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 synthesize 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 NH2-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
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 (Sonnewborn, 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 to those of Prellack, but are different from trichocysts of Pol- lack, 1974) with the ovoid shape of an American football and a highly abortive tip. stubby4 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 a 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 stubby4, 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) (Pines International, Lawrence, Kansas) inoculated with Enterobacter aerogenes and supplemented with ß-sitosterol (0.4 µg/ml) according to standard procedures (Sonnewborn, 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 CaCl₂ to extend the matrices then in distilled H₂O 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 H₂O before homogenization in PBS. Incubations 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 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 Mameddu, personal communication). The serum gives results similar to those obtained with various other polyclonal antibodies on Western blots (Adoutte et al., 1984; Shiib and Nelson, 1991), recognizing a significant proportion of the polypeptides of the mature trichocyst matrix and many different precursor molecules (as determined by two-dimensional 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 35SO₄ (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 three shaped bottles in an oil test centrifuge (OGT, Giovanni Giaccardi, Torino, Italy) to remove parasitacrin 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 30 g. The cell pellets were washed in Volvic (Pay 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 to 800 cells/ml in exhausted WGP medium supplemented with 0.4 µg/ml ß-sitosterol. 5 × 10⁷ 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 × 10⁷ unla- beled bacteria per ml to start the chase. (standard WGP medium contains ~1.5 × 10⁷ bacteria/ml). 6-ml aliquots of the cultures were removed at different times and mixed with 2 ml of a 0.2% solution of MnCl₂, which inhibits caelyary beating, immediately before centrifugation in a clinical cen- trifuge 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 concentra- tion of ethanol.

Immunoprecipitation and Gel Electrophoresis

Contrats 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 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 immunoprecipitation dilutions of wild-type cell lysates with a constant amount of antiserum, and quantifying the results with a Phosphorimage (Molecular Dynamics SA, France).

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 above. 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 tem- perature. (1× lysis buffer is PHEM-1% Triton X-100. PHEM is 60 mM Pipes, 25 mM EGTA, 2 mM MgCl₂, pH 6.9 [Shaw and Van Blarkom, 1981]. 1× protease inhibitors is 50 µg/ml PMSF, 5 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml pepstatin, and 5 mM DTT). The sus- pension was centrifuged 5 min at 12,500 g in a minifuge at room tempera-
ature 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 immunoblotting using anti-ciliary rootlet antigen as control for the insoluble fraction (Sperling et al., 1991) and anti-surface antigen 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 antigen directed against trichocyst matrix proteins was used at 1/10,000 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 collected on nickel grids and saturated and processed with 3% BSA in PBS. The anti-trichocyst serum was used at 1/100 dilution. After washing, the 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 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 35SO4-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-kDa 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 t1/2 of 20 min. This experiment provides the most direct evidence to date for the precursor-product relationship originally postulated by Adoutte et al. (1984).

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 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.

![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.
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.

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 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.

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.
Conversion of precursors to products appears to go to completion in \textit{tam38} cells (Fig. 6 A), \textit{tam33} cells (not shown), and \textit{stubby4} 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 \textit{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 \textit{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 \textit{tam38} cells.

Examination of \textit{tam33} and \textit{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 \textit{stubby4} cells (not shown). Fig. 8 A shows the appearance of ER and adjacent Golgi stacks in wild-type \textit{Paramecium} 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 \textit{tam38} cells.

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Cellular Distribution of Precursors and Products

If, as our data suggest, the primary defect in \textit{tam33}, \textit{tam38}, and \textit{stubby4} 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 \textit{trichless} cells only precursors are present, and they are found exclusively in the soluble fraction. The other mutant cells, \textit{tam38}, \textit{tam33} (results identical to \textit{tam38}, not shown) and \textit{stubby4}, differ from wild-type cells in two significant ways. First, as previously reported for \textit{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
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. 10A. The isolated trichocysts were subjected to SDS-PAGE and the corresponding blots were treated with the antiserum. The results (Fig. 10B) 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-
 nevertheless, 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

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**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.

**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 tam5, 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 (Turkwitz 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 Scheckman, 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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