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# Identification of a Biochemical Marker for the Secretory Pathway in *Tetrahymena thermophila*

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*Tetrahymena thermophila* is a ciliated protozoan studied by investigators from a wide range of disciplines as a model system for a variety of specialized eukaryotic cell functions. The proteinaceous secretory products of *T thermophila* have been isolated and characterized [1] and in this study we identify the major secretory product, a 34,000 Mr polypeptide, and use an antiserum prepared against this secretory protein to (1) demonstrate that this 34,000 Mr polypeptide is truly a secreted protein in *Tetrahymena* and (2) monitor the synthesis and transport of this protein by indirect immunofluorescence and light microscopy during mucocyst biogenesis.

**Key words: protein secretion, ciliates, *Tetrahymena***

The ciliated protozoan *Tetrahymena thermophila* has been studied extensively as a model system of a variety of specialized eukaryotic cell functions, including protein secretion. The introduction of a simple method for obtaining homozygous recessive mutants has greatly enhanced the usefulness of this organism by promoting the genetic dissection of these processes [2]. Numerous ultrastructural studies of *Tetrahymena* have revealed that the secretory pathway in these cells is complex, and in some ways, perhaps, unique [3–5]. *Tetrahymena* is well suited as a model system for the study of the secretory pathway; these cells contain a large number of regularly positioned secretory organelles, and synchronous secretion can be stimulated by a variety of secretagogues. Recently, a series of secretory mutants of *T thermophila* have been isolated by Orias and coworkers [6]. One of these mutants, designated SB 281, has now been characterized; this mutant strain lacks mature secretory organelles and has a reduced level of expression of a 34,000 Mr component [7,8]. This 34,000 Mr component has been isolated from wildtype cells and has been used to raise an

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antiserum in rabbits. This antiserum is precipitating and reacts with a single 34,000 Mr polypeptide species in whole cell extracts of wildtype cells.

The secretory organelles in *Tetrahymena* are referred to as mucocysts. We fixed and processed cells for indirect immunofluorescence, as previously described [9], to (1) establish the identity of the 34,000 Mr polypeptide as a bona fide mucocyst component and (2) to follow the release and biosynthesis of this component during mucocyst regeneration. Our results indicate that the 34,000 Mr component is indeed a mucocyst component, and it is released and synthesized with kinetics consistent with the time course for the reappearance of mucocysts following stimulation of secretion, as previously observed in ultrastructural studies [10,11].

## MATERIALS AND METHODS

### Enrichment of Released Secretory Proteins

*Tetrahymena thermophila* (wt strain SB 210) was grown in an enriched proteose peptone medium (Difco, 2%, 0.1% glucose, 0.1% yeast extract). An enriched "mucus" preparation was obtained by stimulating the cells with 0.025 M dibucaine, a local anesthetic that stimulates synchronous mucocyst secretion, and under these conditions, partial deciliation. Released "mucus" was collected by centrifugation and analyzed by 0.1% SDS, 15% polyacrylamide electrophoresis (SDA PAGE). The 34,000 Mr protein was purified by excising the corresponding band from preparative gels. The protein was electroeluted, dialyzed extensively against water, lyophilized, and resuspended in phosphate-buffered saline (PBS).

### Antiserum Production and Characterization

The isolated electrophoretically homogeneous 34,000 Mr "mucus" protein was used as antigen in immunization of a young female New Zealand rabbit. The rabbit was initially immunized with ten subcutaneous injections of approximately 0.5 mg of the 34,000 Mr protein in complete Freund's adjuvant, and it was boosted with 0.25 mg of the 34,000 Mr protein in incomplete adjuvant by multiple subcutaneous injection on day 14 and subsequently every 2 wk for 2 mo. Preimmune and immune serum were obtained by bleeding the rabbit via ear blood vessels.

Immunoglobulin was characterized by standard techniques, including Ouchterlony double immunodiffusion and immunoelectrophoresis in 1% agarose gels equilibrated in 20 mM Tris-sodium barbitol buffer, pH 8.3. SDS PAGE samples were transferred to nitrocellulose for gel overlay experiments by the use of the protocol outlined by Towbin et al [12]. Nitrocellulose sheets were stained with a 1/500 dilution of primary serum followed by a 1/2,000 dilution of horseradish peroxidase-conjugated IgG (N.L. Cappel Laboratories, Cochranville, PA) and immunoreaction was monitored by incubation with diaminobenzidine in 0.3% hydrogen peroxide.

### Indirect Immunofluorescence Light Microscopy

Cells were fixed in 3% formalin for 30 min postfixed in −20°C absolute acetone for 20 min, and preincubated in 0.1% RIA grade bovine serum albumin (Sigma, St. Louis, MO) before incubation with a 1/200 dilution of the primary antibody, rabbit anti-34,000 "mucus" polypeptide, for 30 min at 37°C. After 3 × 10 min washes in PBS, cells were incubated in a 1/2,000 dilution of sheep anti-rabbit

rhodamine-conjugated IgG (N.L. Cappel Laboratories), and washed as above. The following control experiments were performed: (a) incubation of cells in secondary antibody without prior incubation of cells in primary antibody and (b) incubation of cells in preimmune serum in lieu of primary immune serum.

Wildtype cells were also stimulated to secrete (0.025 M dibucaine) and then returned to fresh medium for mucocyst regeneration before fixation. Cells were incubated in 0.025 M dibucaine hydrochloride (aqueous), pH 6.5, for 30 sec, causing synchronous mucocyst release and partial deciliation as monitored by light microscopy. After cells had been washed twice in a buffer containing 20 mM HEPES and 2.5 mM magnesium sulfate, they were resuspended in prewarmed (27°C) culture medium.

Mucocyst recovery was monitored by fixing 1 ml aliquots of cells at zero time, 10′, 30′, 1 hr, 2 hr, 3 hr, and 6 hr following dibucaine stimulation. Mucocyst recovery following dibucaine stimulation appeared to be uniform within each population of cells fixed and processed for immunofluorescence; however, recovery was not precisely synchronous (cf, Figs. 3, 6h).

## RESULTS

A 34,000 Mr secretory protein has been isolated from preparations of *Tetrahymena*, and this protein has been used to raise a rabbit polyclonal antiserum. The anti-34,000 serum has been characterized by immunodiffusion, immunoelectrophoresis, and western immunoblot analysis (Fig. 1A–C). This antiserum is precipitating and reacts with a single 34,000 Mr polypeptide species in crude "mucus" preparations and in whole cell extracts of wildtype cells.

The results of indirect immunofluorescence experiments with this antiserum are illustrated in Figures 2 and 3. Wildtype cells exhibit a distinctive linear punctate pattern of fluorescent staining in the cell cortex. Many of the point sources of fluorescence are circular and approximately 0.8–1.2 $\mu$m in diameter. Since the diameter of a mucocyst is approximately 0.3 $\mu$m these spots probably correspond to clusters of 3 to 4 mucocysts; these dimensions are consistent with the arrangement of small mucocyst clusters observed in *Tetrahymena* by electron microscopy. Such spots would then represent en face views of mucocysts (ie, looking down on mucocyst tips). Other point sources of fluorescence within a single meridian are oblong in shape, suggesting that within a given focal plane more than one mucocyst orientation is visible. The oblong spots are also approximately 0.8 $\mu$m in diameter and 1.5–2.0 $\mu$m long. The spacing between clusters within a single meridian is variable, but closely spaced points are separated by approximately 1–1.5 $\mu$m. The spacing of fluorescent points is also consistent with the localization of mucocysts in these cells; the distance between meridians at the equator of the cell is approximately 1.5 $\mu$m. Diffuse fluorescent staining is probably due, at least in part, to superimposition of mucocysts, since this staining appears to fade near the periphery of the cells. Control cells processed identically but incubated in preimmune serum rather than antiserum exhibit only a low level of background fluorescent staining (Fig. 2C, C′).

Cells were stimulated to secrete by the use of the local anesthetic dibucaine and then returned to fresh medium for mucocyst regeneration. Representative samples of cells fixed at zero time, 10′, 30′, 1 hr, 2 hr, 3 hr, 6 hr following dibucaine stimulation are illustrated in Figure 3. Ten minutes after dibucaine stimulation, the cells are

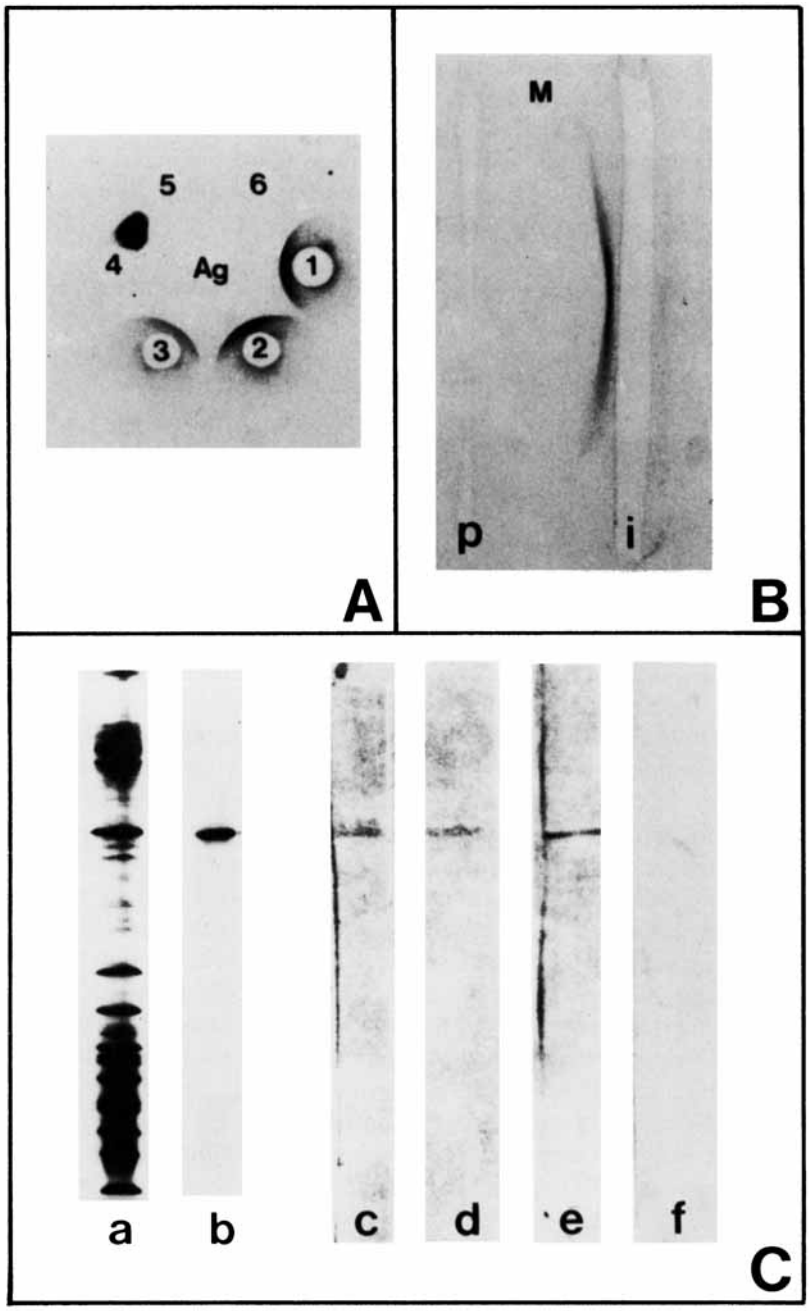


Fig. 1

partially deciliated, have rounded up, and have lost their fluorescent punctate staining (Fig. 3, 10′). Cells are still round after 30 min in fresh culture medium (Fig. 3, 30′), and several small spots (1.3–2.0 μm in diameter) are visible. In addition, larger vesicles approximately 2.5 μm in diameter are labeled. After 1 hr in fresh culture medium (Fig. 3, 60′), most cells have begun to regain their characteristic pyriform shape and to resume swimming. Vesicles of varying dimensions, up to 2.5 μm in diameter, are visible in the cortex, and are particularly evident in the posterior region of the cell below the macronucleus. These vesicles may in some way be related to mucocyst biogenesis; the possibility that some of these organelles are internalized (endocytosed) "mucus", however, cannot be excluded. After 2 hr in fresh medium, a linear punctate pattern of fluorescent staining is visible in the cortex of some cells, as illustrated in Figure 3 (2h). These spots are irregular in shape and spacing and measure approximately 0.4 μm in diameter. Since the diameter of a mucocyst is approximately 0.3 μm, these spots may represent newly synthesized individual mucocysts. If so, many appear to have already been rapidly transported to specific positions within the cell cortex. After 3 hr in fresh transfer medium, fluorescent label is still present in large vesicular structures in the posterior region of the cell, and some cells have at least partially regained regions of linear punctate fluorescent staining (Fig. 3, 3h). By 6 hr most cells have recovered linear punctate fluorescent staining comparable in organization and density to cells at zero time (Fig. 3, 6h, lower micrograph, and 0′). Mucocyst recovery does not appear to be precisely synchronous among the cell population, however, since some cells exhibit cortical fluorescent spots measuring up to 2.0 μm in diameter that are linearly arranged but not closely spaced (Fig. 3, 6h, upper micrograph).

## DISCUSSION

We have identified a proteinaceous secretory product of *T thermophila* with an Mr of 34,000. In indirect immunofluorescence studies, an antiserum raised against this 34,000 Mr mucocyst component specifically localizes in the cell cortex in a linear punctate pattern along the secondary meridians. It is concluded that the linear punctate

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Fig. 1. Characterization of the anti-34,000 Mr serum by double immunodiffusion (A), immunoelectrophoresis (B), and western immunoblot analysis (C). The isolated 34,000 Mr protein was placed in the center well (Ag) of a 1% agarose gel and surrounded by (1) 1/1, (2) 1/10, (3) 1/100, (4) 1/1,000, (5) 1/10,000, and (6) 1/100,000 dilutions of antiserum (wells 1–6, A). In Figure B, a crude mucus (M) sample was resolved by electrophoresis and allowed to diffuse against both preimmune (p) and antiserum (i, immune), resulting in the formation of a single precipitin arc. In Figure C, a crude mucus sample (recovered from the medium following dibucaine stimulation) is shown in lane a (15% polyacrylamide, 0.1% SDS). A prominent component in these preparations, a 34,000 Mr polypeptide, was isolated by preparative polyacrylamide gel electrophoresis and is illustrated in lane b. Lanes a and b are stained with Commassie brilliant blue. Lanes c–f illustrate the results of a western immunoblot analysis, in which crude mucus (c), isolated 34,000 Mr protein (d), and whole cell extracts of a wt strain of *Tetrahymena*, SB 210 (e and f) were transferred from 15% polyacrylamide gels onto nitrocellulose (preadsorbed with bovine serum albumin). The nitrocellulose was overlayed with a 1/500 dilution of antiserum (lanes c–e) or preimmune serum (lane f), washed, and then incubated in a 1/2,000 dilution of horseradish peroxidase-conjugated sheep anti-rabbit IgG. Immunoreaction products were detected by staining with diaminobenzidine. A single band (34,000 Mr) is visible in lanes c–e, whereas no detectable reaction product is visible with the preimmune serum (lane f).

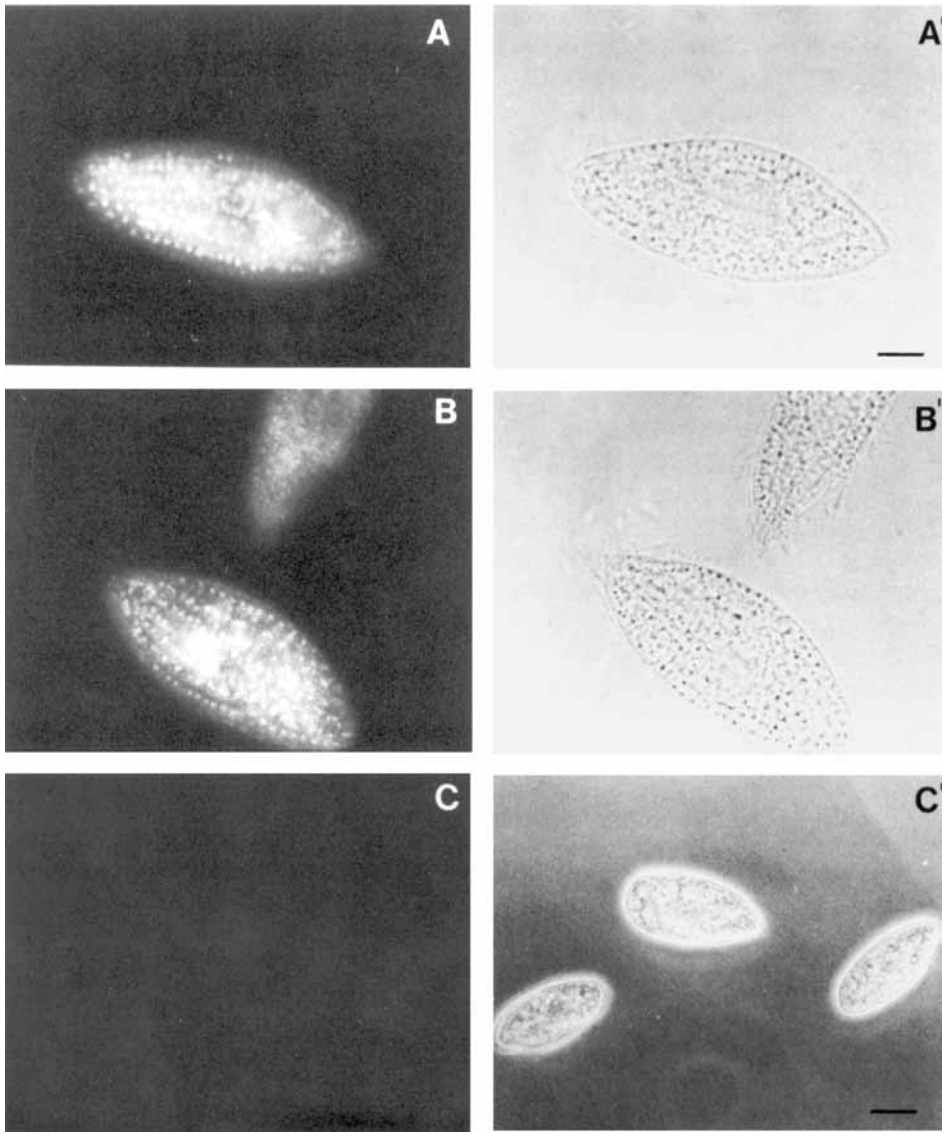


Fig. 2. Indirect immunofluorescent staining of *T thermophila* SB 210 (wt) with anti-34,000 serum. Figure A is a dorsal view of the cell illustrating a linear punctate pattern of fluorescent staining along the secondary meridians. No punctate fluorescence is visible in the region of the oral apparatus (right side of cell). Figure B is a ventral view of the cell, again illustrating the linear punctate pattern of fluorescent staining. A dim region of fluorescence exclusion is seen in all cells in the vicinity of the macronucleus. Figures A′ and B′ are corresponding bright field images (bar equals 10 $\mu$m). Figure C illustrates cells processed identically but incubated in preimmune serum rather than antiserum, and Figure C′ is the corresponding phase image (bar equals 20 $\mu$m).

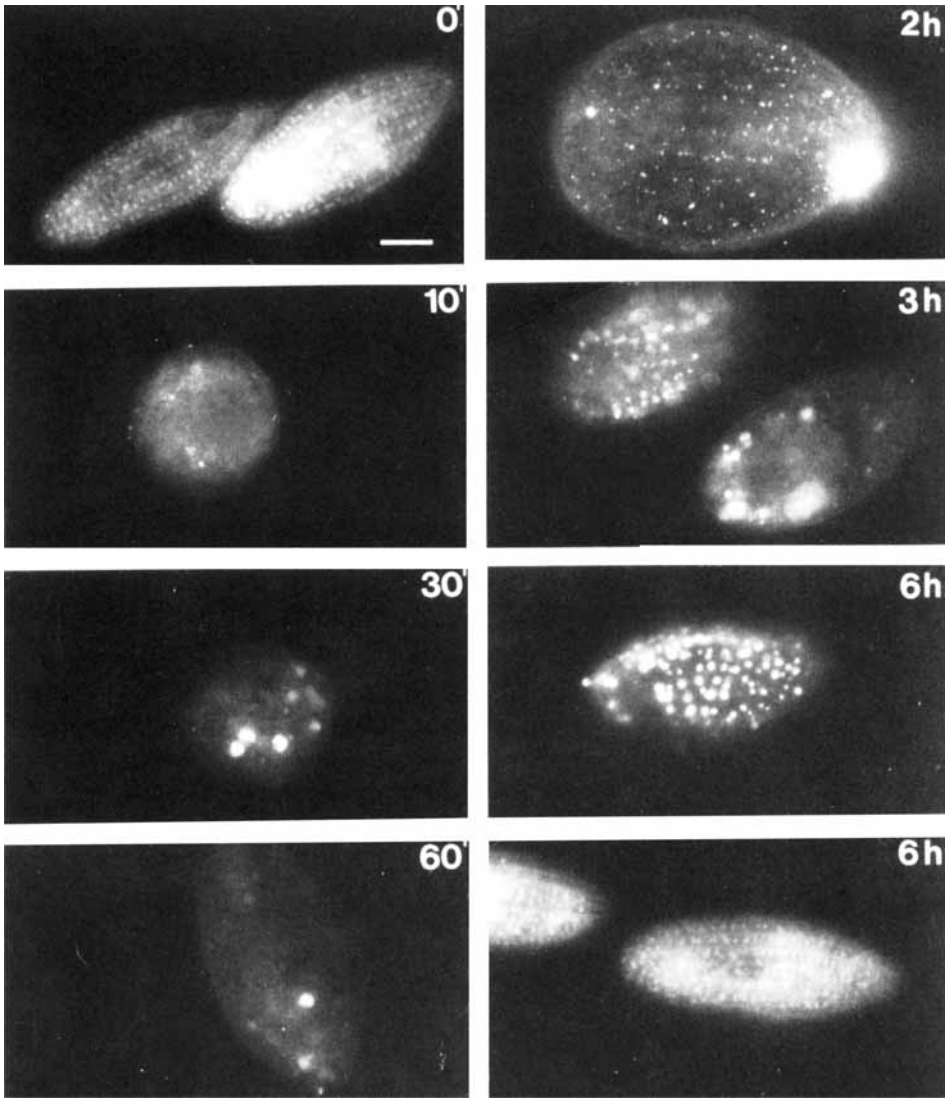


Fig. 3. Indirect immunofluorescent staining of *T thermophila* SB 210 (wt) with anti-34,000 serum during mucocyst regeneration following dibucaine stimulated secretion. At zero time (0′) cells exhibit a linear punctate pattern of fluorescent staining. Ten minutes after dibucaine stimulation (10′) cells have rounded up and lost most point-specific fluorescence. After 30 min (30′) in fresh culture medium, cells are still round, and many display staining in varying sizes of cytoplasmic vesicles. Similar fluorescent inclusions are also seen 60 min after transfer to fresh culture medium (60′). By this time point, fluorescently stained vesicles are located in the posterior region of the cell. Two hours (2h) after transfer to fresh culture medium, small sites (0.4 $\mu$m) of point-fluorescent staining appear in rows near the cortical surface of the cell, probably representing secondary meridians. After 3 hr (3h) in fresh transfer medium some cells (3h, upper left) have regions of linear punctate staining; recovery does not appear to be synchronous, however, since other cells (3h, lower right) have not yet regained a punctate pattern of fluorescence. After 6 hr (6h) of recovery, most cells have regained the fluorescent staining pattern characteristic of unstimulated cells (6h, lower micrograph). A few cells, however, even after 6 hr have not recovered the organization and density of punctate staining characteristic of unstimulated cells (6h, upper micrograph).

pattern of fluorescence corresponds primarily to sites of mature secretory organelles since (1) the spots correspond in shape and dimension to small clusters of mucocysts; (2) spacing of fluorescent spots in a given meridian may be irregular, unlike uniformly positioned basal bodies and cilia; (3) no fluorescent staining is visible in a mutant cell line (SB 281) that is identical in morphology to the wildtype parental strain, with the exception of its lack of mucocysts [8]; and (4) the linear punctate pattern of fluorescent staining is abolished following dibucaine stimulation of secretion and is recovered with kinetics consistent with those previously published for mucocyst recovery, as monitored by electron microscopy [10,11]. The identification of this ciliate secretory protein and the production of an antiserum specific for this component should prove useful in clarification of the function of these organelles and also in defining both morphological and molecular aspects of the secretory pathway in *Tetrahymena*.

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