Evidence for Defects in Membrane Traffic in *Paramecium* Secretory Mutants Unable to Produce Functional Storage Granules

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Abstract. The ciliated protozoan *Paramecium* has a regulated secretory system amenable to genetic analysis. The secretory storage granules, known as trichocysts, enclose a crystalline matrix with a genetically determined shape whose biogenesis involves proteolytic maturation of a family of precursor molecules into a heterogeneous set of small acidic polypeptides that crystallize within the maturing vesicles. We have developed an original pulse-chase protocol for monoxenic *Paramecium* cultures using radiolabeled bacteria to study the processing of trichocyst matrix proteins in wild-type and mutant cells. In wild-type cells, proteolytic processing is blocked in the presence of monensin and otherwise rapidly completed after ~20 min of chase, suggesting that the conversion occurs in the *trans*-Golgi and/or in small vesicles soon after sorting to the regulated pathway, probably before crystallization begins. In *trichless* mutant cells, which contain no visible trichocysts, secretory proteins are synthesized but not processed and we report constitutive secretion of the uncleaved precursor molecules. The mutation thus appears to affect sorting to the regulated pathway and should prove useful for analysis of the sorting machinery and of the relationship between sorting and proteolytic processing of secretory proteins. In mutants bearing misshapen trichocysts with poorly crystallized contents (*tam33*, *tam38*, *stubbyA*), the proteolytic processing of the trichocyst matrix proteins appears to be normal, while both pulse-chase and morphological data indicate that intracellular transport is perturbed, probably between ER and Golgi. Precursor molecules are present in the mutant trichocysts but not in wild-type trichocysts and may account for the defective crystallization. Our analysis of these mutants suggests that the temporal coordination of intracellular traffic plays a regulatory role in granule maturation.

THE unicellular organism *Paramecium*, like other ciliated protozoa (Hausmann, 1978), has a regulated secretory system featuring huge secretory storage granules, several microns in size. These granules, known as trichocysts, have a characteristic shape that can be altered by single gene mutation (Pollack, 1974; for review see Adoutte, 1988). In a wild-type cell, each of the 1,000–2,000 secretable trichocysts resembles a carrot surmounted by an elaborate tip (see Figs. 1 and 10), and it is via the tip that the trichocysts attach to specialized sites in the plasma membrane to await an exocytotic stimulus (Fig. 1 *A*). Recent experiments (Harumoto and Miyake, 1991) support the defensive role for these organelles long postulated (Maupas, 1883) on the basis of their cortical localization and dramatic release: the contents of the trichocysts can be massively and synchronously discharged within tens of milliseconds of stimulation (Knoll et al., 1991) and upon contact with the external medium undergo an irreversible structural transition to a needle-shaped form, literally shooting themselves out of the cell like so many tiny arrows.

The characteristic trichocyst shape reflects the crystalline organization of the protein contents of the vesicles; both the compact intracellular and the extended extracellular forms of the trichocyst matrix are three-dimensional protein crystals at a resolution of ~30 Å (Sperling et al., 1987). However the matrix is composed of a heterogeneous family of small acidic proteins: 30 major and as many as 100 distinct spots can be resolved by high resolution two dimensional gel electrophoresis (Adoutte et al., 1980; Tindall, 1986). Determination of NH_2-terminal protein sequences for a number of the spots has so far revealed unique primary sequences, with probable common secondary structure motifs (Tindall et al., 1989; Peterson et al., 1990; Le Caer et al., 1990).

A new way of looking at the problem of how a heterogeneous set of proteins can assemble into a crystalline structure with a shape, was provided by a study in which it was shown that the small proteins that crystallize are generated from higher molecular mass precursors by proteolytic processing (Adoutte et al., 1984). The evidence consisted in (*a*) the ability of polyclonal antisera raised against the small proteins of the mature trichocyst matrix (15–20 kD) to recognize

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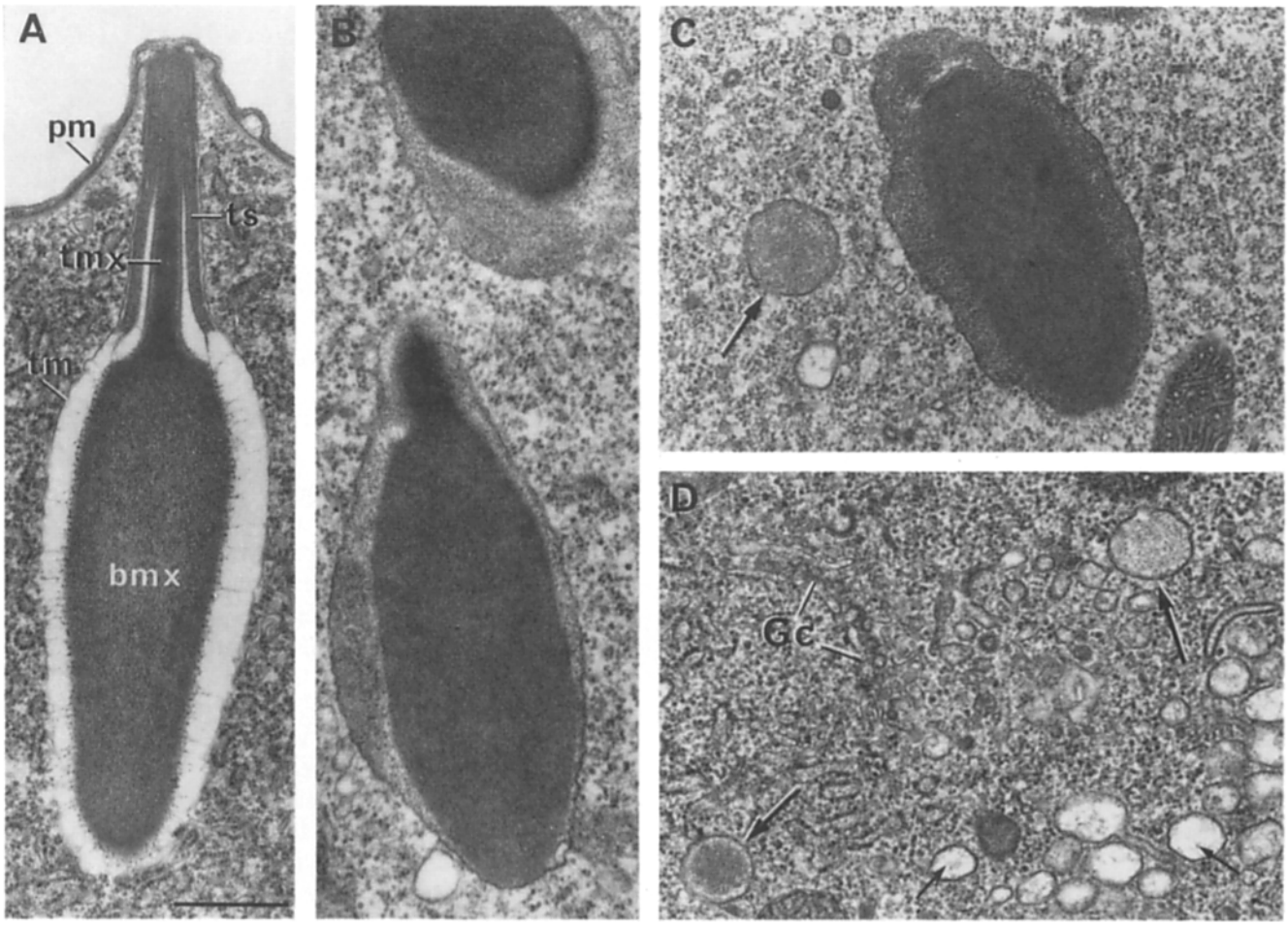


Figure 1. Thin sections of wild-type and mutant cells. (*A*) Longitudinal section through a mature trichocyst, docked at the plasma membrane, in a wild-type cell. *bmx*, body matrix; *tmx*, tip matrix; *ts*, tip sheath; *tm*, trichocyst membrane; *pm*, plasma membrane. The crystalline organization of the carrot shaped body is visualized as ~150 Å striations perpendicular to the trichocyst long axis. The clear space between the body matrix and the vesicle membrane is a fixation artefact. (*B*) Trichocysts in *stubbyA* cells have an irregular, rounded crystalline body and an abortive tip assembly. The lower part of the image shows a mature *stubbyA* trichocyst and the upper part, a maturing *stubbyA* trichocyst. Some amorphous material is visible between the crystalline body and the vesicle membrane. (*C*) Typical *tam38* trichocyst characterized by an oval crystalline core, a highly abortive tip and dense amorphous material between the core and the vesicle membrane. The arrow points to a vesicle representing a very early stage of trichocyst development, before any crystallization. (*D*) Thin section through a *trichless* cell showing clear (*small arrows*) and dense (*large arrows*) post-Golgi vesicles in the vicinity of a Golgi complex (*Gc*). The dense vesicles contain material recognized by anti-trichocyst antisera, as shown in Fig. 5 *B*. Bar, 0.5 μm.

polypeptides of higher molecular mass (40–45 kD) on whole cell blots, and (*b*) variations in the relative abundance of the two families of polypeptides in different physiological and genetic contexts. The authors advanced the hypothesis that proteolytic processing controls the morphogenesis of the trichocyst contents, only the products and not the precursors being able to crystallize. More recent studies involving monoclonal antibodies that recognize subsets of both the precursors and the products (Hausmann et al., 1988; Shih and Nelson, 1991) and biochemical characterization of the precursors (Shih and Nelson, 1992) support the notion that the proteolytic processing is perturbed in trichocyst mutants. In *Tetrahymena*, pulse-chase and cell fractionation experiments with wild-type and mutant cells provide evidence for a similar role of proteolytic processing in mucocyst biogenesis (Turkewitz et al., 1991).

To further characterize the proteolytic processing and to try to understand the steps in the regulated secretory pathway that may be critical for trichocyst morphogenesis, we have developed an original pulse-chase protocol for monoxenic *Paramecium* cultures, using radiolabeled bacteria. The protocol has allowed us to characterize the proteolytic processing of trichocyst proteins in wild-type cells and in mutants in which trichocyst biogenesis is perturbed. The mutants examined, compared with wild type in Fig. 1, are *stubbyA*, irregularly shaped trichocysts with an abortive tip; *tam33* and *tam38*, abortive trichocysts with the ovoid shape of an American football and *trichless*, no visible trichocysts. *Trichless* mutants were found to contain only precursor molecules as previously shown (Adoutte et al., 1984; Shih and Nelson, 1992). However, some of the precursor molecules were secreted constitutively into the medium, indicating that the mutation may affect sorting to the regulated pathway rather than the proteolytic machinery per se. Pulse-chase results coupled with electron microscope observations of trichocysts and Golgi complex indicate that the defect in the

three other mutants is not altered endopeptidase activity, but is situated at an earlier step of transport, probably between the ER and the Golgi apparatus. Cellular fractionation experiments revealed the presence of insoluble precursor molecules in the trichocysts of these mutants, but not in wild-type trichocysts. This observation allows us to suggest how a defect in transport to the Golgi complex can perturb an assembly process that occurs after sorting to the regulated pathway.

Materials and Methods

Cells and Culture Conditions

Wild-type *Paramecium tetraurelia* cells were of stock d4-2 (Sonneborn, 1974). About 30 recessive nuclear mutations affecting trichocyst secretion at different steps have been identified (Pollack, 1974; Cohen and Beisson, 1980). Four biogenesis mutants, containing abortive trichocysts with altered shapes, generally found free in the cytoplasm owing to an abnormal tip assembly, were studied in detail. *trichless* (Pollack, 1974) has the most extreme phenotype as *trichless* cells contain no visible trichocysts. *tam33* (Cohen and Beisson, 1980) and *tam38* (Ruiz et al., 1976) cells contain trichocysts (similar in appearance to the *football* trichocysts studied by Pollack, 1974) with the ovoid shape of an American football and a highly abortive tip. *stubbyA* trichocysts (Pollack, 1974) have an irregular, rounded or dumbbell shaped body and an abortive tip assembly.

tam6 cells (Beisson and Rossignol, 1975) contain functional trichocysts free in the cytoplasm owing to a cortical defect (Lefort-Tran et al., 1981) and were used as reference strain for the preparation of isolated trichocysts, the fractionation being facilitated by the fact that the trichocysts are unattached (Lima et al., 1989).

Two of the above mentioned trichocyst mutants, *trichless* and *stubbyA*, were isolated from stock 51; all the others were isolated from stock d4-2. All of these mutants correspond to distinct genetic loci (Cohen and Beisson, 1980).

Cultures were grown in an infusion of wheat grass powder (WGP)[1] (Pines International, Lawrence, Kansas) inoculated with *Enterobacter aerogenes* and supplemented with β-sitosterol (0.4 μg/ml) according to standard procedures (Sonneborn, 1970). The culture temperature was 27°C unless otherwise stated.

Antibody Preparation and Characterization

Trichocyst matrices were prepared on Percoll gradients as previously described (Sperling et al., 1987). After washing in 1 mM $CaCl_2$ to extend the matrices then in distilled H_2O to remove salt and Percoll, the trichocyst matrices were subjected to SDS-PAGE electrophoresis (~100 μg per minigel) and the 15–20-kD region of unstained gels was cut out and washed in distilled H_2O before homogenization in PBS. Injections of a rabbit were carried out at 3-wk intervals with gel homogenate containing 30–50 μg trichocyst proteins, in complete (first injection) or incomplete (subsequent injections) Freund's adjuvant. The serum obtained after three injections was used for the present study.

The immunizing antigen was a mixture of polypeptides, polypeptides which however not only constitute a periodic structure (the matrix), but have also been shown to be immunologically related (see Adoutte, 1988). We furthermore now have direct evidence that these polypeptides are encoded by a large multigene family (Gautier and Madeddu, personal communication). The serum gives results similar to those obtained with various other polyclonal antibodies on Western blots (Adoutte et al., 1984; Shih and Nelson, 1991), recognizing a significant proportion of the polypeptides of the mature trichocyst matrix and many different precursor molecules (as determined by two-dimesional Western blots, not shown). Most important, the serum efficiently and reproducibly immunoprecipitates both precursor and product molecules.

Pulse Chase

The bacteria used to feed *Paramecium*, *Enterobacter aerogenes*, were metabolically labeled by growth in M9 minimal medium supplemented with 200 μCi/ml carrier free $^{35}SO_4$ (Amersham International, Amersham, UK), essentially as described in Harlow and Lane (1988).

Exhausted WGP medium was prepared from stationary cultures of wild-type cells. The cultures were centrifuged in pear-shaped bottles in an oil testing centrifuge (GGT, Giovanni Giaccardo, Torino, Italy) to remove paramecia then filtered twice on ash-free filter paper to remove any remaining bacteria. The pH was adjusted (~pH 7.0) by adding Tris base to a final concentration of 2 mM before autoclaving.

Log phase *Paramecium* cultures (~1,200 cells/ml) were filtered on surgical gauze and centrifuged 5 min at 50 *g*. The cell pellets were washed in Volvic (Puy de Dôme, France) and incubated for 30 min in Volvic at the desired temperature to allow the cells to finish digestion and release existing food vacuoles. The cells were concentrated by centrifugation and taken up at 800 cells/ml in exhausted WGP medium supplemented with 0.4 μg/ml β-sitosterol. 5×10^7 labeled bacteria were added per ml of cell culture and incubation was carried out for 10 min in a thermostated water bath, at 27°C unless otherwise stated. After centrifugation, the cells were resuspended at 800 cells/ml in standard WGP medium, supplemented with 5×10^7 unlabeled bacteria per ml to start the chase. (standard WGP medium contains ~$1–5 \times 10^7$ bacteria/ml). 6-ml aliquots of the cultures were removed at different times and mixed with 2 ml of a 0.2% solution of $MnCl_2$, which inhibits ciliary beating, immediately before centrifugation in a clinical centrifuge to pellet the cells for immunoprecipitation.

Pulse-chase experiments in the presence of monensin (Calbiochem-Novabiochem, Lajolla, CA) were carried out after preincubation of the cell culture for 2 h in 20 μM monensin, added to the culture from a 20 mM stock solution in ethanol. Control cultures contained an equivalent concentration of ethanol.

Immunoprecipitation and Gel Electrophoresis

Conditions for solubilization of trichocyst proteins for immunoprecipitation were determined by Western blot experiments (not shown). The cell pellets were lysed in two steps. First, to solubilize the crystalline matrix proteins, each pellet (resuspended in the remaining drop of chase medium: 100 μl volume) was projected into an equivalent volume of hot (95°C) 0.4% SDS. Second, a standard immunoprecipitation buffer (RIPA; Harlow and Lane, 1988) was immediately reconstituted by addition of an equal volume (200 μl) of 2× RIPA (without SDS) containing protease inhibitors. 1× RIPA is 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0. 1× protease inhibitors is 50 μg/ml PMSF, 5 μg/ml leupeptin. At this stage, the cell lysates can be frozen.

Before immunoprecipitation, the cell lysates were cleared by centrifugation at 12,500 *g* in a refrigerated minifuge for 20 min. The cleared lysates were incubated for 90 min at 4°C with a saturating quantity of the polyclonal antiserum (2 μl antiserum per 400 μl cell lysate). To collect the immunocomplexes, 100 μl of a 10% suspension of protein A-sepharose beads (Pharmacia, Uppsala, Sweden) were added to each lysate and gentle mixing was carried out for 3 h at 4°C. The beads were collected by brief centrifugation and washed three times in RIPA buffer. Final pellets were incubated 10 min at 95°C in sample buffer before electrophoresis on 12% SDS-PAGE gels according to Laemmli (1970). After Coomassie blue staining, the gels were dried and were either exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) at room temperature (24–72 h) or quantified with a Phosphorimager (Molecular Dynamics SA, France).

The efficiency of the immunoprecipitation as a function of antigen concentration was determined by immunoprecipitating dilutions of wild-type cell lysates with a constant amount of antiserum, and quantifying the results with a Phosphor Imager. We found a linear relationship over two logs of sample dilution (not shown).

Cell Fractionation and Western Blots

Exponential cell cultures were washed and resuspended at 200,000 cells/ml in Volvic. To make whole cell lysates, the cell suspension was projected into an equal volume of boiling 4% SDS and incubated for 2 min before snap freezing. To examine proteins secreted into the culture medium, cells were removed by centrifugation and aliquots of the medium were precipitated with methanol, as described below. To make Triton X-100 soluble and insoluble fractions, the cell suspension was mixed with an equal volume of 2× lysis buffer, 2× in protease inhibitors, and incubated 5 min at room temperature. (1× lysis buffer is PHEM–1% Triton X-100. PHEM is 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9 [Schliwa and Van Blerkom, 1981]. 1× protease inhibitors is 50 μg/ml PMSF, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 mM DTT). The suspension was centrifuged 5 min at 12,500 *g* in a minifuge at room tempera-

1. *Abbreviation used in this paper*: WGP, wheat grass powder.

ture and the pellet and supernatant separated. The supernatant was precipitated by addition of nine volumes of ice cold methanol followed by a 20-min incubation on ice. The precipitate was collected by centrifugation at 12,500 *g* for 20 min in a refrigerated minifuge. Cell fractionation was monitored by immunoblot using anti-ciliary rootlet antiserum as control for the insoluble fraction (Sperling et al., 1991) and anti-surface antigen antiserum as control for the soluble fraction.

Trichocysts with their membranes were isolated on Percoll density gradients according to Lima et al. (1989) except that the homogenization buffer contained 0.1% BSA delipidated on activated charcoal according to Chen (1967) instead of 0.5% BSA. The trichocysts were washed by diluting the Percoll gradient bands eight times with homogenization buffer and centrifuging the suspension 10 min at 10,000 rpm in 15 ml corex tubes in the JA-20 rotor of a Beckman MSE 21 centrifuge, at 15°C (Beckman Instruments, Inc., Fullerton, CA). The loose pellets were carefully taken up in fresh homogenization buffer, transferred to Eppendorf tubes and the final pellets were obtained by centrifugation in a minifuge (10 min at 12,500 *g*, at room temperature).

After 12% SDS-PAGE electrophoresis of the different cell fractions (Laemmli, 1970), proteins were electroblotted to nitrocellulose filters according to Towbin et al. (1979). The filters were saturated and processed in 5% low fat milk powder in TBS pH 8.0–0.1% Tween 20. The antiserum directed against trichocyst matrix proteins was used at 1/1,000 dilution. The second antibody was affinity purified anti-rabbit IgG coupled to alkaline phosphatase and was used according to the supplier's instructions (Promega, Madison, WI).

Electron Microscopy and Immunolocalization

Whole cell pellets were fixed in 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.2, for 90 min at 4°C. After washing in the same buffer, the samples were post-fixed in 1% osmium tetroxide in 0.05 M cacodylate buffer, for 60 min at 4°C. Post-fixed cells were dehydrated by passages through a series of ethanol and propylene oxide baths before embedding in Epon. Thin sections were contrasted with ethanolic uranyl acetate and lead citrate, then examined with a Philips EM 201 or EM 301.

For immunolocalization, samples were fixed in 2% paraformaldehyde–0.15% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, at room temperature for 2 h. After washing in the same buffer, cells were dehydrated by passage through a series of ethanol baths before embedding in LRW (London Resin Ltd.). Thin sections were collected on nickel grids and saturated and processed with 3% BSA in PBS. The anti-trichocyst serum was used at a dilution of 1/200. After washing, the sections were incubated with 10 nm colloidal gold-conjugated anti-rabbit immunoglobulins (GAR b10, Amersham) at 1/100 dilution. After extensive washing, the sections were contrasted with ethanolic uranyl acetate.

Results

We have used pulse-chase experiments to examine the role of the postulated proteolytic processing of trichocyst matrix proteins in the biogenesis of trichocysts. Such experiments had not previously been attempted as *Paramecium* cultures are nourished with bacterized herb infusion, the classic medium for *Paramecium* genetics (Sonneborn, 1970). Defined axenic media have been developed for biochemical studies, but they provide only poor incorporation of labeled amino acids, as discussed by Shih and Nelson (1992). Our protocol involves feeding $^{35}SO_4$-labeled bacteria to paramecia for 10 min, followed by a chase with cold bacteria. As it takes ~15 min for a digestive vacuole to travel through a *Paramecium* (Fok et al., 1982), our experiments are "blurred" by a 15-min window corresponding to the time it takes the cell to digest the bacteria and add the bacterial amino acids to the pool available for protein synthesis.

Rapid Conversion in Wild-type Cells

Fig. 2 shows a typical experiment carried out with wild-type cells at 27°C. The family of 40–45-kD precursor molecules, strongly labeled at the beginning of the chase period, are completely converted to the lower molecular mass products by 40 min of chase with an apparent $t_{1/2}$ of 20 min. This experiment provides the most direct evidence to date for the precursor-product relationship originally postulated by Adoutte et al. (1984).

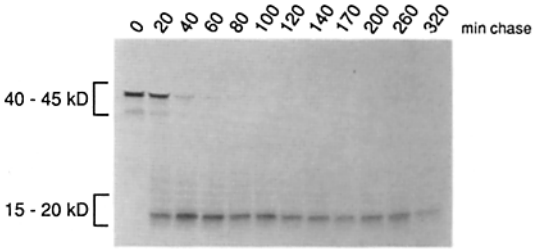


Figure 2. Pulse–chase experiment, wild-type cells at 27°C. A log phase culture of wild-type cells was fed with metabolically labeled bacteria for 10 min, washed, and resuspended in chase medium containing unlabeled bacteria. Aliquots of the culture were removed, lysed, and immunoprecipitated with the anti-trichocyst antiserum over a 5-h chase period. The immunoprecipitated proteins were separated on a 12% SDS-polyacrylamide gel, which was dried and exposed to autoradiographic film.

To obtain information on the compartment where processing occurs, we examined the effects of the drug monensin on the conversion of precursors to products. Wild-type cells were treated with 20 μM monensin starting 2 h before the pulse-chase experiment, and a total block in the proteolytic processing was observed (Fig. 3 *A*). The effects of the drug are reversible: if the monensin is washed out after 1 h of chase, the precursors are then completely converted to products, with approximately normal kinetics (Fig. 3 *B*). Although monensin is known to affect many different cellular processes (Mollenhauer et al., 1990), the transport of the secretory proteins is clearly blocked before they reach the processing compartment. As monensin has been shown to block transport in mammalian cells between *medial-* and *trans*-Golgi (Griffiths et al., 1983; Quinn et al., 1983), these results indicate that the proteolytic conversion occurs in the *trans*-Golgi or after sorting to the regulated pathway (see Discussion).

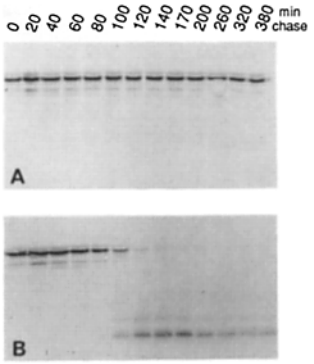


Figure 3. Pulse-chase profiles of wild-type cells treated with monensin. (*A*) A culture was treated with 20 μM monensin starting 2 h before the pulse–chase experiment. Total absence of conversion of precursors is observed throughout the 6-h chase period. (*B*) A parallel culture of wild-type cells was treated as in *A*, but the monensin was removed after 60 min of chase. Once the monensin is removed, the precursors are rapidly converted to products, showing that the effects of the drug are reversible.

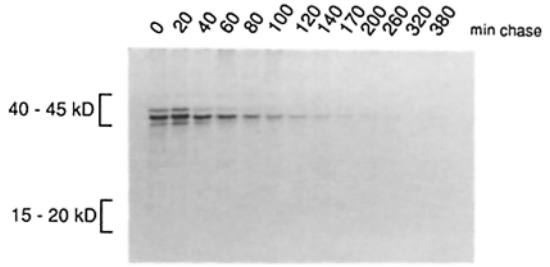


Figure 4. Pulse-chase profile of a *trichless* cell culture at 27°C. No products are detected, however the precursors disappear progressively, with a $t_{1/2}$ of ~40–50 min.

No Proteolytic Processing in the Mutant Trichless

Fig. 4 shows a pulse-chase experiment carried out with *trichless* cells at 27°C. As expected, absolutely no conversion of the precursors to products was found. The maximal amount of the precursors is detected at 20 min rather than at 0 min of chase and this illustrates the limitations of using the radiolabeled bacteria. In this slow growing mutant, the true chase has begun as much as 20 min after the radiolabeled bacteria were removed and the cells transferred to the chase medium.

Although no polypeptides the size of products appear, the quantity of the precursors diminishes with time, with a $t_{1/2}$ of 40 min, or less if we try to correct for the true beginning of the chase. It is possible either that the precursor molecules which cannot be processed are degraded or that they are secreted into the medium. To test the latter possibility, pulse-chase experiments were carried out in parallel on *trichless* and wild-type cells, and both cells and medium were analyzed at different times after the beginning of the chase. The result is shown in Fig. 5 *A*. In the *trichless* culture, but not in the wild-type culture, precursor molecules can be immunoprecipitated from the medium by 40 min of chase. Moreover, the same pattern of precursor bands is observed for intracellular and secreted molecules, as best illustrated by Western blots of cells and medium (Fig. 5 *B*).

The pulse-chase data show that 10–15% of the intracellular precursor molecules are secreted. This value should be corrected for proteolytic degradation in the culture medium, estimated from incubation experiments carried out with immunopurified radiolabeled precursor molecules to be ~50% (Madeddu, L., unpublished data).

Immunocytochemical examination of *trichless* cells at the ultrastructural level reveals the presence of 250–400 nm vesicles in the vicinity of the plasma membrane that are labeled by antibodies directed against trichocyst matrix proteins (Fig. 5 *C*). Vesicles of this size that contain trichocyst material can also be observed in wild-type cells (see Fig. 8 *A*), usually near Golgi stacks, where they fuse with other vesicles in an early step of granule maturation (Hausmann et al., 1988; Garreau de Loubresse, 1993). Although caution is required in interpreting these images of *trichless* cells since constitutive secretion has not been characterized in *Paramecium* (see Discussion in Capdeville et al., 1993), it is tempting to suggest that these are the vesicles responsible for the secretion of precursor molecules.

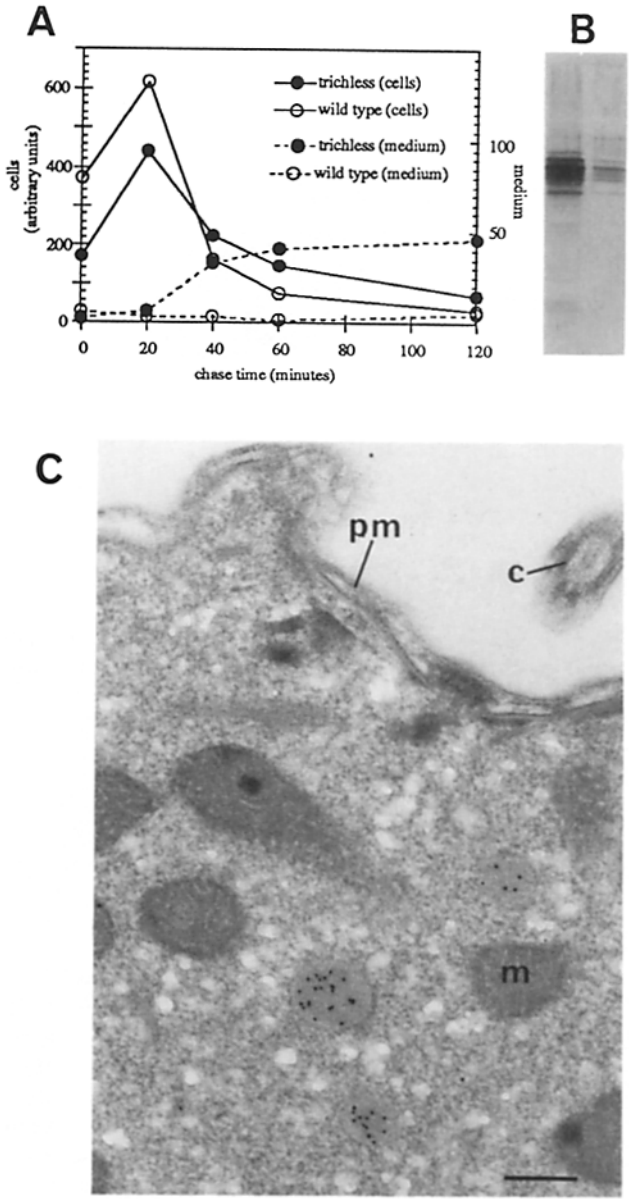


Figure 5. trichless cells secrete precursor molecules into the culture medium. (*A*) A pulse–chase experiment was carried out in parallel on wild-type and *trichless* cultures, and after immunoprecipitation of cell lysates and of aliquots of the culture medium, the radioactivity in the precursor bands separated by SDS-PAGE was quantified with a Phosphorimager (Molecular Dynamics). The values are represented in arbitrary units as a function of the chase time. (*B*) Western blot, with the anti-trichocyst antiserum, of *trichless* cells (*left*) and corresponding medium (*right*) after separation on an SDS-PAGE gel, showing the pattern of the 40–45-kD precursor bands. (*C*) immunocytochemical image of a cortical cross section of a *trichless* cell, showing several 250–350-nm vesicles near the plasma membrane whose contents are recognized by the anti-trichocyst antiserum. *c*, cilia; *m*, mitochondrion; *pm*, plasma membrane. Bar, 0.25 μm.

Impaired transport in tam33, tam38, and stubbyA Mutants

We expected *tam33*, *tam38*, and perhaps also *stubbyA* cells to be defective in processing the precursor molecules. The pulse–chase results were not consistent with this hypothesis.

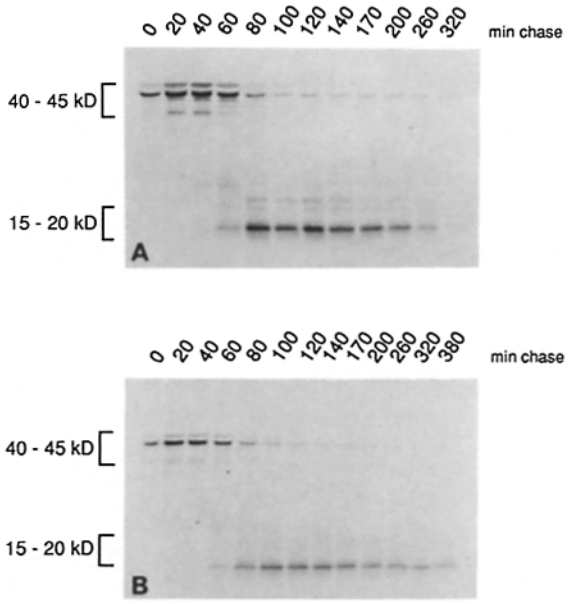


Figure 6. Pulse-chase profiles of *tam38* and *stubbyA* mutant cells. (*A*) Pulse-chase experiment, *tam38* culture at 27°C. (*B*) Pulse-chase experiment, *stubbyA* culture at 27°C. In both *A* and *B*, ~50% of the precursors have been converted to products after 80 min of chase.

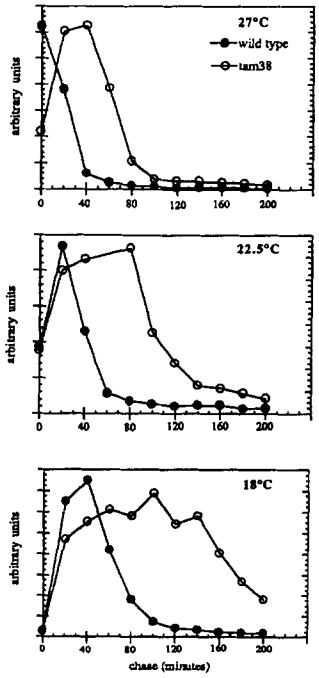


Figure 7. Pulse-chase profiles of precursor molecules in wild-type and *tam38* cultures. The radioactivity contained in each band was quantified with a Phosphorimager and is represented in arbitrary units as a function of chase time. The experiments were carried out in parallel, at three temperatures, from top to bottom: 27, 22.5, and 18°C.

Conversion of precursors to products appears to go to completion in *tam38* cells (Fig. 6 *A*), *tam33* cells (not shown), and *stubbyA* cells (Fig. 6 *B*). However, the $t_{1/2}$ of the precursors is much longer than in the wild type, consistent with delayed processing in these mutant cells.

To try to determine whether the enzymatic machinery responsible for processing the secretory proteins is indeed normal in these mutants, we performed pulse-chase experiments on *tam38* and wild-type cells at three different temperatures (18, 22.5, and 27°C) and quantified the results (Fig. 7). Reducing the temperature increases the time required for conversion of the precursors in both wild-type and *tam38* cells. Significantly, at each temperature the curves are shifted with respect to each other but their slopes, indicative of the approximate rates of proteolytic conversion, are the same. This experiment speaks for normal endopeptidase activities in *tam38* cells.

Examination of *tam33* and *tam38* cells by electron microscopy reveals the accumulation of transport vesicles with an electron-dense coat between the ER and the Golgi complex, the same vesicles recently identified by Allen and Fok (1993) as nonclathrin coated vesicles (a preliminary account of these results may be found in Garreau de Loubresse, 1993). Similar images of vesicle accumulation were obtained for *stubbyA* cells (not shown). Fig. 8 *A* shows the appearance of ER and adjacent Golgi stacks in wild-type *Paramecium* cells. In *tam33* and *tam38* cells at 27°C, the usual culture temperature, many Golgi stacks appear grouped together, and numerous vesicles of diverse types are seen around them, with a predominance of nonclathrin coated transport vesicles.

tam38 cells were examined at different temperatures. At 33°C, the effect is even more dramatic than at 27°C: the cells display enormous clusters of vesicles, with ER and Golgi membranes visible at the periphery of the clusters (Fig. 8, *C* and *D*). At 14°C there is no notable accumulation of the vesicles and the *tam38* Golgi complex has an almost normal appearance, although the trichocysts still have the mutant phenotype (Fig. 8 *B*). As lowering the temperature reduces the rate of many cellular processes, such as budding of transport vesicles from donor membranes (Rothman and Orci, 1992), the net result we observe may be partial resynchronization of vesicle transport with the restoration of a nearly wild-type ultrastructure. In conclusion, the delayed proteolytic conversion taken together with nonclathrin-coated vesicle accumulation lead us to suggest that the primary defect in these mutants is in intracellular vesicle-mediated transport, probably between the ER and the Golgi complex.

Cellular Distribution of Precursors and Products

If, as our data suggest, the primary defect in *tam33*, *tam38*, and *stubbyA* mutant cells affects transport to or through the Golgi complex and not proteolytic processing, then why are the trichocysts, which assemble later in the pathway within maturing secretory granules, aberrantly shaped? As it had already been shown that these cells contain a high precursor to product ratio (Adoutte et al., 1984), we decided to investigate the cellular distribution of the precursor molecules.

We first performed Western blot experiments on extracts of whole cells and on Triton-soluble and Triton-insoluble fractions (Fig. 9). In wild-type cells, the 40–45-kD polypeptides are found in the soluble fraction and the 15–20-kD polypeptides in the insoluble fraction. In *trichless* cells only precursors are present, and they are found exclusively in the soluble fraction. The other mutant cells, *tam38*, *tam33* (results identical to *tam38*, not shown) and *stubbyA*, differ from wild-type cells in two significant ways. First, as previously reported for *tam38* (Adoutte et al., 1984), the precursor/product ratio is much higher than in wild-type cells, consistent with an accumulation of transport vesicles containing precursor molecules. Second, the insoluble fraction from

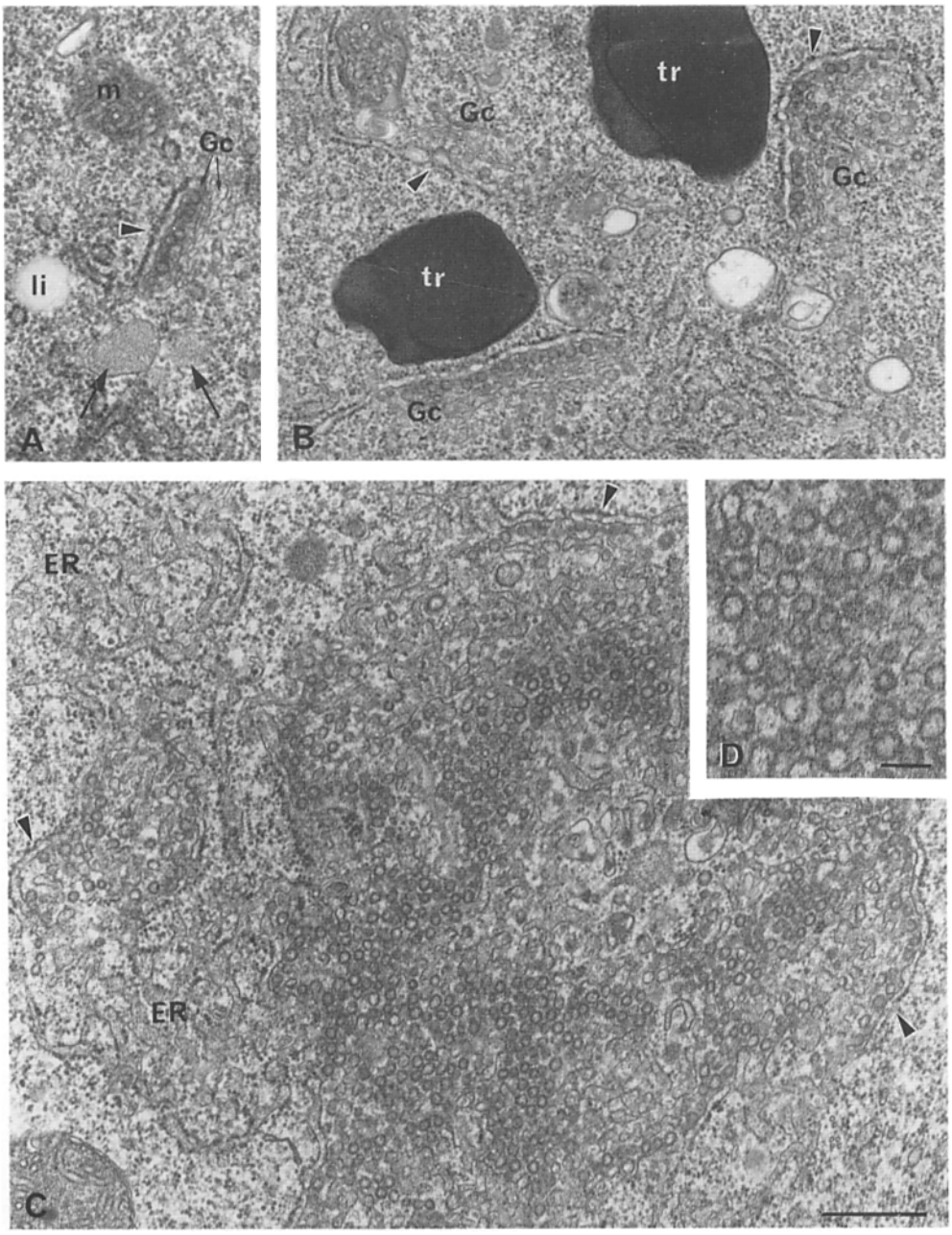


Figure 8. Thin sections of wild-type and *tam38* cells. Arrowheads point to transitional ER cisternae. *Gc*, Golgi complex; *li*, lipid droplet; *tr*, trichocyst. (*A*) Wild-type cell grown at 27°C showing a typical *Paramecium* Golgi complex. The arrows point to vesicles representing early stages of trichocyst development, before any crystallization. (*B*) *tam38* cell grown at 14°C, showing subnormal Golgi complex and football shaped trichocysts. (*C*) *tam38* cell grown at 33°C, showing massive accumulation of membranous structures, mostly nonclathrin-coated transport vesicles. At the periphery of the cluster, patches of Golgi complexes with recognizable transitional ER cisternae can be seen (*arrows*). (*D*) Enlarged view of the coated transport vesicles that accumulate in *tam38* cells at 27 and 33°C. They have a diameter of ~55–60 nm. Bars: (*A*, *B*, and *C*) 0.5 μm; (*D*) 0.1 μm.

each of the mutant cell populations contains 40–45-kD precursor molecules.

To see whether the 40–45-kD polypeptides present in the insoluble fraction are in fact trapped in the abortive trichocyst matrices, we isolated trichocysts with their membranes from *tam6*, *tam38*, and *stubbyA* cells. *tam6* cells contain functional trichocysts free in the cytoplasm which we consider to be "wild type," the mutation affecting the cortical docking sites (see Materials and Methods). Phase-contrast images of aliquots of the trichocyst fractions are shown in Fig. 10 *A*. The isolated trichocysts were subjected to SDS-PAGE and the corresponding blots were treated with the antiserum. The results (Fig. 10 *B*) show that 40–45-kD precursor molecules are present in the vesicles isolated from *tam38* (at either temperature) and *stubbyA* cells but are not present in the "wild type" trichocysts from *tam6* cells. Trichocysts isolated from *tam38* cells were further fractionated according to their solubility in Triton X-100 and the results confirm that the precursor molecules present in the trichocysts are insoluble (not shown). The explanation of these observations we favor is that the presence of precursor molecules in maturing granules perturbs the crystallization process and can account for the aberrantly shaped non-functional trichocysts.

Discussion

Studies of storage granule biogenesis in a number of exocrine and neuroendocrine cell types have led to the generally accepted notion that sorting of secretory proteins to the regulated pathway involves their aggregation within the TGN. The aggregated contents then bud from the TGN as immature secretory granules (for review see Tooze et al., 1993). None-

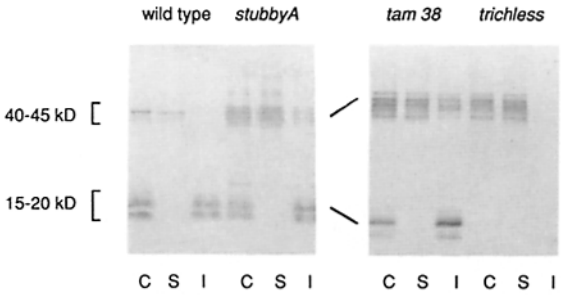


Figure 9. Western blots of wild-type and mutant cells. Whole cell lysate (*C*), Triton X-100 soluble fraction (*S*) and Triton X-100 insoluble pellet (*I*) were prepared for each cell culture. The polypeptides were separated by SDS-PAGE and transferred to nitrocellulose filters for treatment with the anti-trichocyst antiserum. The gel samples were adjusted so that the 15–20-kD product bands contain equivalent quantities of protein.

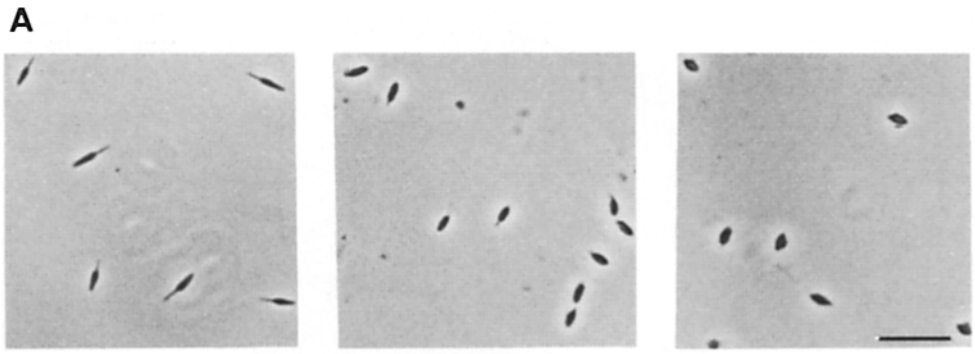
theless, the molecular mechanisms of the aggregation-dependent sorting and of subsequent steps in granule maturation (i.e., fusion and budding to add or remove contents and membranes from the maturing granules; condensation of contents) remain to be clarified.

Paramecium provides a model system for the study of regulated secretion (Adoutte, 1988). Although best exploited to date for studies of stimulus-secretion coupling (Plattner et al., 1991; Cohen and Kerboeuf, 1993), the system has great potential for analysis of the above mentioned problems, since mutants are available in which secretory storage granule biogenesis is defective. In the present article, we have analyzed several trichocyst biogenesis mutants using a novel pulse-chase protocol to identify the steps in the secretory pathway that are affected by each mutation. We report that one mutant, *trichless*, is of particular interest as it is most likely affected at the level of the sorting machinery. In the three other mutants analyzed, we find that primary defects which slow down vesicle-mediated transport early in the secretory pathway are sufficient to perturb late steps in granule maturation and yield aberrantly shaped trichocysts, suggesting that the temporal coordination of membrane traffic may be an important parameter in granule biogenesis.

Trichocyst Biogenesis and the Golgi Complex

Ultrastructural studies have traced trichocyst maturation starting from small post-Golgi vesicles containing amorphous material to the mature organelle with fully crystallized carrot-shaped contents and an elaborate tip involved in docking at sites in the plasma membrane (Yusa, 1963; Hausmann et al., 1988). However the earlier part of the pathway, through the Golgi complex, is less well characterized. Only recently has a careful ultrastructural study of *Paramecium* (Allen and Fok, 1993) provided a clear description of the organization of ciliate Golgi stacks and in particular of the ER–Golgi transitional zone, with identification of non-clathrin coated transport vesicles.

On the biochemical side, the trichocyst matrix proteins are only slightly glycosylated as judged by lectin-binding studies (Glas-Albrecht et al., 1990), and no other posttranslational modifications that would allow the secretory proteins to be



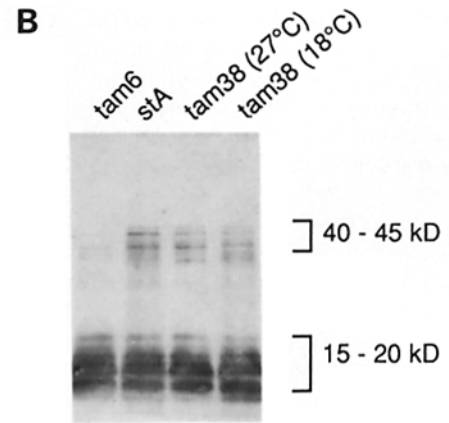


Figure 10. Insoluble precursor molecules are present in trichocysts isolated from *tam38* and *stubbyA* cells. (*A*) Phase-contrast light microscope images of trichocyst preparations. From left to right, the trichocysts were prepared from *tam6*, *stubbyA*, and *tam38* cultures grown at 27°C. (*B*) Western blot of the different trichocyst preparations using the anti-trichocyst antiserum. Bar, 10 μm.

followed as they travel through the Golgi have been characterized. The only modification detected by our pulse–chase experiments is extensive proteolytic maturation.

Evidence that the secretory proteins do travel through the Golgi complex has been obtained by a morphological and immunolocalization study in which proliferation of ER and Golgi elements, recognized by antibodies directed against trichocyst matrix proteins, was observed after stimulation of massive trichocyst exocytosis (Garreau de Loubresse, 1993). Thus, 20–30 min after stimulation of massive exocytosis, proliferation of ER, Golgi membranes and small post-TGN vesicles was observed. Larger vesicles with a small core of crystalline material were most numerous 40–60 min after stimulation and mature (fully crystallized) trichocysts appeared in the cytoplasm starting 60–90 min after stimulation. It therefore seems likely that the proteolytic processing of trichocyst matrix proteins in wild-type cells, which is (*a*) blocked by monensin and (*b*) occurs ~20 min after synthesis of the precursors according to the pulse-chase data, is complete before the beginning of crystallization, taking place in the TGN and/or in the small amorphous vesicles that bud from the TGN.

Secretory Mutants Defective in Intracellular Traffic

Proteolytic processing of secretory proteins is involved in the maturation of dense core vesicles in many systems (Steiner et al., 1992) and previous work in *Paramecium* suggested that the different trichocyst biogenesis mutants would turn out to be defective in proteolytic processing. Contrary to expectations, the *Paramecium* secretory mutants we have examined appear to be affected not in the activity of processing enzymes (with the possible exception of *trichless*), but rather in membrane traffic.

trichless cells are characterized by the absence of any visible trichocysts and contain only uncleaved precursor molecules. However, the observation that the precursor molecules are secreted constitutively into the culture medium indicates that sorting of secretory proteins to the regulated pathway is affected in some way in these cells. A *Tetrahymena* mutant very similar to *trichless* has been described (*SB281*; Orias et al., 1983); the mutant cells lack visible mucocysts and have been reported to constitutively secrete mucocyst precursors into the culture medium (Turkewitz et al., 1991).

In metazoan cells, few examples of mutations that affect sorting to the regulated secretory pathway are available. Constitutive secretion of proinsulin has been reported, in the case of a familial proinsulinemia which results from a single amino acid change in the proinsulin molecule (Gross et al., 1989), and the physicochemical properties of the mutant proinsulin are thought to be responsible for the sorting defect. Given that the trichocyst precursor molecules are the products not of a single gene but of a multigene family, it seems unlikely that a recessive mutation in a single precursor molecule could account for the *trichless* phenotype.

Current views on sorting favor a mechanism involving aggregation, within the particular environment provided by the lumen of the TGN, of those molecules that constitute the dense core of the granule (Chanat and Huttner, 1991; Chanat et al., 1993). A recessive mutation could alter the physicochemical environment of the TGN in *trichless* cells so as to affect the specific interactions between secretory proteins necessary for sorting to the regulated pathway. For example, it has been shown in many systems that the TGN is an acidic (or acidifying) compartment (Orci et al., 1987; Anderson and Orci, 1988); vacuolar pH could be affected in *trichless* cells.

Although missorting of secretory proteins as they exit the TGN would preclude any proteolytic processing that occurs later in the pathway and account for the *trichless* phenotype, the inverse could also be true. In a number of metazoan systems, some or all of the cleavage events involved in maturation of proteins destined for dense core vesicles occur in the TGN (Davidson et al., 1988; Lepage-Lesin et al., 1991; Sossin et al., 1990). This may also be the case in *Paramecium*, and if only processed or partially processed molecules successfully aggregate and sort to the regulated pathway, then a primary defect in the processing endopeptidase could affect sorting. Future experiments will aim to clarify the relationship between proteolytic processing and sorting of trichocyst matrix proteins.

The other secretory mutants we have studied, *tam33*, *tam38*, and *stubbyA*, are characterized by trichocysts with altered shape of the crystalline body matrix and an abortive tip assembly. Our pulse–chase analysis indicates that the activity of the endopeptidase(s) in these cells is most probably normal while transport of newly synthesized precursor molecules to the compartment where proteolytic conversion occurs is significantly retarded. Electron microscope images of *tam38* cells show accumulation of vesicles which we presume on the basis of their morphology and localization to be the nonclathrin coated transport vesicles identified in *Paramecium* by Allen and Fok (1993). In this respect, *tam38* is reminiscent of yeast *sec* mutants that accumulate transport vesicles (Kaiser and Sheckman, 1990). The ensemble of our data thus strongly support the notion that *tam38*, and probably the two other mutants as well, are affected in vesicle-mediated transport to and/or through the Golgi complex.

How can a defect in transport through the Golgi complex affect the assembly of trichocysts, a process which occurs after sorting to the regulated secretory pathway? Our data indicate that the enzyme activities involved in proteolytic processing are normal in these cells. Yet the proteolytic processing is perturbed, as a result of the traffic defect, since some incorrectly processed/unprocessed precursor molecules show up in the mutant trichocysts. The presence of these incorrectly processed molecules may be sufficient to sabotage the assembly process that normally leads to a mature functional trichocyst, providing an example of how the state of posttranslational modification of structural proteins can affect the shape of an organelle.

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References

Adoutte, A. 1988. Exocytosis: biogenesis, transport and secretion of trichocysts. *In* Paramecium. H.-D. Görz, editor. Springer-Verlag, Heidelberg.

325–362.

Adoutte, A., N. Garreau de Loubresse, and J. Beisson. 1984. Proteolytic cleavage and maturation of the crystalline secretion products of *Paramecium*. *J. Mol. Biol.* 180:1065–1080.

Adoutte, A., R. Ramanathan, R. M. Lewis, R. R. Dute, K. Y. Ling, C. Kung, and D. L. Nelson. 1980. Biochemical studies on the excitable membrane of *Paramecium tetraurelia*. III. Proteins of cilia and ciliary membranes. *J. Cell Biol.* 84:717–738.

Allen, R. D., and A. K. Fok. 1993. Nonclathrin vesicle coats and filament networks in the transition zone and trans–Golgi region of the Golgi complex of Paramecium. *J. Struct. Biol.* 110:215–226.

Anderson, R. G. W., and L. Orci. 1988. A view of acidic intracellular compartments. *J. Cell Biol.* 106:539–543.

Beisson, J., and M. Rossignol. 1975. Movements and positioning of organelles in *Paramecium aurelia*. *In* Molecular Biology of Nuclear Cytoplasmic Relationships. S. Puiseaux-Dao, editor. Elsevier, North Holland Amsterdam. 291–294.

Capdeville, Y., R. Charret, C. Antony, J. Delorme, P. Nahon, and A. Adoutte. 1993. Ciliary and plasma membrane proteins in *Paramecium*: description, localization and intracellular transit. *In* Membrane Traffic in Protozoa. H. Plattner, editor. JAI Press Inc., Greenwich, CT. 181–226.

Chanat, E., and W. B. Huttner. 1991. Milieu-induced, selective aggregation of regulated secretory proteins in the *trans*-Golgi network. *J. Cell Biol.* 115:1505–1519.

Chanat, E., U. Weiss, W. B. Huttner, and S. A. Tooze. 1993. Reduction of the disulfide bond of chromogranin *B* (secretogranin I) in the trans-Golgi network causes its missorting to the constitutive secretory pathway. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:2159–2168.

Chen, R. F. 1967. Removal of fatty acids from serum albumin by charcoal treatment. *J. Biol. Chem.* 242:173–181.

Cohen, J., and J. Beisson. 1980. Genetic analysis of the relationships between the cell surface and the nuclei in *Paramecium tetraurelia*. *Genetics*. 95: 797–818.

Cohen, J., and D. Kerboeuf. 1993. Calcium and trichocyst exocytosis in *Paramecium*: genetic and physiological studies. *In* Membrane Traffic in Protozoa. H. Plattner, editor. JAI Press Inc., Greenwich, CT. 61–81.

Davidson, H. W., C. J. Rhodes, and J. C. Hutton. 1988. Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic β cell via two distinct site–specific endopeptidases. *Nature (Lond.)*. 333:93–96.

Fok, A. K., Y. Lee, and R. D. Allen. 1982. The correlation of digestive vacuole pH and size with the digestive cycle in *Paramecium caudatum*. *J. Protozool.* 29:409–414.

Garreau de Loubresse, N. 1993. Early steps of the secretory pathway in *Paramecium*: ultrastructural, immunocytochemical and genetic analysis of trichocyst biogenesis. *In* Membrane Traffic in Protozoa. H. Plattner, editor. JAI Press Inc., Greenwich, CT. 27–59.

Glas–Albrecht, R., A. Németh, and H. Plattner. 1990. Secretory proteins and glycoproteins from Paramecium cells. *Eur. J. Protistol.* 26:149–159.

Griffiths, G., P. Quinn, and G. Warren. 1983. Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from medial to trans Golgi cisternae in baby hamster kidney cells infected with Semliki Forest Virus. *J. Cell Biol.* 96:835–850.

Gross, D. J., P. A. Halban, C. R. Kahn, G. C. Weir, and L. Villa-Komaroff. 1989. Partial diversion of a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells. *Proc. Natl. Acad. Sci. USA*. 86:4107–4111.

Harumoto, T., and A. Miyake. 1991. Defensive Function of Trichocysts in *Paramecium*. *J. Exp. Zool.* 260:84–92.

Hausmann, K. 1978. Extrusive organelles in protists. *In* International Review of Cytology. G. H. Bourne and J. F. Danielli, editors. Academic Press, New York. 197–276.

Hausmann, K., A. K. Fok, and R. D. Allen. 1988. Immunocytochemical analysis of trichocyst structure and development in *Paramecium*. *J. Ultrastruct. Mol. Struct. Res.* 99:213–225.

Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 726 pp.

Kaiser, C. A., and R. Schekman. 1990. Distinct sets of *SEC* genes govern transport vesicle formation and fusion early in the secretory pathway. *Cell.* 61:723–733.

Knoll, G., C. Braun, and H. Plattner. 1991. Quenched flow analysis of exocytosis in *Paramecium* cells—time course, changes in membrane structure, and calcium requirements revealed after rapid mixing and rapid freezing of intact cells. *J. Cell Biol.* 113:1295–1304.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680–685.

Le Caer, J.-P., J. Rossier, and L. Sperling. 1990. Crystalline contents of paramecium secretory vesicles: N–terminal sequences of a family of polypeptides separated on 2–D minigels and blotted onto immobilon. *J. Protein Chem.* 9:290–291.

Lepage-Lesin, A., P. Joseph-Bravo, G. Devilliers, L. Benedetti, J.-M. Launay, S. Gomez, and P. Cohen. 1991. Prosomatostatin is processed in the Golgi apparatus of rat neural cells. *J. Biol. Chem.* 266:1679–1688.

Lima, O., T. Gulik–Krzywicki, and L. Sperling. 1989. *Paramecium* trichocysts isolated with their membranes are stable in the presence of millimolar Ca^{2+}. *J. Cell Sci.* 93:557–564.

Lefort–Tran, M., K. Aufderheide, M. Pouphile, M. Rossignol, and J. Beisson. 1981. Control of exocytotic processes: cytological and physiological studies of trichocyst mutants in *Paramecium tetraurelia*. *J. Cell Biol.* 88:301–311.

Maupas, E. 1883. Contribution à l'étude morphologique et anatomique des infusoires ciliés. *Arch. Zool. Exp. Gen.* 1:427–664.

Mollenhauer, H. H., D. J. Morré, and L. D. Rowe. 1990. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta.* 1031:225–246.

Orci, L., M. Ravazzola, M. Storch, R. G. W. Anderson, J. D. Vassilli, and T. A. Perrele. 1987. Proteolytic maturation of insulin is a post-golgi event which occurs in acidifying clathrin-coated secretory vesicles. *Cell.* 49: 865–868.

Orias, E., M. Flacks, and B. Satir. 1983. Isolation and ultrastructural characterization of secretory mutants of *Tetrahymena thermophila*. *J. Cell Sci.* 64:49–67.

Peterson, J. B., D. L. Nelson, and R. H. Angeletti. 1990. Relationships of *Paramecium* and endocrine secretory proteins. *In* Current Research in Protein Chemistry. J. J. Villafranca, editor. Academic Press, New York. 79–85.

Plattner, H., C. J. Lumpert, G. Knoll, R. Kissmehl, B. Hohne, M. Momayezi, and R. Glas-Albrecht. 1991. Stimulus-secretion coupling in *Paramecium* cells. *Eur. J. Cell Biol.* 55:3–16.

Pollack, S. 1974. Mutations affecting the trichocysts in *Paramecium aurelia*. I. Morphology and description of the mutants. *J. Protozool.* 21:352–362.

Quinn, P., G. Griffiths, and G. Warren. 1983. Dissection of the Golgi complex. II. Density separation of specific Golgi functions in virally infected cells treated with monensin. *J. Cell Biol.* 96:851–856.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature (Lond.)*. 355:409–415.

Ruiz, F., A. Adoutte, M. Rossignol, and J. Beisson. 1976. Genetic analysis of morphogenetic processes in *Paramecium*. I. A mutation affecting trichocyst formation and nuclear division. *Genet. Res.* 27:109–122.

Schliwa, M., and J. Van Blerkom. 1981. Structural interactions of cytoskeletal components. *J. Cell Biol.* 90:222–235.

Shih, S. J., and D. L. Nelson. 1991. Multiple families of proteins in the secretory granules of *Paramecium tetraurelia*—immunological characterization and immunocytochemical localization of trichocyst proteins. *J. Cell Sci.* 100:85–97.

Shih, S. J., and D. L. Nelson. 1992. Proteolytic processing of secretory proteins in paramecium—immunological and biochemical characterization of the precursors of trichocyst matrix proteins. *J. Cell Sci.* 103:349–361.

Sonneborn, T. M. 1970. Methods in *Paramecium* research. *Methods Cell Physiol.* 4:241–339.

Sonneborn, T. M. 1974. *Paramecium aurelia*. *In* Handbook of Genetics. R. King, editor. Plenum Publishing Corp., New York. 469–594.

Sossin, W., J. Fisher, and R. Scheller. 1990. Sorting within the regulated secretory pathway occurs in the *trans*-Golgi network. *J Cell Biol.* 110:1–12.

Sperling, L., A. Tardieu, and T. Gulik-Krzywicki. 1987. The crystal lattice of *Paramecium* trichocysts before and after exocytosis by x-ray diffraction and freeze–fracture electron microscopy. *J. Cell Biol.* 105:1649–1662.

Sperling, L., G. Keryer, F. Ruiz, and J. Beisson. 1991. Cortical morphogenesis in *Paramecium*: A transcellular wave of protein phosphorylation involved in ciliary rootlet disassembly. *Dev. Biol.* 148:205–218.

Steiner, D. F., S. P. Smeekens, S. Ohagi, and S. J. Chan. 1992. The new enzymology of precursor processing endoproteases. *J. Biol. Chem.* 267:23435–23438.

Tindall, S. H. 1986. Selection of chemical spacers to improve isoelectric focusing resolving power: implication for use in two-dimensional electrophoresis. *Anal. Biochem.* 159:287–294.

Tindall, S. H., L. D. De Vito, and D. L. Nelson. 1989. Biochemical characterization of the *Paramecium* secretory granules. *J. Cell Sci.* 92:441–447.

Tooze, S. A., E. Chanat, J. Tooze, and W. B. Huttner. 1993. Secretory granule formation. *In* Mechanisms of Intracellular Trafficking and Processing of Proproteins. Peng Loh, editor. CRC Press, Boca Raton, FL. 157–177.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA*. 76:4350–4354.

Turkewitz, A. P., L. Madeddu, and R. B. Kelly. 1991. Maturation of dense core granules in wild type and mutant *Tetrahymena thermophila*. *EMBO (Eur. Mol. Biol. Organ.) J*. 10:1979–1987.

Yusa, A. 1963. An electron microscope study on regeneration of trichocysts in *paramecium caudatum*. *J. Protozool.* 10:253–262.