Fig. 3A). By live imaging, such cells showed strong GFP signals in docked mucocysts (Fig. 3B and C). In addition, live cells also bore a number of GFP-positive cytoplasmic puncta, whose number and appearance depended on culture density. We imaged such cells after incubation with either FM4-64, which labels endocytic compartments (Fig. 3D), or Lysotracker Red (Fig. 3D and E). In both cases, the GFP-positive puncta showed clear albeit incomplete overlap with the endolysosomal markers.

**Monomeric GFP, but not Cth4p-GFP, accumulates in docked mucocysts.** The Western blot results in Fig. 2A indicate that puncta in cells expressing Cth4p-GFP report the localization of both the full-length protein and also monomeric GFP generated by cleavage. These species may not colocalize entirely, if they undergo separate sorting following the cleavage. To determine whether full-length Cth4p-GFP is present in docked mucocysts, we stimulated cells expressing Cth4p-GFP (cth4-2p, from the endogenous locus) to release their docked mucocysts via exocytosis and analyzed the poststimulated, mucocyst-depleted cells by Western blotting using anti-GFP and anti-Grl3p antibodies. There was no detectable release of GFP into the cell culture medium of nonstimulated cells (data not shown). For the stimulated samples, we found that monomeric GFP, but not full-length Cth4p-GFP, was depleted from cells by dibucaine stimulation, indicating that only the former is present in mature mucocysts (Fig. 4). These results explain the difference between the intensity of mucocyst labeling in cells continuously expressing Cth4p-GFP (Fig. 3) versus those in which short-term expression was induced (Fig. 2), since in the former there is a large pool of accumulated monomeric GFP cleaved from the full-length protein.

Taken together, our results show that Cth4p localizes partially to endolysosomal compartments, while GFP that has been cleaved from Cth4p-GFP accumulates in mature mucocysts. Importantly, GFP with an N-terminal signal sequence shows no localization to mucocysts (22). Therefore, the targeting of GFP to mucocysts in strains expressing Cth4p-GFP must depend on signals present in Cth4p, prior to cleavage of the chimeric protein. These results can be explained if some cleavage of GFP from Cth4p occurs in immature mucocysts and free GFP is retained during subsequent mucocyst maturation while Cth4p-GFP and Cth4p are retrieved. In addition, our results do not rule out the possibility that some cleavage of GFP from Cth4p-GFP occurs in docked mucocysts, in which Cth4p may also be retained.

**CTH4 knockout results in a marked defect in mucocyst exocytosis.** To determine whether CTH4 is required for mucocyst function, we replaced the open reading frame of the gene with a drug resistance cassette via homologous recombination (Fig. 5A). We obtained cells with no detectible CTH4 transcript, indicating that all copies of the gene in the somatic macronucleus had been disrupted (Fig. 5B). Therefore, the gene is not essential, and such Δcth4 cells had a doubling time indistinguishable from that of the wild type (data not shown).

We exposed Δcth4 cells to dibucaine, a secretagogue that induces rapid synchronous mucocyst exocytosis (Fig. 5C) (41). In wild-type cells, the core proteins in mucocysts assemble as a crystal roughly 1 μm in length, which upon exocytosis undergoes dramatic expansion to ~7 μm (20). As a result, stimulation followed by low-speed centrifugation results in a visible layer of sedimenting flocculent, made up of the expanded and released mucocyst cores (Fig. 5D). In contrast, we found that stimulation of Δcth4 cells resulted in a much smaller flocculent layer, indicating that the gene is required for efficient mucocyst synthesis or exocytosis. Together with the aspartyl cathepsin encoded by CTH3, the enzyme encoded by CTH4 is thus a second enzyme implicated in mucocyst function.

**CTH4 is not required for formation and accumulation of docked mucocysts but is required for efficient core extrusion upon exocytosis.** T. thermophila that lacks CTH3 accumulates a reduced number of mucocysts, which are also morphologically aberrant (27). These defects explained why Δcth3 cells show negligible release of mucocyst contents upon exocytic stimulation. Surprisingly, Δcth4 cells showed no apparent defect in mucocyst formation, notwithstanding the clear exocytosis phenotype. We immunostained the cells with antibodies against two different
FIG 1 (A) The expression profile of CTH4 is very similar to those of genes required for mucocyst biogenesis: GRL1, GRT1, and CTH3. In contrast, the expression profiles of two genes that are sequence related to CTH4, CTH30 (TTHERM_00502690) and CTH32 (TTHERM_01248970), show less overlap with those of mucocyst-associated genes. The profiles of transcript abundance under a variety of culture conditions, derived via hybridization of cDNAs to whole-genome microarrays, were downloaded from the Tetrahymena Functional Genomics Database (http://tfgd.ihb.ac.cn/). In the plots shown here, each trace was normalized to that gene’s maximum expression level. The culture conditions sampled at successive time points represent growing (Ll, Lm, and Lh), starved (S0, S3, S6, S9, S12, S15, and S24), and conjugating (C0, C2, C4, C6, C8, C10, C12, C14, C16, and C18) cultures (28). (B) Phylogenetic reconstruction of cysteine cathepsin genes. The maximum likelihood tree illustrates the phylogenetic relationship between cysteine cathepsins in ciliates, apicomplexans, Arabidopsis species, and Homo sapiens. The T. thermophila cysteine cathepsin CTH4 is emboldened. For key to color blocks, see the bottom. Abbreviations: Tg, Toxoplasma gondii; P, Plasmodium; A, Arabidopsis; Hs, Homo sapiens; Tt, Tetrahymena thermophila; Pt, Paramecium tetraurelia; Im, Ichthyophthirius multifilis. The tree shown was assembled with aspartyl cathepsins as the outgroup; see Fig. SA1 in the supplemental material for complete tree. See Table SA2 for accession numbers.
mucocyst markers, and both revealed docked mucocysts indistinguishable from those of the wild type at the level of light microscopy (Fig. 6A). Similarly, electron microscopy of thin sections revealed uniformly elongated mucocysts, demonstrating that Cth4p activity is not required for the assembly of the crystalline mucocyst core (Fig. 6B).

Since Δcth4 cells accumulate mucocysts, their secretion defect suggested that Δcth4 mucocysts are incapable of undergoing efficient exocytosis. To investigate this possibility, we visualized both wild-type and mutant cells that were fixed immediately following stimulation with the secretagogue alcaline blue (42), using anti-mucocyst antibodies and immunofluorescence or electron microscopy (Fig. 7A, B, and D). Because this secretagogue also binds tightly to secreted mucocyst proteins, a subset of the alcaline blue-treated wild-type Tetrahymena becomes trapped in capsules formed by cross-linked, extruded mucocyst cores, which are highly immunoreactive (Fig. 7B, 3rd row). A second pool of stimulated wild-type cells showed virtually no antibody reactivity in the cells, consistent with previous observations that synchronous exocytosis of all docked mucocysts is complete within seconds (43) (Fig. 7B, 4th row). These two pools could be clearly resolved by flow cytometry (Fig. 7C). The Δcth4 samples appeared identical to the wild type prior to stimulation (Fig. 7B, top rows) but still showed very substantial immunostaining within the cells after stimulation, indicating that many mucocyst cores had not been fully released (Fig. 7B, bottom row). This was confirmed by flow cytometry (Fig. 7C). In addition, Δcth4 cells failed to form any alcaline blue-staining capsules (Fig. 7B, bottom row, and Fig. 7C). The Δcth4 mucocysts that were retained in stimulated cells no longer appeared elongated by light microscopy (Fig. 7B, bottom row, inset). Electron microscopy of Δcth4 cells after stimulation confirmed that cores had become roughly spherical (Fig. 7D).

Thus, while Δcth4 mucocysts respond to cell stimulation by secretagogues, both the core expansion and its functional consequences are distinct from those in wild-type mucocysts.

The partial release of contents from Δcth4 cells could be accounted for by complete failure of some mucocysts to undergo exocytosis, or partial failure of all mucocysts. Both light and electron microscopy data suggested the latter, implying that individual Δcth4 mucocysts only released a portion of their cargo upon stimulation. One striking feature of a wild-type mucocyst core is that as it undergoes ∼7-fold expansion upon exocytosis from stimulated cells, the Grl proteins remain organized in an extended, very stable lattice (20). Thus, partial release of a mucocyst core could occur only if the lattice itself was unstable and could fragment during exocytosis.

To investigate this possibility, we exploited the fact that even following release, wild-type Grl proteins persist in large stable complexes (44). Indeed, this property is the basis of the formation of the sedimenting flocculent layer described above. To determine if this property is conserved in Δcth4 mucocyst cores, we stimulated Δcth4 cells to undergo exocytosis and then centrifuged the sample using conditions that sediment Grl proteins in wild-type samples. We then used Western blotting to analyze the distribution of Grl proteins in the flocculent layer versus the supernatant. In the Δcth4 samples, much of the protein was found in the nonpelleting fractions (Fig. 8). The difference between wild-type and Δcth4 samples therefore suggests that some interactions between Grl proteins depend on the activity of Cth4p during core assembly. In addition, the Western blots suggested that processing of mature Grl proteins was incomplete in Δcth4 cells, as indicated by slight shifts in electrophoretic mobility and as detailed below.

**CTH4 is required for full processing of proGrl proteins.** Formation of mucocyst cores is controlled by proteolytic maturation...
Localization of endogenous Cth4p-GFP. (A) Expression of cth4-2: GFP-tagged Cth4p from the endogenous CTH4 locus, under the control of its native promoter. cth4-2p was immunoprecipitated from detergent lysates using polyclonal rabbit anti-GFP antiserum, and immunoprecipitates were Western blotted with monoclonal anti-GFP Ab. One immunoreactive band is of the size expected for the Cth4p-GFP fusion, while a second is the size expected for monomeric GFP. (B) Live immobilized cells expressing cth4-2p were imaged to capture cell surface and cross sections and show the expected array of docked mucocysts as well as intracellular puncta. (C) Immunostaining of fixed cells to simultaneously localize mucocyst protein Grl3p and cth4-2p. There is extensive colocalization in docked mucocysts, while some puncta deeper in the cytoplasm are positive for cth4-2p but not Grl3p. (D) Cells were incubated for 5 min with 200 nM Lysotracker, and live images were captured within 30 min. Optical sections shown are cell cross sections. (E) Cells were incubated for 5 min with 5 μM FM4-64, an endocytic tracer and then pelleted and resuspended in tracer-free medium. The times shown represent minutes after resuspension. Scale bars = 10 μm.
of the Grl proteins (45). Since Cth4p is predicted to act as a protease, the defects in ∆cth4 mucocysts may be due to defects in pro-Grl processing. Consistent with this idea, Western blots of ∆cth4 whole-cell lysates with antibodies against Grl1p revealed clear differences from the wild type. First, the ∆cth4 cells accumulated higher levels of the unprocessed precursor than did the wild type (Fig. 9A). Second, ∆cth4 cells also accumulated a processed Grl1p product, but this species had slightly lower mobility on
SDS-PAGE than the corresponding band in wild-type lysates (Fig. 9A). The same pattern was also seen for a second Grl protein, Grl3p (Fig. 9B).

The accumulation of unprocessed proGrl precursors has been noted in a variety of mutants affecting mucocyst function (46; unpublished data), but accumulation of a larger-than-wild-type final product has not previously been observed, and it suggested the existence of a previously overlooked processing step. To investigate this possibility, we isolated the secreted contents of both wild-type and Δcth4 cells, i.e., the flocculent layer following stimulation. The material was separated by SDS-PAGE, from which we excised gel regions that were expected to contain processed Grl

FIG 6 CTH4 is not required for formation and accumulation of docked mucocysts. (A) (Top) docked mucocysts in fixed wild-type cells, immunolabeled using MAb 4D11, which recognizes Grt1p (left two images), and MAb 5E9, which recognizes Grl3p (right two images). Shown are optical surface and cross sections. (Bottom) parallel immunostaining of Δcth4 cells shows patterns indistinguishable from the wild type. Scale bars = 10 μm. (B) Electron micrographs of mucocysts (labeled with asterisks) in wild-type and Δcth4 cells. The mucocyst cores in both wild-type and Δcth4 cells are organized as visible lattices. Scale bars = 0.2 μM.
**FIG 7** *CTH4* is required for efficient cargo release upon exocytosis. (A) Illustration of qualitative assay for mucocyst discharge. Cells are stimulated by addition of alcian blue, which triggers mucocyst exocytosis and binds to released mucocyst proteins, resulting in two distinct pools of cells. Cells in pool 1 are individually trapped by translucent capsules formed by released mucocyst contents, while cells in pool 2 are not entrapped because they have already escaped from their capsules. (B and C) Cells, before and after stimulation with alcian blue, were fixed, detergent permeabilized, immunolabeled with MAb against Grl3p, and then analyzed by confocal microscopy or flow cytometry. (B) Wild-type and Δ*c*th4 cells prior to stimulation display identical docked mucocysts, visible as discrete puncta (top two rows). After stimulation, wild-type cells are surrounded by translucent capsules of released mucocyst contents, distinct from the punctate
proteins (Fig. 9C). This material was analyzed by mass spectrometry. We focused on peptides derived from pro-Grl proteins, which are encoded by a small gene family (22). Importantly, we had previously used Edman degradation to establish the N termini of five of the mature Grl products, derived from proGrl1p, -3p, -4p, -5p, and -7p (20).

Concentrating on these five Grl proteins, we found additional peptides in the Δcth4 samples compared to the wild type. These peptides, mapped to the full-length protein sequence, corresponded to extensions from the mature Grl N termini characterized in wild-type cells (Fig. 9D). We found such extensions for five of the six previously mapped N termini. Thus, the absence of Cth4p leads to N-terminally elongated Grl products, which is consistent with the shift to larger sizes seen by SDS-PAGE and Western blotting. Since CTH4-related cathepsins in other organisms can act as aminopeptidases, one possibility is that Cth4p is required for N-terminal trimming of Grl processing intermediates. Our data do not establish the precise extensions present in Δcth4 cells, since the amino termini of the peptides detected are produced by trypsin treatment during preparation of the mass spectrometry samples.

**In vivo CTH4 function depends on conserved enzymatic residues.** The polypeptide corresponding to CTH4 includes the residues, conserved among related papain-family enzymes, which are directly involved in substrate hydrolysis (39) (Fig. SA2). If Cth4p acts in vivo as an enzyme, mutating the conserved enzymatic residues should be functionally equivalent to gene disruption. We therefore used site-specific mutagenesis to substitute the conserved active site cysteine and histidine and replaced the endogenous gene with the mutated allele via homologous recombination followed by drug selection (Fig. 10A). In addition, we added a C-terminal GFP tag to detect the novel product. We compared this construct to a similarly GFP-tagged allele of the wild-type gene, also expressed via gene replacement at the endogenous locus.

Both the wild-type and enzymatically disabled alleles were expressed as products of the expected sizes, judging by SDS-PAGE (Fig. 10B). Western blotting of whole-cell lysates demonstrated that cells expressing the site-specifically mutated allele showed the same upward shift in the size of processed Grl products as seen in Δcth4 cells (Fig. 10C and D). In addition, cells expressing mutated Cth4p showed a clearly defective secretory response upon secretagogue stimulation, indistinguishable from the response of Δcth4 cells (Fig. 10E). The processing and secretion defects were not due to mistargeting of the disabled protein, since localization was not distinguishable from that of the wild-type, GFP-tagged protein (Fig. 10F). These results are therefore consistent with the ideas that Cth4p acts enzymatically and that the defects in Δcth4 cells are due to the absence of Cth4p catalytic activity during mucocyst formation.

**Cth4p undergoes CTH3-dependent processing.** A model based on studies with Cth3p and Cth4p, the two enzymes now implicated in proGrl processing, is shown in Fig. 11. Based primarily on the gene knockout phenotypes, CTH3 appears to act upstream of CTH4, and we propose that Cth4p directly trims the products generated by Cth3p. Because the proGrl sites recognized and cleaved by Cth3p are as yet unknown, we are not yet in a position to test such products in vitro as potential Cth4p substrates. The precise sites of Cth3p cleavage cannot be inferred from mucocyst staining prior to stimulation, that stain brightly for Grl3p (pool 1, 3rd row) or are largely devoid of Grl3p staining (pool 2, 4th row). Stimulated Δcth4 cells do not form capsules and still display abundant docked mucocysts (bottom row), though different in appearance from prestimulation samples. Scale bars = 10 μm. Insets in right-hand panels: rows 1 and 2, elongated docked mucocysts in WT and Δcth4 cells prior to stimulation; row 3, irregular mucocyst profiles in Δcth4 cells following stimulation. (C) Flow cytometry of wild-type and Δcth4 cells, before and after stimulation. (Left graph) prior to stimulation, WT and Δcth4 cells show similar labeling with anti-Grl3p Ab. (Right graph) after stimulation, WT cells show two distinct populations, corresponding to pools 1 and 2. Poststimulation Δcth4 cells instead show a single population, whose staining intensity suggests that roughly half of Grl3p was released by exocytosis. For flow cytometry, 10⁴ cells/sample were analyzed. MFI, mean fluorescence intensity. (D) Electron micrographs of docked mucocysts (*) in Δcth4 cells, fixed before and after alcian blue stimulation. Scale bars = 0.2 μm.
the mass spectrometry results shown above, since the N termini of the newly identified $\Delta$cth4-dependent peptides were generated by trypsin used in processing the gel samples, rather than by proGrl maturases in vivo.

In many organisms, cathepsins and other proteolytic enzymes are synthesized as zymogens and attain full activity toward their substrates only after they are themselves processed (47). Cth4p cannot be essential for activation of Cth3p, since CTH3 disruption resulted in more profound processing defects than CTH4 disruption (27). Another possibility is that Cth3p is involved in activation of Cth4p, consistent with their proposed order of action in Fig. 11. Cathepsin C family members, including the Toxoplasma gondii enzyme most closely related to Cth4p, are comprised of 5 recognized domains: a signal peptide, an exclusion domain, an internal proregion, and finally a catalytic heavy and light chain (48). In order to detect potential processing intermediates of $T.$ thermophila Cth4p, we expressed a copy that

**FIG 9** CTH4 is required for full processing of Grl proteins. Cell lysates ($5 \times 10^5$ cell equivalents in panel A and $10^5$ cell equivalents in panel B) were resolved by SDS-PAGE (10% [A] and 4 to 20% [B]), transferred to PVDF, and Western blotted with antibodies against Grl proteins. The unprocessed (proGrl) and processed (Grl) bands are labeled. (A) Blotting with anti-Grl1p antibody. The predominant band in WT lysates (lane 1) is mature processed Grl1p. The corresponding band in $\Delta$cth4 lysates is shifted slightly in mobility, and the lysate also shows a high level of unprocessed precursor. (B) Same as panel A, but blotting with anti-Grl3p antibody. (C) Gel (4 to 20%) stained with Coomassie brilliant blue, containing flocculent samples from dibucaine-stimulated WT and $\Delta$cth4 cultures. Molecular mass standards are shown on the left. The gel regions excised for mass spectrometry are shown. (D) The processed Grl products retain N-terminal extensions in $\Delta$cth4 cells. Each of the proGrl genes shown begins with an N-terminal signal sequence (gray rectangle) and has either one (for Grl1, -3, -5, and -7) or two (for Grl4) sites (blue circles) that were previously established as N termini of processed Grl products isolated from extruded wild-type mucocysts (20). The red rectangles represent the positions of peptides present in $\Delta$cth4 samples but not the wild-type samples. In each case, the peptide constitutes an amino-terminal extension to the N terminus in proteins isolated from wild-type cells. The amino acids within the $\Delta$cth4-specific peptides are shown below the corresponding rectangles in red, while the adjacent amino acids in blue are those at the beginning of the mature polypeptide in wild-type cells.
was C-terminally tagged with 6×His, with the idea that a small epitope tag might be less subject to removal by cytosolic proteases than a globular protein tag like GFP. Indeed, we found that His-tagged Cth4p, expressed in wild-type cells, accumulated as three major bands in growing cells (Fig. 12, lane 2). These could tentatively be assigned to three different processed forms of Cth4p, although the precise junctions between domains in the Tetrahymena protein cannot be predicted due to the relatively low sequence conservation in those regions (Fig. 12B). The same three bands were present in wild-type and mutant cell lysates, though there were relatively lower levels of the HL species than of the wild type. The apparent difference in the processing of Cth4p-6×His in cth3 cells was more striking when cultures were sampled during starvation. Importantly, starvation is known to accelerate proGrl processing and also induce expression of both CTH3 and CTH4 (Fig. 1A) (45). Starved wild-type cells showed accumulation of the EIHL and HL species (see Fig. 12A), the latter predicted to represent the active enzyme. In contrast, in starved cth3 cells virtually all cth4p-6×His accumulated as the unprocessed EIHL species, which is predicted to be inactive. These results suggest that Cth3p activity is involved, directly or indirectly, in proteolytic activation of Cth4p zymogen.

### DISCUSSION

The proteinaceous cores of secretory granule-like organelles in oligohymenophorean ciliates, best studied for *Tetrahymena thermophila* and *Paramecium tetraurelia*, are remarkable structures that assemble as complex multicomponent crystals and then are transformed into projectiles during exocytosis, by undergoing rapid ordered expansion. Pioneering studies in the 1980s established that the proteolytic processing of proproteins was likely to be a key mechanism in controlling core assembly, and this was substantiated by subsequent identification and analysis of the proprotein-encoding genes as well as detailed characterization of the secreted polypeptides (24–26, 50). Nonetheless, the proteases themselves could only be inferred until recently, when expression profiling led to an aspartyl cathepsin, Cth3p (27). The failure of cth3 cells to process *Tetrahymena* mucocyst proproteins and to assemble ordered mucocysts provided strong direct confirmation that proteases were key determinants in this pathway. In the current experiments, we show that Cth3p is able to process Cth4p zymogen in vitro and also in vivo in wild-type cells.
From the image, we can extract the following text:

**FIG 11** Model for proteolytic processing of mucocyst proGrp proteins in *Tetrahymena*. We propose that processing of proGrp proteins during mucocyst maturation initiates with endoproteolytic cleavage by Chth3p at a site upstream of the mature N termini. The products are competent to form highly ordered complexes that assemble to create the elongated mucocyst core. Chth3p subsequently acts to trim the amino termini. This step is proposed to be essential for reinforcing the lattice in a way that promotes stability during expansion. Since removal of the peptides results in stabilization during expansion, we have termed them “destabilizing peptides” (DP). The relative timing of DP removal versus assembly is unknown. Cores that have not undergone DP trimming are defective during the directional expansion required for efficient core extrusion during exocytosis.

**FIG 12** Cth4p undergoes Cth3p-dependent processing. (A) Proposed organization of *T. thermophila* Cth4p, based on established structures of cathepsin C homologs in other eukaryotes. S, signal sequence; E, exclusion domain; I, internal proregion; H, heavy chain; L, light chain. The catalytic domains are the heavy and light chains. (B) Processed forms of Cth4p in *T. thermophila*. Cth4p-6×His expression was induced for 2 h in wild-type or Δcth3 cultures. Cells were induced in either growth or starvation media, by adding 1 or 0.1 μg/ml of CdCl2, respectively. Cell lysates (3 × 10^6 cell equivalents/lane) were separated by SDS-PAGE and Western blots were probed with anti-His MAb. Three major His-tagged species are present, and their tentative relationship with the domains indicated in panel A is indicated. Particularly in starvation, the large majority of Cth4p in Δcth3 cells remains in an unprocessed form that is predicted to be inactive. Note that starvation is a state in which transcription of Cth3p and Cth4p, as well as processing of proGrp, is induced.
activity than the mature forms on Western blots (unpublished data). For this reason, we instead propose that destabilization of the cores chiefly results from the uniformly untrimmed amino-terminal peptides in the mature Grl polypeptides. These additional peptides are unlikely to interfere with proper folding, since the Grl products still assemble into visible lattices, and we did not detect any morphologically aberrant mucocysts in Δcth4 samples. However, amino-terminal trimming may be required to permit additional interactions that are required to maintain the integrity of the lattice, especially during expansion. Carboxy-terminal trimming of Grl products is also likely to occur during mucocyst biogenesis, judging by the identification of a carboxypeptidase, Car1p, which localizes to mucocysts (27; unpublished data). However, disruption of CAR1 did not produce a secretion phenotype, so such trimming may serve a nonstructural purpose (unpublished data).

In wild-type cells, the amino-terminal extensions must be removed by endoproteolytic cleavage and/or exoproteolytic trimming, and this may be accomplished directly by Cth4p. Cells expressing a variant of CTH4, in which the conserved catalytic residues were mutated, were phenotypically equivalent to Δcth4 cells, which strongly suggests that all in vivo functions of Cth4p rely on its predicted enzymatic activity. In other organisms, proteins in the cathepsin C subfamily demonstrate both endoproteolytic and exoproteolytic activities, depending on the enzyme oligomerization state (55). Among cathepsin C family members whose activities have been characterized, the one most closely related to Cth4p is an enzyme from T. gondii (TgCPC2, annotated as Tg6 in Fig. 1B) (48). T. gondii is an apicomplexan and therefore belongs to a sister group of the ciliates (56). This enzyme showed exoproteolytic activity in vitro, but endoproteolytic activity was not examined.

The simplest model consistent with our data is that Cth3p and Cth4p act sequentially on the same substrates. Cth3p is required for the primary endoproteolytic cleavage of Grl proproteins, and Cth4p acts on products of that cleavage. However, our data do not rule out the possibility that Cth3p and/or Cth4p also has important non-Grl substrates. In addition, the current data do not distinguish between Cth4p acting directly upon proGrl substrates or being indirectly responsible for processing, e.g., by activating another enzyme which directly acts upon proGrls. In T. gondii, a cathepsin L enzyme, TgCPL, processes dissimilar substrates that are moreover targeted to two different compartments: secretory organelles (micronemes) and an acidic compartment termed the plant-like vacuole (PLV) (57, 58).

Cathepsin C family members were initially considered lysosomal hydrolases, and the role of Cth4p in mucocyst biogenesis is therefore consistent with the idea that secretory organelles in ciliates are related to lysosome-related organelles and underscores the idea that adapting the machinery of lysosome biogenesis has been a major evolutionary mechanism driving organelar diversity in different tissues and lineages (39) (16, 59, 60). Interestingly, the functions provided by cathepsin C members in animals extend beyond proteolytic degradation of lysosome contents. In particular, the aminopeptidase activity can activate serine proteases by removing inhibitory short amino-terminal peptides (61). Proteolytic activation by cysteine proteases also occurs in T. gondii (58). Thus, the defects in Δcth4 cells may reflect the failure to activate additional enzymes, rather than the direct action of Cth4p on Grl proproteins. Our survey of protease genes in T. thermophila revealed five that are coregulated with the GRL genes, and all are targeted to mucocysts (27; unpublished data). However, we disrupted each of these genes, and only CTH3 resulted in a secretion phenotype of severity comparable to that of CTH4 (unpublished data). However, as noted above, Cth4p cannot be required for activation of Cth3p since Δcth3 manifests the more severe exocytosis deficiency. Therefore, if Cth4p acts primarily by activating a second processing enzyme, that protein is likely to be novel. Whether or not Cth4p acts on downstream enzymes, our data suggest that it is itself activated by Cth3p under starvation conditions, while other functionally overlapping cathepsins encoded by this large gene family may be more relevant during growth conditions.

Cth4p acts during mucocyst maturation, and the simplest hypothesis is that it functions in an immature mucocyst compartment, for which we are currently developing molecular markers. Cth4p, like Cth3p, showed significant overlap in localization studies with endolysosomal markers. Whether the immature mucocyst compartment is itself labeled with FM4-64, i.e., whether it receives vesicular traffic from endosomes, is not known. One interesting possibility is that Cth4p may not be directly transported from the TGN to immature mucocysts, since we detected numerous cytoplasmic puncta that are positive for Cth4p-GFP but not Grl3p in cells that have been induced for short times to express the tagged gene. A related question is whether Cth3p and Cth4p are active in the same compartment. One notable difference between the two is that Cth3p, but not Cth4p, was readily detected in cell culture supernatants, suggesting that only the former accumulates in a compartment that releases its contents to the cell medium (27). In the future, these issues may be clarified by determining the targeting mechanisms used for the different proteases.

Endoproteolytic cleavage followed by obligatory trimming is a feature of many proproteins packaged in animal secretory granules, such as proinsulin (3). The similarity with proprotein processing in secretory organelles in ciliates, as revealed by analysis of Cth3p and Cth4p, offers a striking example of convergent solutions based on evolutionarily unrelated enzymes, to the problem of generating and packaging bioactive peptides.

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