Proteolytic processing of secretory proteins in Paramecium: immunological and biochemical characterization of the precursors of trichocyst matrix proteins

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Summary

We used polyclonal serum raised against mature trichocyst matrix proteins to detect their unprocessed precursors, a group of proteins (45-55 kDa) present in the whole-cell extract. These precursor proteins were partially purified from the soluble fraction of wild-type cells by ammonium sulfate precipitation and anion-exchange chromatography. Using monoclonal antibodies against each of four families of mature (processed) matrix proteins, we showed that each family was derived from a separate group of precursors. Our results also suggest that in three of four precursors, those in which the mature proteins consist of disulfide-linked heterodimers, intrachain disulfide bonds form before proteolytic processing.

Purified precursors eluted from preparative SDS-gels were used to raise rabbit antiserum, which after preadsorption with mature processed proteins specifically recognized precursors, as judged by ELISA and immunoblots. In cross-sections of developing trichocysts, the anti-precursor serum after preadsorption no longer stained the central, paracrystalline region, but still stained the peripheral as well as the structureless region of the secretory granule. In trichocyst-developing mutants tl (trichless) and ftA (football A), the precursors for all four groups of mature proteins were present but their processing was affected: severely blocked in tl (which has no recognizable crystalline trichocyst matrix), and partially blocked in ftA (which has some abnormal trichocyst matrices with crystalline centers). These observations constitute further evidence that proteolytic processing of precursors occurs in parallel with crystallization.

Key words: trichocyst, Paramecium tetraurelia, secretion.

Introduction

The unicellular ciliate Paramecium tetraurelia has thousands of secretory granules in each cell. These secretory granules, trichocysts, are docked beneath the plasma membrane and await stimulated exocytosis. Although the function of trichocysts is still unclear, regulation of exocytosis and the signal transduction of stimuli in this system are similar to the regulated secretion in higher organisms in many ways, including the increase in intracellular Ca\textsuperscript{2+} concentration (for reviews, see Adoutte, 1988; Satir, 1989). Three different types of mutants that do not secrete trichocysts are available: (1) signal transducing and exocytosis mutants, which have normal trichocysts docked at correct sites; (2) transporting mutants, which have normal trichocysts in the cytosol, which are not attached to the plasma membrane; and (3) developmental mutants, which do not have normal trichocysts (Lefort-Tran et al., 1981; for reviews, see Adoutte, 1988 and Satir et al., 1988). Paramecium can be grown in large quantities in the laboratory, and exocytosis of trichocysts is easy to monitor by light microscopy, which makes trichocysts suitable for the study of mechanisms of regulated exocytosis, biogenesis of secretory granules, processing and packing of secretory proteins in the secretory granules, transport and targeting of secretory granules and release of the contents of the secretory granules.

The contents of resting secretory granules, primarily the condensed trichocyst matrix (ctmx), are a compact, highly ordered array of proteins, all about 14-25 kDa and acidic (PI 4-6) (Steers et al., 1969; Adoutte et al., 1980; Tindall et al., 1989). Exocytosis results in a remarkable expansion of this compact structure into another paracrystalline structure, the extruded and extended trichocyst matrix (xtmx), with an eight-fold increase in length and two-fold decrease in diameter. This extension is triggered by Ca\textsuperscript{2+}, as can be shown by Ca\textsuperscript{2+}-induced in vitro extension of ctmx isolated in the presence of EDTA (Matt et al., 1978; Garofalo and Satir, 1984; Lima et al., 1989). Because the tmx is extruded out of the cell without cell damage, it is easy to isolate.
The tmx is remarkably stable toward dissociation by detergents, salt, pH and chelating agents (Peterson et al., 1987a). Heat dissociates the structure, but large aggregates reform on cooling (Pollack and Steers, 1973; Peterson et al., 1987a). The temperature-dependent dissociation/reassociation is affected by Ca\(^{2+}\) or La\(^{3+}\), which stabilize the xtmx against heat-induced dissociation, and by reagents that break disulfide bonds, which destabilize trichocyst structure (Peterson et al., 1987a).

The tmx has 34 major and more than 80 minor proteins when resolved by narrow pH range two-dimensional gel electrophoresis (Tindall, 1986). These are very similar in size, pI and chromatographic properties (Tindall et al., 1989). Monoclonal antibodies raised against them define four different groups of proteins, each of which contains several members that differ slightly in size and pI value (Shih and Nelson, 1991). Most tmx proteins are disulfide-linked dimers; some are heterodimers. Three of the four families identified by monoclonal antibodies (mAbs) are in this category, but the fourth family of tmx proteins does not have interchain disulfide bonds and exists as monomers in the intact tmx. These four immunologically distinct groups of proteins are localized in different parts of both condensed and extruded tmx (Shih and Nelson, 1991).

Mature tmx proteins are produced by proteolytic processing of large precursors with molecular mass 40-45 kDa to intermediate forms of about 25 kDa (Adoutte et al., 1984). Several lines of evidence suggest that these 40-45 kDa proteins are precursors of tmx proteins. Firstly, antiserum raised against mature tmx recognizes these proteins (Adoutte et al., 1984). Secondly, processing of these precursors can be blocked by monensin, an ionophore which inhibits proteolytic processing of several secretory proteins (Mollenhauer et al., 1990); treatment of both wild type and the biogenesis mutant tam8 with monensin decreases the amount of the mature size tmx proteins, while simultaneously causing accumulation of 40-45 kDa proteins (Adoutte et al., 1984). Adoutte et al. (1984) also reported that the amount of mature size tmx proteins in the whole cell extract is correlated with the extent of partially crystallized tmx in monensin-treated cells observed in the thin-sections for electron microscopy.

Two-dimensional gel electrophoresis studies indicate that there are more than one hundred tmx proteins (Tindall et al., 1989; Shih and Nelson, 1991). Their highly ordered structure and similar biochemical properties, however, suggest that they are encoded by a limited number of genes. We want to determine whether this heterogeneity is generated by post-translational modification. Proteins in the same immunologically defined group have similar molecular masses and slightly different pI, which suggests they may be derived from the same precursors by slightly different processing. Given the size of precursor proteins (45-55 kDa) and of the mature proteins (most are 14-22 kDa), it is possible that one precursor protein is processed to yield two or three smaller proteins, all of which end up in the mature tmx. Alternatively, proteins in each immunologically defined family might be derived from a separate precursor, the product of a separate gene. As part of our continuing efforts to characterize trichocyst matrix protein synthesis and processing, we set out to determine how many different precursors there are for tmx proteins, to purify and characterize these precursors, and to raise antibodies specific for them.

Materials and methods

**Materials**

*Paramecium tetraurelia* was the wild-type stock 51S. Acrylamide, N,N'-methylenebisacrylamide, and SDS were from Bethesda Research Laboratories (Bethesda, MD), Tris, ultra pure urea, and pepstatin A were purchased from Boehringer-Mannheim (Indianapolis, IN). Nitrocellulose paper (0.45 µm), molecular mass markers for SDS-PAGE, and Bio-Lyte 3/10 and 4/6 (40%) were from Bio-Rad (Richmond, CA). Goat anti-mouse IgG (H+L), affinity purified and coupled to alkaline phosphatase, was from Kirkegaard and Perry (Gaithersburg, MD). Goat anti-rabbit IgG conjugated to 10 nm gold was from Janssen Life Science Products (Piscataway, NJ). DEAE high-capacity anion-exchange cellulose was from Pierce (Rockford, IL). Phenyl-Sepharose CL-4B, goat anti-rabbit IgG (H+L) coupled to alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt), nitro blue tetrazolium (grade III), Ponceau S concentrate, and all other reagents were from Sigma (St. Louis, MO). Rabbit anti-tmx serum was the generous gift of Dr. J. B. Peterson in this laboratory, and two *Paramecium* mutants tl and ftA were kindly provided by Dr. Karl Aufderheide (Texas A & M University, College Station, Texas).

Cell culture and preparation of high-speed supernatant of whole-cell extract

*Paramecium tetraurelia* was grown and harvested as described in Gundersen and Nelson (1987). Cell bodies were resuspended in homogenization buffer (50 mM HEPES, 250 mM sucrose, 5 mM EDTA, pH 7.2) with protease inhibitors (0.2 units/ml aprotinin, 10 µg/ml leupeptin, 2 µg/ml pepstatin, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), and 0.3 mM N-p-tosyl-L-arginine methyl ester (TAME)). Cells were homogenized and subjected to centrifugation at 30,000 *g* for 30 min to sediment cell debris and trichocysts. This low-speed supernatant (S1) was centrifuged again at 100,000 *g* for 60 min to generate the high-speed supernatant (S2).

Partial purification of precursors - ammonium sulfate fractionation and DEAE chromatography

(NH₄)₂SO₄ was added to S2 to 40% saturation and stirred at 4°C for 60 min. Precipitated proteins were pelleted by centrifugation at 30,000 *g* for 30 min. This pellet, which represented about 1/3 of the total proteins in S2, contained all of the proteins recognized by anti-tmx serum. The pellet was dissolved in 20 mM Tris-HCl, 5 mM EDTA, pH 7.2 (buffer A) to final protein concentration of 1 to 1.5 mg/ml and clarified by centrifugation at 20,000 *g* for 20 min. The resulting supernatant was loaded onto a DEAE anion-exchange column (13 cm × 2.8 cm) with a 20 ml/hour flow rate, and unbound proteins were washed away with buffer A containing 75 mM NaCl. Bound proteins were eluted with a linear gradient from 75 to 400 mM NaCl in buffer A containing 75 mM NaCl. Bound proteins were eluted with a linear gradient from 75 to 400 mM NaCl in buffer A. Every fraction was assayed for protein concentration by the method of Bradford (Bradford, 1976). Equal volumes (20 µl) of each fraction were assayed by immunoblotting after separation on a 12% reducing SDS polyacrylamide gel. The affinity-purified rabbit anti-trichocyst antibody (0.1 µg/ml in 0.5% nonfat dried milk in Tris-buffered saline (TBS: 10 mM Tris-HCl, 150 mM NaCl, pH 8.0)) and goat anti-rabbit IgG (H+L) coupled to alkaline phosphatase (0.1 µg/ml) were used as primary and secondary antibodies,
respectively, in the immunoblots. All other conditions were as described (Shih and Nelson, 1991). The fractions showing positive response in immunoblotting were collected, and usually this DEAE chromatography step gave greater than 30-fold purification. This DEAE pool of tmx precursors was resolved by 12% SDS-PAGE and the proteins were stained with 0.5% Coomassie brilliant blue C-250 and destained according to Coleman and Raetz (1988). The region of the gel containing precursor proteins (45-55 kDa range) was excised, rinsed briefly with distilled water, and incubated with elution buffer (50 mM Tris-HCl, 5 mM EDTA, 0.1% SDS, pH 8.0). Proteins were extracted with four changes of the elution buffer or until no blue color was left in the gel pieces. The inclusion of SDS is important for keeping precursor proteins soluble; without it, the yield of the precursors is very poor. The eluted proteins were pooled and recovered by precipitation with four volumes of acetone at -20°C for 1 h or 10% trichloroacetic acid on ice for 30 min.

**SDS-PAGE of reduced and unreduced tmx precursors**

SDS-PAGE (Bio-Rad Mini-Protein II) was performed as described by Laemmli et al. (1970). Samples were boiled in SDS sample buffer without 2-mercaptoethanol. Reduced samples were generated from the same sample vial by subsequent addition of 2-mercaptoethanol (final concentration of 0.5%).

**Two-dimensional gel electrophoresis**

Partially purified tmx precursors were subjected to two-dimensional gel electrophoresis with an expanded pH range from 4 to 6 as described by Tindall (1986).

**Heat treatment of tmx precursors**

The DEAE pool of partially purified tmx precursors (in 20 mM Tris-HCl, 5 mM EDTA, pH 7.2) was heated in a heating block whose temperature slowly increased from 60 to 98°C in approximately 40 min. The resulting precipitate was sonicated for 1 min to release any trapped soluble proteins, and pelleted by centrifugation at 30,000 g for 20 min.

**Phenyl-Sepharose chromatography**

The Ca²⁺-dependent adsorption of S2 proteins to phenyl-Sepharose was performed according to Gundersen and Nelson (1987), except that the tightly bound proteins were eluted from the column by 20% (v/v) ethanol in 20 mM Tris-HCl, pH 7.5.

**Preparation of polyclonal antibodies specific to tmx precursors**

To prepare antiserum against tmx precursors, a rabbit was immunized with SDS preparative gel-eluted precursor proteins (100 µg for primary injection; 50 µg for boosts). All sera were tested using both partially purified precursors and purified tmx (Adoutte et al., 1980) in immunoblots and ELISA as described (Shih and Nelson, 1991). The antibodies against tmx were removed from diluted serum by incubating with tmx-coated nitrocellulose paper at least six times, and completion was judged by immunoblot and ELISA against tmx. The serum was found also to contain antibodies against tubulin, so a preparation of crude axonemes of *Paramecium* (Adoutte et al., 1980) was used to adsorb these anti-tubulin antibodies. However, the preadsorption with tubulin did not change either the staining pattern of this serum in immunoblots against tmx proteins or immunolocalization inside the secretory granule (data not shown). For the adsorption of anti-tmx and anti-tubulin antibodies, both the tmx and axonemes were treated with 6 M urea (to dissociate all subunits) or 1% SDS followed by boiling (to mimic the proteins in immunoblots) before being coated onto nitrocellulose papers. The concentration of proteins coated onto the paper was 40-60 µg/cm².

**Immunolocalization study of precursor-specific antibodies**

Wild-type *Paramecium tetraurelia* was fixed, dehydrated, and embedded in LR-White, and thin-sectioned for immunolabeling with antibodies as described (Shih and Nelson, 1991). Sections were incubated in the rabbit tmx precursor-specific polyclonal antiserum (400-fold dilution), followed by incubation with goat anti-rabbit IgG conjugated to 10 nm colloidal gold (30-fold dilution). Micrographs were taken with a Philips EM410 electron microscope at 60 kV.

**Results**

**Partial purification of precursors of trichocyst matrix proteins**

To purify tmx precursors, the high-speed supernatant (S2) of whole-cell homogenate was used as starting material, because it contains soluble tmx precursors (although a substantial amount of precursors remained in the pellets in insoluble forms; data not shown), and it is free of mature tmx proteins, as judged by immunoblotting with rabbit anti-tmx serum (lanes 1 and 1' in Fig. 1). This serum, which also recognizes the precursors of tmx proteins, was used to test all subsequent steps in the purification (Fig. 1). S2 was fractionated by 40% (NH₄)₂SO₄ saturation, under conditions that precipitated most of the tmx precursors (lanes 2 and 2' in Fig. 1). The pelleted proteins were resolubilized and applied to a DEAE anion-exchange column. tmx precursors bound to DEAE-cellulose, and could be eluted with 200-300 mM NaCl (the eluted fraction is shown in lanes 3 and 3' in Fig. 1). This DEAE pool was run on an SDS preparative gel, and the part of the gel containing tmx precursors (45-55 kDa range) was excised. The 45-55 kDa proteins eluted from the preparative gel (lanes 4 and 4' in Fig. 1) were subjected to isoelectric focusing gel electrophoresis followed by immunoblotting with rabbit anti-tmx serum; approximately 70% of the proteins were recognized by the serum (data not shown). Because further purified precursor proteins appeared to adhere to the test tube walls in the absence of SDS, the DEAE pool (lanes 3 and 3' in Fig. 1) was used for the following biochemical and immunological characterization of precursors of the tmx proteins. The precursors of tmx proteins eluted from SDS preparative gel (lane 4 and 4' of Fig. 1) were used to raise rabbit anti-precursor serum (see below). In this fraction of proteins, there are high molecular mass bands (above 100 kDa) in both lanes 4 and 4' of Fig. 1, which presumably are aggregates of tmx precursors.

**Unreduced precursors for dimeric proteins migrate faster on SDS-PAGE than reduced ones**

Most tmx proteins are disulfide-linked heterodimers; the rest are monomeric proteins (Shih and Nelson, 1991). We tried to determine whether disulfide bonds also exist in the precursors. For easy comparison, the DEAE partially purified fraction was resolved in different lanes of the same gel in the presence or absence of reducing agent (Fig. 2). We ran 5 lanes with 2-mercaptoethanol present only in the samples in the two outside lanes (lanes 1 and 5 in every panel in Fig. 2). Under our electrophoresis conditions, 2-mercaptoethanol appeared to diffuse half-way into the adjacent
In the absence of reducing agent, some of the tmx precursors migrated faster in the gel (lane 3, along with part of lanes 2 and 4, in Fig. 2B-D, and 2F); others migrated more slowly (lane 3 in Fig. 2B). We added 2-mercaptoethanol into the same sample immediately prior to electrophoresis, to exclude the possibility of proteolysis occurring in the absence of reducing agent.

Monoclonal antibodies raised against mature tmx proteins have defined four groups of immunologically related proteins: three groups consist of dimeric proteins, while the fourth consists of monomeric proteins (Shih and Nelson, 1991). Here, we tried to ask if the precursors of the dimeric tmx protein have disulfide bonds, by immunoblotting the samples shown in Fig. 2A with monoclonal antibodies against tmx proteins. The precursors recognized by mAb B5-5 (group I, Fig. 2C), B4-4 (group II, Fig. 2D), and B2-5 (group IV, Fig. 2F) all showed faster migration in gels in the absence of reducing agent, producing a “U”-shaped pattern on the immunoblots. Group I antibody (B5-5) recognized 50, 52 and 56 kDa proteins under reducing conditions and 37, 41 and 43 kDa proteins under nonreducing conditions. Group II antibody (B4-4) gave an immunoblot pattern similar to that of group I: reduced proteins at 53 and 56 kDa, and unreduced proteins at 38, 41 and 43 kDa. B2-5, the group IV antibody that had much higher affinity for the reduced proteins than the unreduced ones, labeled 51 and 54 kDa reduced proteins and a 35 kDa unreduced protein band (very weakly). The 50 and 49 kDa proteins recognized by B7-4 (group III, Fig. 2E), the only antibody that recognizes monomeric tmx proteins, did not change their migration in the gel under reducing or nonreducing conditions.

**Different precursors for different groups of trichocyst matrix proteins defined by monoclonal antibodies**

The immunoblots of reduced and unreduced precursors with the four monoclonal antibodies helped to distinguish the precursors of group III from the other three groups, which all gave similar U-shaped patterns (Fig. 2). To distinguish further the precursors of the immunologically different mature tmx proteins, the DEAE fraction was resolved by two dimensional gel electrophoresis which did show distinct immunostained patterns for these four groups. However, these stained proteins were too close to each other to allow clear interpretation (data not shown; cf. Fig. 3). Therefore, we took advantage of the fact that limited proteolysis of precursors occurred spontaneously on storage to obtain proteolytic peptide maps. This was done by immunoblotting after resolution of the partial proteolytic digest by two-dimensional gel electrophoresis. Since the chemical spacer was included to expand the pH 4-6 range of the electrophocusing gel in the first dimension (Tindall, 1986), proteins with pl from 4-6 were well separated at the middle of the gel, but the proteins with higher pl were focussed at the left side of the gel (the protein pattern was not shown in this figure). The immunoblot patterns of the partially digested DEAE fraction with four different monoclonal antibodies were different in (1) the stained precursors in the 45-55 kDa range and (2) the stained proteolytic fragments of precursors (group I, shown in Fig. 3A; group II, Fig. 3B; group III, Fig. 3C; group IV, Fig. 3D). In every immunoblot, there were proteins with pl around 5 (appearing at the middle of the gel) and those with pl higher than 6.5 (at the left side of the gel). These results strongly suggested that the four different mature tmx proteins are derived from different precursors which are distinguishable by their positions in 2-D gels as well as their different immuno-peptide maps. Precursors labeled by mAb B7-4 did not show smaller fragments, which suggested that they were more resistant to proteolysis or that the epitope was destroyed by proteolysis (Fig. 3C).

**Precursor-specific antibodies did not label the crystalline center of immature trichocysts**

To study further the processing of tmx proteins, polyclonal antibodies raised against the tmx precursors eluted from SDS preparative gels were used in some immunological characterization. First, this serum recognized purified extruded tmx in immunoblots (unreduced tmx proteins shown in lane 6, and reduced ones in lane 7 of Fig. 4). Secondly, this serum labeled the condensed tmx in a mature trichocyst as well as the partially condensed tmx in a developing trichocyst in immunolocalization (Fig. 5B). The pre-
Precursors of trichocyst matrix proteins

Precursors of dimeric trichocyst matrix proteins have intrachain disulfide bonds. Precursors partially purified by DEAE chromatography after being resolved by SDS-PAGE (12%), electrotransferred to nitrocellulose paper, and stained with Ponceau S (A). (B-F) are immunoblots of the same samples shown in A. (B) was blotted with rabbit anti-tmx serum (see Materials and methods); (C) with mAb B5-5 (group I); (D) with mAb B4-4 (group II); (E) with mAb B7-4; and (F) with mAb B2-5 (group IV). Lanes 1-5 contained the same amount of partially purified precursors (15 µg), but 0.5% 2-mercaptoethanol was added into lanes 1 and 5 just prior to the start of electrophoresis.

Immune rabbit serum did not stain the tmx in immunoblots (lanes 4 and 5 in Fig. 4) or in the immunolocalization study (Fig. 5A). Antibodies against mature tmx proteins were removed from the serum by immunoadsorption (see Materials and methods), and the remaining antiserum was tested by ELISA (data not shown) and immunoblots (lanes 8 and 9 in Fig. 4). The ELISA results showed that the titer of the resulting immunological reagent for the mature proteins after adsorption decreased at least 1000-fold, but the titer for the DEAE fraction decreased only about 2-fold (data not shown). In immunoblotting, the serum after preadsorption still recognized the 45-55 kDa proteins in the DEAE fraction (lane 3 in Fig. 4), but no longer labeled the reduced mature proteins (lane 9). Although it still stained the unreduced mature proteins, the intensity was much weaker than with the original serum (lane 8); further adsorption did not help to abolish completely this staining. When this precursor-specific antiserum was used in the immunolocalization in wild-type cells, it no longer labeled mature trichocyst matrix (Fig. 5C; cf. Fig. 5B), but in the immature trichocyst it still labeled, although not heavily, the outer edge of the paracrystalline structure of tmx as well as the structureless, uncrystallized part of the secretory granule (Fig. 5D-H). The density of gold label (particles/µm²) over the uncrystallized region and the edge of the paracrystalline structure is about 10× greater than that over the central part of paracrystalline matrices.

tl and ftA are defective in processing precursors of trichocyst matrix proteins

The developmental mutant tl (trichless) does not have a recognizable tmx and another mutant ftA (football A) has an abnormal tmx (Pollack, 1974; Hausmann et al., 1988). It has been shown that in tl the processing of most of the tmx precursors is blocked (Adoutte et al., 1984) Our mAbs also defined these mature tmx proteins, by immunolocalization, as core- or cortex-specific or in-whole-matrix proteins (Shih and Nelson, 1991). We wanted to determine whether tl and ftA mutants are defective in the expression or in the processing and maturation of all, or only of certain tmx proteins, using mAbs against tmx proteins as tools. Homogenates of these two mutants as well as wild-type cells were immunoblotted with rabbit anti-tmx serum and mAbs (Fig. 6). Fig. 6B, which was immunoblotted with rabbit serum, showed that in the tl mutant, most of the tmx precursors were at 42-52 kDa and some were processed into 39 kDa intermediate fragments, while in ftA, some were further processed into smaller products (25 kDa, 14-20 kDa). Immunoblots using each monoclonal antibody against
mature tmx proteins (Fig. 6C-E) showed that (1) the precursors of these four groups of tmx proteins all existed in both mutants; (2) there were no mature size products for these four groups in *tl; (3) there were processing intermediate as well as mature size products for each group of tmx proteins in *ftA. The proteins recognized by mAb B5-5 were processed from 47 and 45 kDa bands, to 39 kDa (weakly seen) and 21 kDa fragments, and to 17 kDa in *ftA, or in

Fig. 3. Different groups of tmx proteins have different precursors. The fractions of DEAE-resolved proteins containing tmx precursors were pooled, and stored frozen for more than one month to allow spontaneous proteolysis to occur. The proteins were subjected to two-dimensional electrophoresis, in the presence of 8 M urea and the chemical spacer which was used to expand the pH range of 4-6 in the isoelectric focusing gel, as described by Tindall (1986). (A) represents the immunoblot with mAb B5-5 (group I); (B) with B4-4 (group II); (C) with B7-4 (group III); and (D) with B2-5 (group IV), respectively. The asterisks represent two proteins (62 kDa, pl 5.4; 30 kDa, pl 5.6) whose positions were marked with pencil after being visualized by Ponceau S stain.

Fig. 4. Immunoblots with precursor-specific polyclonal antibodies. Precursors partially purified on DEAE-cellulose were resolved by SDS-PAGE (12%) and electrotransferred to nitrocellulose paper for immunoblotting (lanes 1, 2 and 3; 10 µg each). The purified extruded tmx (5 µg on each lane) was resolved in SDS-PAGE under nonreducing (lanes 4, 6, 8) and reducing (lanes 5, 7, 9) conditions and electrotransferred as above. Lanes 1, 4 and 5 were immunoblotted with preimmune rabbit serum (PIS); lanes 2, 6, 7, with rabbit anti-precursor (45-55 kDa) serum (RαP); and lanes 3, 8, and 9 were immunoblotted with the precursor-specific antiseraum after preadsorption (RαP'); see Materials and methods). NR, non-reduced; R, reduced trichocyst proteins.
wild type to 17 and 15 kDa (Fig. 6C). The proteins labeled by mAb B4-4 were processed from 53, 50 and 47 kDa to some intermediate fragments at 39, 24 and 21 kDa, then to 17 kDa which is also the major product in wild type (Fig. 6D). The proteins stained by B7-4 derived from a major band at 47 kDa and some minor bands (51 and 50 kDa) to intermediate fragments at 39 and 25 kDa, and then to the final products 21 and 22 kDa (Fig. 6E). The proteins stained by B2-5 were processed from 47 and 43 kDa to 23, 18 and 17 kDa in wild type (lane 2 in Fig. 6F), but from 50, 47 and 39 kDa to 17 kDa in ftA (lanes 3 and 4 in Fig. 6F).

Calcium affects properties of tmx precursors

It is believed that the tmx contains Ca\(^{2+}\)-binding proteins, based on the fact that Ca\(^{2+}\) alone can induce the in vitro expansion of ctmx (Matt et al., 1978; Garofalo and Satir, 1984; Lima et al., 1989). We have detected weak Ca\(^{2+}\)-binding activity for some mature tmx proteins, but not with partially purified precursors (Shih, 1991). However, in the purification we noticed that Ca\(^{2+}\) played an important role in some of the physical properties of tmx precursors. First, tmx precursors are resistant to heat denaturation in the absence of Ca\(^{2+}\). The DEAE pool was heated in the presence of 5 mM EDTA in a heating block whose temperature increased from 60°C to 98°C within approximately 40 min. After heat treatment and centrifugation, less than 10% of the total proteins remained in the supernatant (lanes 1 and 2 in Fig. 7). However, this heat-stable supernatant contained most of the precursors of tmx proteins, although some proteolysis was evident (lane 2′ in Fig. 7). To test whether Ca\(^{2+}\) affected the heat-stability of tmx precursors, Ca\(^{2+}\) was added to the same DEAE pool to final concentrations of 1 and 2.5 mM. We found that the addition of Ca\(^{2+}\) alone, without heating, precipitated most of the proteins including tmx precursors (Fig. 8). (The change of pH by the addition of Ca\(^{2+}\) was less than 0.2 unit, which should not account for protein precipitation.)

Phenyl-Sepharose chromatography has been used to purify Ca\(^{2+}\)-binding proteins including calmodulin and Ca\(^{2+}\)-dependent protein kinases (Walsh et al., 1984). We tried to determine if the tmx precursors have Ca\(^{2+}\)-binding activity by using phenyl-Sepharose chromatography according to an established procedure (Walsh et al., 1984). The S2 was loaded onto the column in the presence of 0.1 mM Ca\(^{2+}\), and unbound proteins and non-specifically bound proteins were removed by buffer and buffer with 1 M salt. The Ca\(^{2+}\)-binding proteins could be eluted by 1 mM EGTA in Ca\(^{2+}\)-free buffer. Each fraction was immunoblotted with rabbit anti-tmx serum. As shown in Fig. 9, the starting material (S2) contained a small amount of tmx precursors which gave a barely recognizable band (see lanes 1 and 1′ in Fig. 9), and the unbound proteins (representing more than 98% of the proteins in the S2; cf. Gundersen and Nelson, 1987) contained very small amounts of tmx precursors (lanes 2 and 2′). Most of the tmx precursors could be eluted by 1 mM EGTA (lanes 3 and 3′), although there were still some precursors tightly bound to the phenyl-Sepharose which were eluted with 20% ethanol (lanes 4 and 4′). In a similar experiment, immunoblots with monoclonal antibodies showed that precursors for group I, II and IV tmx proteins were in the EGTA eluant, but the precursors for group III were missing in the EGTA eluant and the ethanol wash (data not shown).

### Discussion

Because Paramecium appears to lack efficient mechanisms for the uptake of amino acids from the growth medium, pulse-chase experiments to establish precursor-product relationships are impractical. We have instead used several well-characterized mAbs against trichocyst matrix proteins to study the processing of secretory proteins of Paramecium.

### Precursor-product relationship between 45-55 kDa proteins and mature tmx proteins

We describe here the partial purification of the precursors

| Table 1. The similarity between chromogranins and trichocyst matrix proteins |
|-----------------------------|-----------------------------|-----------------------------|
| Proteins                     | Chromogranins               | tmx proteins                |
|                             | (A, B and C)                | (precursors and mature products) |
| Heterogeneity               | (Winkler et al. 1986)       | Precursors: Fig. 1           |
| Enrichment in acidic amino acid residues | (Benedum et al., 1986, 1987; Iacangelo et al., 1986; Fisher-Colbrie et al., 1990) | Mature proteins (Steers et al., 1969; Rauh and Nelson, 1981) |
| Heat-stability               | (Gorr et al., 1988, 1989)   | Precursors: Fig. 7           |
| Calcium binding activity     | (Gorr et al., 1988, 1989, Gerdes et al., 1989) | Mature products (Peterson et al., 1987a) |
| Calcium-induced aggregation  | (Gorr et al., 1988, 1989, Gerdes et al., 1989) | Dissociated mature proteins (Peterson et al., 1987a) |
| Calcium-induced conformational change (possibly exposure of hydrophobic sites) | (Yoo and Albanesi, 1990) | Precursors: Fig. 8 |
| pH-dependent calcium binding | (Gorr et al., 1988, 1989)   | Mature proteins (Garofalo and Satir, 1984) |
Fig. 5. Immunolocalization studies using antiserum specific to tmx precursors. Wild-type *Paramecium tetraurelia* was fixed, embedded in LR white, thin-sectioned and immunolabeled with different antisera. The bound antibodies were decorated with goat anti-rabbit IgG conjugated to 10 nm gold particles (see Materials and methods). (A) shows the immunolabeling of mature tmx with rabbit preimmune serum; (B) mature and immature tmx with rabbit antiserum against tmx precursors; (C to H) were labeled with the precursor-specific antiserum. (C) shows the cross-sections of mature tmx. (D, E, F, G and H) are the sections of immature tmx. T, trichocyst matrix; M, mitochondria; A, amorphous, uncrystallized part of a developing trichocyst; C, crystallized trichocyst matrix in a developing trichocyst. Bars, 0.5 µm.
Precursors of trichocyst matrix proteins

of tmx proteins and the biochemical and immunological characterization of these proteins. Consistent with Adoutte’s observation (Adoutte et al., 1984), we found that tmx precursors are a group of 45-55 kDa proteins with a similar degree of heterogeneity as noted for the mature tmx proteins. We obtained four lines of evidence to prove that these 45-55 kDa proteins are, or at least contain, the precursors of tmx proteins: (1) murine mAbs raised against tmx proteins also recognized these proteins; (2) these proteins recognized by mAbs accumulated in two trichocyst developmental mutants \( tl \) and \( ftA \); (3) antiserum raised against these 45-55 kDa proteins not only recognized the mature tmx on immunoblots and ELISA, but also labeled the ctmx in immunolocalization studies; and (4) after preadsorption to remove the antibodies recognizing mature tmx proteins, the remaining antiserum did not label the ctmx in thin sections.

Under some circumstances, we saw large proteins (with molecular mass 100 kDa and higher) which were also recognized by both the polyclonal serum raised against tmx (Figs 1B and 6) and the serum against purified precursors (data not shown), as well as by some monoclonal antibodies (Figs 2C and 6). The detection of these high molecular mass “precursors” in the whole cell extract is weak, even in the mutants in which precursors accumulated. The 45-55 kDa precursors in DEAE partially purified fractions appeared as large aggregates with a wide range of size (from >200 kDa to 50 kDa) in gel filtration chromatography (data not shown). Moreover, the precursors tended to self-aggregate even in the presence of SDS; in the fraction which was eluted from the 45-55 kDa range of preparative SDS gels and reloaded on a second gel, some precursors migrated as proteins larger than 100 kDa (Fig. 1B). We think that these high molecular mass forms are stable aggregates of 50 kDa and smaller forms, rather than being very large precursors.

The processing of some precursors is probably insulin-like

Three groups of precursors for dimer mature proteins seem to have a more compact structure under nonreducing conditions than when reduced (Fig. 2). These are the same precursors that yield mature proteins that are disulfide-linked heterodimers. The size of these three groups of precursors (about 38-44 kDa) under nonreducing conditions is similar
Fig. 6. The processing of tmx precursors is defective in two trichocyst mutants: tl and ftA. Low-speed supernatants and pellets were prepared from whole-cell extracts of wild-type Paramecium tetraurelia and mutants tl and ftA (see Materials and methods). The proteins in the low speed supernatants were resolved in 12% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose papers. (A) shows a Ponceau S-stained paper. (B) represents the immunoblot with rabbit anti-mature tmx serum; (C) mAb B5-5 (group I); (D) mAb B4-4 (group II); (E) mAb B7-4 (group III); and (F) mAb B2-5 (group IV). Each panel shows the low-speed supernatant (S) and pellet (P) (approximately 20 μg protein) from wild-type cells in lanes 1 and 2, from ftA in lanes 3 and 4 and from tl in lanes 5 and 6.
Precursors of trichocyst matrix proteins

to that of the unreduced mature dimeric proteins (32-40 kDa; Shih and Nelson, 1991). Our interpretation is that these precursors have intrachain disulfide bond(s), and that one precursor gives rise to a heterodimer after being processed at the region between the two subunits of the heterodimer. The maturation of heterodimeric tmx proteins would thus be like that of insulin. However, we have no information about the processing of precursors for monomeric proteins or the number of mature products that are derived from one precursor. Efforts are underway to clone the trichocyst matrix proteins. The amino acid sequences and localization of cysteine residues should clarify the processing pathways.

**tl and ftA mutants are defective in processing all precursors**

In both wild type and mutants, the most abundant pool of precursors is the 45-55 kDa proteins, and the second most abundant one contains proteins of about 39 kDa and 25 kDa, as judged by immunoblotting with anti-tmx serum (Adoutte et al., 1984). This processing path is also similar in the ftA mutant for all four different groups of tmx proteins recognized by monoclonal antibodies, including dimeric, monomeric, core-, cortex- and whole-matrix-localized mature products (Fig. 6). Adoutte et al. also showed that in the ftl mutant the processing of tmx proteins is blocked. The immunoblot results presented here indicated that in mutants defective in the formation of trichocyst matrices, tl and ftA, the expression of tmx precursors in at least four immunologically defined groups is not defective. The processing of precursors of these four groups appears to be affected about equally: blocked completely in tl, severely in ftA (Fig. 6). There may therefore be a single or...
et al., 1986). Trichocysts in wild-type cells are found to be the processing of tmx precursors is coupled to the crystallization of tmx. Firstly, the immunoblots of ftA showed partially blocked processing of four groups of tmx precursors (Fig. 6). Secondly, the removal of antibodies labeling mature tmx proteins from the serum raised against 45-55 kDa precursors dramatically reduced the labeling by this serum of the central part of the trichocyst matrix in both mature and developing trichocysts (Fig. 5D-H). However, this antisera specific to tmx precursors still labeled the outer edge of the crystalline tmx and the amorphous, uncrystallized material around it. The antigenic epitopes for this antisera are presumed to include the fragments present in the precursors only, or the proteolytic processing sites or the conformational structures that exist only in precursors. These epitopes seem not be present in the center of the crystalline tmx. However, further characterization of the processing and assembly mechanisms in both wild-type and mutant cells is essential. Combined with the observation that immunologically distinct proteins are localized in different parts of the condensed tmx (Shih and Nelson, 1991), this hypothesis raises a question about how the order of processing is regulated to assure that core proteins are condensed before cortex proteins.

**Fig. 9.** Some tmx precursors bind phenyl-Sepharose in a Ca\(^{2+}\)-dependent manner. S2 from whole-cell extract was subjected to phenyl-Sepharose chromatography as described in Materials and methods. Proteins were resolved on a 12% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper. (A) Ponceau S-stained paper. (B) Immunoblot of the same paper with rabbit anti-tmx serum. Lane 1, column load (S2, 10 µg); lane 2, 1 mM EGTA eluant (15 µg, precipitated by 5% trichloroacetic acid); and lane 3, 20% ethanol wash (15 µg, also precipitated by 5% trichloroacetic acid), respectively.

A limited number of processing proteolytic enzyme(s) responsible for generating mature size proteins. In ftA, the putative enzyme is not functional, whereas in ftA it is mutated to a less active form or expressed at a lower level. This protease should be present in the isolated condensed trichocyst granules, unless it commits proteolytic suicide during trichocyst maturation.

Another possible explanation for the different extent of processing of tmx precursors in ftA and ftA is that the machinery responsible for acidification of trichocysts is defective in these two mutants. It has been suggested that acidification of secretory granules is required for the processing and/or condensation of proteins in secretory granules (Orci et al., 1986). Trichocysts in wild-type cells are found to be acidic (Busch and Satir, 1989), and monensin inhibited the processing of tmx precursors in wild type and tam8 cells (Adoutte et al., 1984). Considering all the evidence, we suggest that ftA and ftA are defective in the acidification of trichocysts.

**Processing occurs simultaneously with formation of paracrystallized matrix**

Adoutte et al. (1984) suggested, based on immunological and structural studies of wild type and tam8 mutants, that the processing of tmx precursors is coupled to the crystallization of trichocyst matrix and that complete processing of tmx precursors may establish some interactions among the subunits, providing an irreversible step of assembly of the tmx crystalline structure. Hausmann et al. (1988) reported that a mAb specific to core tmx proteins labeled small vesicles with no crystallized structure in ftA. In ftA it labeled the core of the crystallized tmx as well as uncrystallized material in the same trichocyst. Our results support the hypothesis that proteolytic processing of tmx precursors is coupled to the crystallization of tmx. Firstly, the immunoblots of ftA showed partially blocked processing of four groups of tmx precursors (Fig. 6). Secondly, the removal of antibodies labeling mature tmx proteins from the serum raised against 45-55 kDa precursors dramatically reduced the labeling by this serum of the central part of the trichocyst matrix in both mature and developing trichocysts (Fig. 5D-H). However, this antisera specific to tmx precursors still labeled the outer edge of the crystalline tmx and the amorphous, uncrystallized material around it. The antigenic epitopes for this antisera are presumed to include the fragments present in the precursors only, or the proteolytic processing sites or the conformational structures that exist only in precursors. These epitopes seem not be present in the center of the crystalline tmx. However, further characterization of the processing and assembly mechanisms in both wild-type and mutant cells is essential. Combined with the observation that immunologically distinct proteins are localized in different parts of the condensed tmx (Shih and Nelson, 1991), this hypothesis raises a question about how the order of processing is regulated to assure that core proteins are condensed before cortex proteins.

**Similarity of trichocyst matrix proteins with chromogranins**

Chromogranin A (CgA) proteins, acidic secretory proteins in mammalian cells, are immunocrossreactive with tmx proteins (Peterson et al., 1987b, 1990). Proteins immunorelated to CgA are also found in mucocysts of *Tetrahymena*, which are the organelles homologous to the trichocysts of *Paramecium* (J. B. Peterson and R. Angeletti, unpublished result; Y. Ding and B. Satir, unpublished result). These two families of secretory proteins, one from mammalian cells and one from protozoa, share some common properties (Table 1). Both CgA (Gorr et al., 1988) and tmx precursors (Fig. 7) are resistant to heat denaturation. Secondly, CgA (Gorr et al., 1988) and the precursors of tmx proteins (Fig. 8) aggregate upon the addition of Ca\(^{2+}\); in both cases other proteins in the same fraction were co-precipitated with these secretory proteins after centrifugation. CgA has been shown to undergo a conformational change upon binding to Ca\(^{2+}\) (Yoo and Albanesi, 1990). The binding of some tmx precursor proteins to phenyl-Sepharose in a Ca\(^{2+}\)-dependent manner (Fig. 9) also suggests that these precursor proteins change conformation upon binding to Ca\(^{2+}\). It is very likely that the mature tmx proteins also undergo Ca\(^{2+}\)-dependent conformational changes to achieve the eight-fold expansion that occurs during exocytosis. Ca\(^{2+}\)-binding proteins have also been found in the mucocysts of *Tetrahymena* (Turkevitz et al., 1991). Although the partial amino-terminal sequences of certain mature tmx proteins reveal no significant sequence homology between tmx proteins and CgA, or other proteins (Tindall et al., 1989; Peterson et al., 1990; J. B. Peterson, R. Angeletti, and D. L. Nelson, unpublished result), these interesting similarities between these two groups may help to unveil the physiological function of trichocyst matrices.
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