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Proteolytic Processing and Ca^{2+}-binding Activity of Dense-Core Vesicle Polypeptides in *Tetrahymena*

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Formation and discharge of dense-core secretory vesicles depend on controlled rearrangement of the core proteins during their assembly and dispersal. The ciliate *Tetrahymena thermophila* offers a simple system in which the mechanisms may be studied. Here we show that most of the core consists of a set of polypeptides derived proteolytically from five precursors. These share little overall amino acid identity but are nonetheless predicted to have structural similarity. In addition, sites of proteolytic processing are notably conserved and suggest that specific endoproteases as well as carboxypeptidase are involved in core maturation. In vitro binding studies and sequence analysis suggest that the polypeptides bind calcium in vivo. Core assembly and postexocytic dispersal are compartment-specific events. Two likely regulatory factors are proteolytic processing and exposure to calcium. We asked whether these might directly influence the conformations of core proteins. Results using an in vitro chymotrypsin accessibility assay suggest that these factors can induce sequential structural rearrangements. Such progressive changes in polypeptide folding may underlie the mechanisms of assembly and of rapid postexocytic release. The parallels between dense-core vesicles in different systems suggest that similar mechanisms are widespread in this class of organelles.

INTRODUCTION

Dense-core secretory vesicles, also called secretory granules, are specialized vesicles with a condensed protein core (Halban and Irminger, 1994). The vesicle core can be described as a dynamic aggregate. First, it is assembled within the secretory pathway from previously soluble proteins. Conversion of a set of soluble proteins to a temporarily insoluble form can serve diverse functions, including their efficient storage in a state that imposes a reduced osmotic burden. Secondly, when an appropriate exocytic stimulus triggers the fusion of the vesicle membrane with the plasma membrane, the dissemination of the secreted contents involves re- or dis-assembly of the core. Granule core dynamics exemplify the potential for compartment-specific regulation of protein interactions in the secretory pathway (Arvan and Castle, 1992), and offer the opportunity to investigate the underlying mechanisms.

Interactions among proteins in transit may be influenced by their microenvironments, as for example gradients of pH and calcium concentration within and between secretory compartments. The potential importance of such modulation is not limited to granule assembly. For example, the assembly of T-cell receptor subunits may be controlled by their calcium-specific binding to BiP (Suzuki *et al.*, 1991), and ligand-binding by the ER retrieval receptor may be regulated by the pH difference between the ER and the Golgi (Wilson *et al.*, 1993). For proteins forming the dense cores of neuroendocrine granules, a combination of these factors in the TGN and newly formed secretory vesicles have been implicated in the induction of aggregation/condensation (references in Natori and Huttner, 1996).

The regulation of core assembly and dispersal may involve several mechanisms, two of which are considered here. The first is direct modulation of protein folding by the succession of ionic environments experienced during transport. Such conformational maturation has been suggested in the case of newly synthe-

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sized proinsulin and several other secreted proteins, on the basis of progressive changes in the accessibility of protein disulfide bonds (references in Huang and Arvan, 1995). Second, regulation may be indirect, as exemplified by the action of granule-specific endoproteases called prohormone convertases (Rouille *et al.*, 1995). Many well-studied neuroendocrine granule core proteins are synthesized as proproteins and cleaved during granule maturation by prohormone convertases, to generate the biologically active polypeptides.

Ciliated protozoa are useful organisms for studying granule synthesis and function (Hutton, 1997), in part because the sequential transitions that characterize neuroendocrine granules have also been well-described in several of these organisms (Adoutte, 1988; Peck *et al.*, 1993). In addition, several species are genetically and biochemically accessible. In both *Tetrahymena thermophila* and *Paramecium tetraurelia*, granule core synthesis occurs by assembly of soluble proteins into an insoluble, well-ordered lattice (Anderer and Hausmann, 1977). Organization of core proteins as a crystalline lattice is also a feature of some metazoan granules (Tooze *et al.*, 1989). The second core transition is postexocytic decondensation. In ciliates this takes the form of a rapid spring-like expansion of the core lattices, projecting them from the cell (Anderer and Hausmann, 1977). In an analogous manner, granule core expansion in several mammalian cell types can also facilitate rapid dispersal of core contents (reviewed in Rahaminoff and Fernandez, 1997).

Normal core assembly, in both neuroendocrine cells and ciliates, is strongly correlated with proteolytic processing in the maturing granule (Adoutte *et al.*, 1984; Orci *et al.*, 1987; Turkewitz *et al.*, 1991). The mechanisms by which processing facilitates or drives core assembly are not known, but may be related to structural differences between proproteins and their products. Little information is available addressing these issues, particularly regarding changes as prohormones are processed to mature forms. Mechanisms involved in postexocytic decondensation are also largely unknown. The mechanism of lattice expansion in *Paramecium* involves binding of extracellular calcium (Bilinski *et al.*, 1981; Lima *et al.*, 1989), but the protein targets are difficult to analyze because the lattice is estimated to consist of more than 100 proteins (Madeddu *et al.*, 1995). Lattice composition in *Tetrahymena* appears less complex, although only the pattern of polypeptide bands has thus far been described (Maihle and Satir, 1986). Expansion has been shown to depend in vivo on the presence of the abundant core protein, *Gr*anule *l*attice *p*rotein *1* (Grl1p)[1], although its precise role is unknown (Chilcoat *et al.*, 1996). A suggestive hint is that Grl1p binds calcium in vitro. Any model explaining how extracellular calcium triggers expansion must take account of the fact that early compartments of the secretory pathway, through which granule proteins must initially be transported, are also likely to contain high levels of free calcium (Pozzan *et al.*, 1994; Sambrook, 1990).

Understanding granule assembly and disassembly will require analysis of the set of core proteins in a manipulable model system. The range of powerful molecular genetic tools including gene replacement that have recently been developed in *T. thermophila* make this organism an attractive choice (Cassidy-Hanley *et al.*, 1997; Gaertig and Gorovsky, 1995; Sweeney *et al.*, 1996; Yao and Yao, 1991). We have begun by identifying the set of abundant core polypeptides that bind calcium in vitro. Their cloning and initial characterization are described in this article. To understand how these proteins and their interaction with calcium may contribute to the dynamics of the granule core, we have tried to correlate the morphological transitions during both core synthesis and disassembly with changes in individual proteins. The availability of characterized exocytosis mutants facilitated the analysis of granule proteins before and after both proteolytic processing and exocytosis (Orias *et al.*, 1983)(Melia *et al.*, in press). The results are consistent with the idea that one function of compartmentalization in the secretory pathway is the regulation of protein interactions through programmed changes in their folding.

MATERIALS AND METHODS

Reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Cells and Cell Culture

Cells were grown at 30°C with agitation in 2% proteose peptone, 0.2% yeast extract (both from Difco Laboratories, Detroit, MI) with 0.003% ferric EDTA. All *Tetrahymena* strains were derived from the inbred B strain. *Tetrahymena* strains are described by their micronuclear diploid genotype, followed by their macronuclear-determined phenotype in parentheses (Bruns, 1986). The heterokaryon strain CU428.1, *mpr1–1/mpr1–1* (mp-s, VII) was provided by Peter Bruns (Cornell University, Ithaca, NY) and is wild type with respect to exocytosis. Strain MN173, *mpr1–1/mpr1–1* (mp-r, V) is an exocytosis-defective strain in which granules accumulate in the cytoplasm rather than docking at the plasma membrane (Melia *et al.*, in press). Strain SB281, *gal1–1/gal1–1* (dg-r, III), isolated by (Orias *et al.*, 1983) and provided by Ed Orias (UC Santa Barbara) is a mutant in which granule proproteins fail to undergo normal proteolytic processing (Ding *et al.*, 1991)(Turkewitz *et al.*, 1991).

In Vitro Calcium Binding

^{45}Ca was incubated with nitrocellulose transfers of granule proteins as described by (Volpe *et al.*, 1988). Samples were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

[1] The abbreviations used are: dg, 6-deoxygalactose; GRL, granule lattice; Grlp, granule lattice protein; mp, 6-methylpurine; Tmp, trichocyst matrix protein.

Cloning of Genes Encoding Granule Proteins

On the basis of findings presented in this article, it appears that single granule protein precursors may generate at least two stable fragments. To indicate these, we will adopt the following convention. The precursor of Grl4p before processing (not including removal of the signal peptide) will be referred to as proGrl4p. The amino-terminal fragment of Grl4p will be referred to as N-Grl4p, and the carboxyl-terminal fragment as C-Grl4p.

Granule proteins were isolated, separated by SDS-PAGE on 20% gels, transferred to a polyvinylidene difluoride membrane, and lightly stained with Coomassie blue as previously described (Chilcoat *et al.*, 1996). Bands corresponding to those which bound calcium in vitro on nitrocellulose membranes were excised and subjected to amino-terminal sequencing (Carol Beach, University of Kentucky; Mark Crankshaw, Washington University, St. Louis, MO). Degenerate oligonucleotide primers were designed from these peptide sequences on the basis of known codon usage (Martindale, 1989) and paired with vector-specific primers for PCR amplification from a cDNA library kindly provided by Tohru Takemasa (Tsukuba, Japan). The vector-specific forward primer was: AGCAAGTTCAGCCTGGTTAAG; the reverse primer was CTTATGAGTATTTCTTCCAGGGTA. Peptide-based primers were designed corresponding to the following sequences: in proGrl3p, residues 234–239; in proGrl4p, residues 246–252 and 228–234; in proGrl5p, residues 75–80; in proGrl7p, residues 71–76. PCR conditions were as follows: 94°C, 15 s; 5 × [92°C, 30 s; 50°C, 30 s; 72°C, 60 s]; 24 × [92°C, 15 s; 50°C, 30 s; 72°C, 60 s]; 72°C, 10 min. The buffer was 50 mM KCl, 50 mM Tris-HCl pH 9, 1% Triton X-100 (Boehringer Mannheim, Indianapolis, IN), 2.5 mM $MgCl_2$, 1 μM in each primer, 0.25 mM dNTPs and 2.5U *Taq* polymerase. A limited number of bands were produced in each reaction, which were then cloned and sequenced to confirm the presence of the expected peptide sequence adjacent to the peptide-based primer sequence. Nondegenerate oligonucleotides were designed on the basis of the sequences of the positive clones, and used to amplify the remaining portions of the cDNAs from the library. Clones were sequenced by primer walking. Potential sequencing errors resulting from the degeneracy of the initial primers or incorporation of errors in the initial clones were rectified by direct sequencing of the amplification products of genomic and cDNA. Sequence analysis was using the Lasergene software (DNAStar, Madison, WI).

Preparation of Cell Lysates and Secretory Granules

Two hundred milliliters of cells were grown overnight to stationary phase, then rapidly chilled and pelleted at 150 × *g* for 5 min in conical bottles. All subsequent operations were performed cold. Cells were washed once in 10 mM K-HEPES pH 7.0; the pellet was then measured, washed, and resuspended in 2 volumes of buffer A [0.3 M sucrose, 10 mM K-HEPES pH 7, 28.8 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA (Fluka, Ronkonkoma, NY)] containing 0.1% gelatin and the following protease inhibitors: leupeptin (0.5 μg/ml), antipain (12.5 μg/ml), E-64 (10 μg/ml) and chymostatin (10 μg/ml). The resuspended pellet was homogenized by ~15 passages through a ball-bearing cell cracker (Hans Issel, Palo Alto, CA) with a nominal clearance of 0.0005 in. The entire homogenate was pelleted at 200,000 × *g* for 2 h. The supernatant was withdrawn and the pellet was resuspended in 1–2 ml of Buffer A lacking sucrose, to induce hypotonic lysis of membrane-bound organelles. Aliquots were frozen in liquid nitrogen and stored at −80°C.

Isolated secretory granules were prepared from lysates of MN173 cells as previously described (Chilcoat *et al.*, 1996). Protein concentrations were determined using bicinchoninic acid (Pierce, Rockford, IL).

Calcium Concentrations

All noncalcium-containing buffers included 2 mM EGTA (Fluka, Ronkonkoma, NJ). To adjust calcium concentrations, samples were mixed with equal volumes of the same buffer but containing $CaCl_2$. Compositions of calcium buffers were calculated following Fabiato and Fabiato (1979). The pH of calcium-containing stocks was preadjusted with KOH to balance the acidification that occurs when calcium is added to EGTA-containing buffers. The pH in these experiments did not shift by more than 0.05 units.

Heat Solubilization

Isolated DCGs in Buffer A (with added calcium where noted) were incubated for 30 min at 95°C, and then centrifuged at room temperature, either for 20 min at 17,000 × *g* in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY), or for 2 h at 150,000 × *g* in an SW50.1 rotor (Beckman Instruments, Carlsbad, CA).

Protein Electrophoresis and Antibody Blotting

SDS-PAGE was performed according to (Laemmli, 1970). Quantitation of Coomassie Blue-stained bands was using a computing densitometer (Molecular Dynamics, Sunnyvale, CA). Antibody blotting was according to (Towbin *et al.*, 1979), and antibodies were visualized using the ECL system (Amersham, Arlington Hts, IL). The rabbit antiserum specific for Grl1p (previously called p40) has been described (Turkewitz *et al.*, 1991).

Proteolytic Digestion

Preincubations of samples with Triton X-100, $CaCl_2$ (Fluka, Ronkonkoma, NJ) or EGTA were for 10 min on ice. Chymotrypsin (Worthington, Freehold, NJ) was added from a stock solution of 10 mg/ml in 50% glycerol. Digestion was on ice for 45 min, and was terminated by addition of PMSF to a concentration of 2 mM. After 20 min, samples were solubilized by addition of an equal volume of hot SDS-PAGE sample buffer containing 2% SDS and 10% 2-mercaptoethanol, and incubated for 3 min at 100°C.

Electron Microscopy

All reagents were from EM Sciences (Ft. Washington, PA). Cells were fixed in 2% glutaraldehyde, 0.5% osmium tetroxide, 1.3 mM sodium phosphate pH 7.0, 0.5% sucrose for 1 h at room temperature. After partial dehydration, cells were stained for 1 h with 1% uranyl acetate in 70% ethanol. After dehydration through a graded series of ethanol solutions and propylene oxide, samples were embedded in EMbed 812. Sections were stained with uranyl acetate and lead citrate, and viewed using a JEOL CX100 electron microscope. Purified granules were fixed overnight as pellets in 2% glutaraldehyde, 1% osmium tetroxide in buffer A, and processed as above. Purified granules were adsorbed to carbon-coated grids and stained with 2% sodium phosphotungstate near pH 7. Negative staining of expanded granules was with 2% uranyl acetate.

RESULTS

Expansion of Granule Cores in the Presence of Calcium

The secretory granules in *Tetrahymena*, called mucocysts, are docked near the plasma membrane in resting cells (Figure 1A). Granules can be readily isolated from the exocytosis-defective strain MN173, where they fail to dock but instead accumulate in the cytoplasm. Negative staining of such isolated condensed granules shows a dense protein lattice that constitutes their core (Figure 1B). Cores are exposed to the high concentration of extracellu-

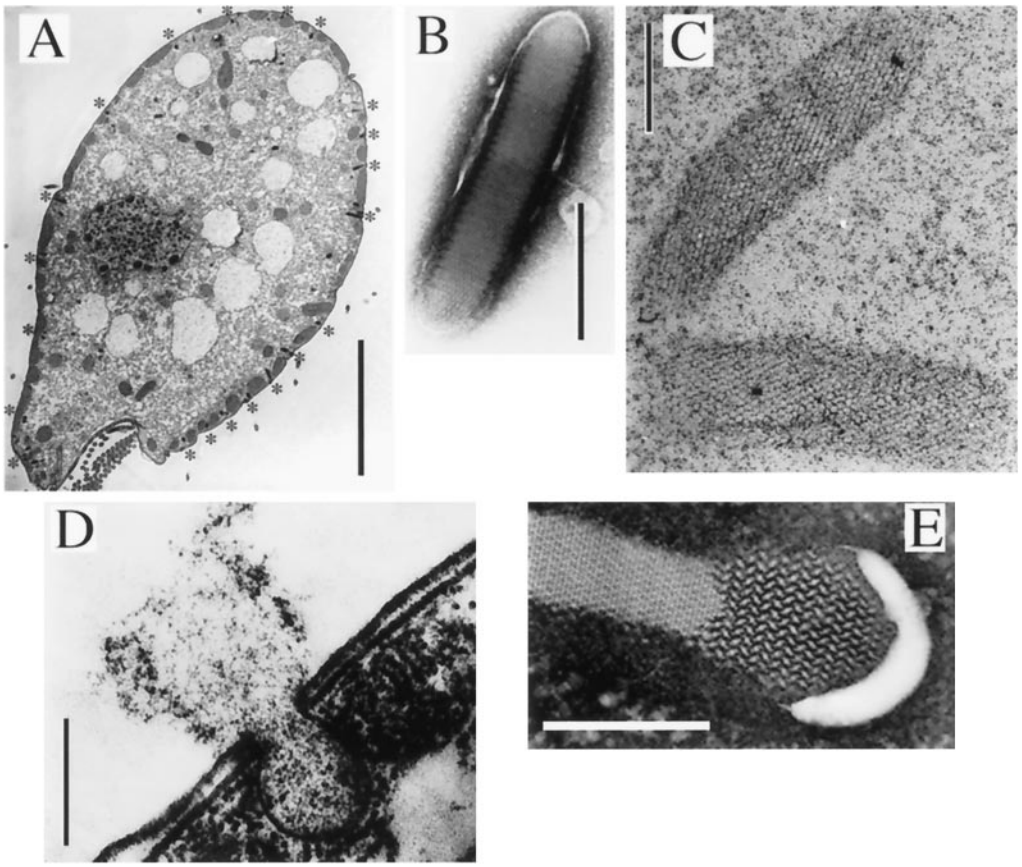

Figure 1. Electron micrographs. (A) Thin section of *Tetrahymena thermophila*. Dense core secretory vesicles docked at the cell surface are marked by *. The larger bodies lying near the cell surface are mitochondria. Bar = 10 μm. (B) Isolated vesicle, in which the granule lattice core has been visualized by negative staining by phosphotungstate. Bar = 0.4 μm. (C) Two expanded granules, negatively stained with uranyl acetate. Note difference in scale between B and C: the expanded lattices are >sixfold longer than in the condensed state. The particulate matter is residual Percoll. Bar = 2 μm. (D) Fusion of a secretory vesicle and release of contents. Such fusion can be triggered by glutaraldehyde during cell fixation, producing such exocytic Figures. Bar = 0.8 μm. (E) Isolated granule in a state of partial expansion, negatively stained with phosphotungstate. Bar = 0.4 μm.

lar calcium after exocytic membrane fusion of the granule and plasma membranes. To test whether calcium could trigger lattice expansion, we first incubated pellets of isolated granules with nonionic detergent to remove the limiting membrane, and then exposed them to 1 mM calcium. This treatment led to notable expansion of the pellet size, suggesting that expansion of the lattices had occurred (our unpublished observations). Negative staining of isolated granules using uranyl acetate allowed visualization of the expanded granules, which increase in length by >sixfold while maintaining a lattice structure (Figure 1C). Expansion upon exocytosis in vivo drives the rapid release of core proteins within seconds, as illustrated in Figure 1D. The process may occur through a series of ordered states, since intermediate expanded forms can be identified within individual granules (Figure 1E).

Identification of Calcium-binding Proteins

To identify components that underlie this dramatic expansion and/or the process of core assembly, isolated granules were subjected to SDS-PAGE, transferred to nitrocellulose, and overlaid with ^{45}Ca. Previous work had demonstrated in vitro calcium binding activity of Grl1p (*Gr*anule *l*attice protein *1)*, a 24-kDa polypeptide that migrates, however, with a MW_a of 40 kDa (Turkewitz *et al*., 1991). Subsequently, a change in gel running conditions to resolve smaller species revealed a set of abundant polypeptides that also show in vitro calcium binding activity (Figure 2). In contrast, none of the proteins of $MW_a > 40$ kDa, of which three are prominent, shows calcium binding. We prepared seven prominent bands below 40 kDa for amino terminal sequencing. On the basis of densitometry of Coomassie Blue-stained gels, these polypeptides make

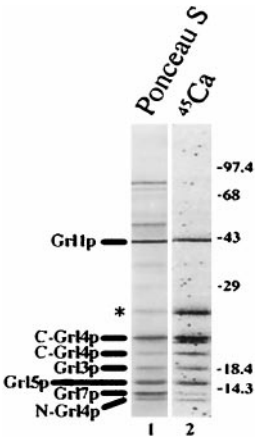

Figure 2. In vitro calcium binding to proteins in granule cores. Proteins in isolated granules were resolved by SDS-PAGE (17.5% polyacrylamide, 40 μg/lane) and transferred to nitrocellulose. Lane 1 was stained for protein with Ponceau S; lane 2 was incubated with ^{45}Ca, and bands were visualized after overnight exposure to a PhosphorImager screen. Identies of characterized species are indicated on the left. A prominent calcium-binding band, which appears to be a minor species by protein staining, is marked by $*$. Molecular weight standards are indicated on the right.

up more than 80% of total granule protein. With the exception of the single smallest band, which gave more than one residue at each position, each polypeptide yielded a unique sequence. Sequences obtained are shown in Table 1, along with the MW_a of the fragments from which they were derived.

Cloning and Sequence Analysis of Granule Lattice Proproteins

PCR using degenerate oligonucleotide primers, in combination with vector-based primers, was used to amplify the corresponding genes from a λgt10 cDNA library. Sequencing of the cloned genes established unique open reading frames encoding the expected polypeptide sequence, and each conformed to the expectations for *Tetrahymena* codon usage and AT-rich nontranslated regions. Sequence analysis revealed that the six polypeptides are derived from four precursors that range in size from 377–402 residues. Together with the previously described *GRL1* gene product, these proteins can be considered to constitute a family of granule lattice proproteins (proGrlps). Each begins with a signal sequence, and features of interest are diagrammed in Figure 3, while the sequences themselves are shown in Figure 4. For the purposes of comparison, proGrl1p is also included. A panel of features is generally duplicated within each precursor. First, each proGrlp contains two large blocks of predicted coiled-coil-forming residues. The heptad repeats that are predicted to form these coiled-coils are nearly continuous in some cases, and more fragmented in others. These blocks have a high density of negatively charged residues, with the result that these proteins are all calculated to be strongly acidic, and predicted to bind calcium with low affinity. The calculated acidity of granule polypeptides is consistent with previous observations (Maihle and Satir, 1986)(Turkewitz, unpublished data). Preceding each block of coiled-coils is a small stretch with a high concentration of positively charged residues. In proGrl1p and proGrl3p, the basic block near the amino terminus is absent. The amino termini of granule core polypeptides derived from these precursors are located directly following the basic stretches, suggesting that these regions are directly or indirectly recognized by processing proteases. A notable feature of these stretches is that they contain virtually all of the methionine residues in the proteins. Every internal methionine in the proGrlps lies within or very close to a basic stretch, and only one basic stretch lacks a methionine. The conservation of a methionine residue strongly suggests its functional significance, since it, along with tryptophan, is the amino acid most susceptible to change by point mutation.

Overall sequence identity between the proGrlps is not greater than 22% between any two members, a substantial part derived from common residues that define the coiled-coil-forming heptad repeats (Figure 4). The similarities between the proGrlps suggest that they may fold into related structures. The approximate repeat of features noted in each protein may reflect gene fusion or duplication. ProGrl5p in particular contains a suggestive internal repeat between the amino- and carboxyl-terminal halves, as shown in Figure 5.

In general, conservation of structure rather than specific sequence is consistent with a role for the Grlps in forming the architectural units of the dense core lattice. This impression was reinforced by a search of the protein sequence database. The most similar sequences were in granule proteins of *Paramecium*, called trichnins or trichocyst matrix proteins (Tmps), which also form an expandable lattice structure (Gautier *et al.*, 1996). Examination of the three known Tmp sequences revealed that they encode similarly sized proteins with comparable blocks of coiled-coil regions,

Table 1. Amino-terminal sequences of granule polypeptides

Name	Sequence	MW_a (kDa)
N-Grl4p	GLVDDVIDLVKQAQEDVA	12.3
C-Grl4p (i)	FDEQRLAEVISKLQTIQAAIQASYIED	21.5
C-Grl4p (ii)	-DEQRLAEVI	22.5
Grl3p	EVDQSSLAKVVSLINDLLEEL	19
Grl5p	YDYEELYDAFAALKNQL?NNLDLE	16
Grl7p	ANIIVNILALLNDLLNDQST	14

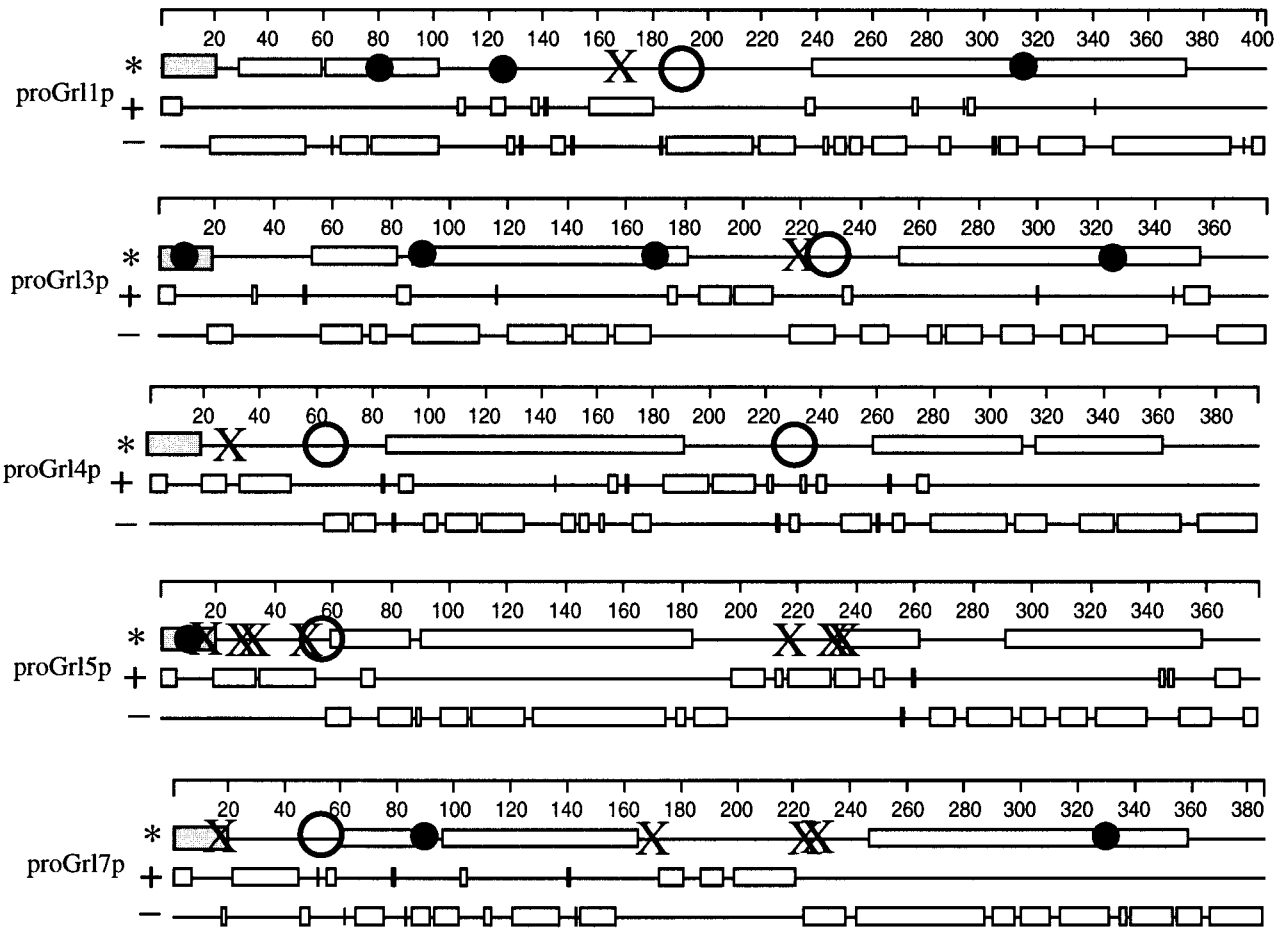

Figure 3. Predicted features of proGrlps. On the basis of the deduced amino acid sequences and the known N-termini of isolated polypeptides, a number of features are predicted. Features of each Grlp are shown on three consecutive lines. On the first of each set of three lines are shown the regions forming coiled-coils (as open rectangles), sites corresponding to known amino termini of processed polypeptides (as large open circles), cysteine residues (as small solid circles), methionines (as X's), and signal sequences (as shaded rectangles). On the second line, stretches of basic residues are shown as open rectangles. On the third line, stretches of acidic residues are shown as open rectangles.

also preceded by methionine-rich basic stretches (Figure 4). The proGrlps share approximately the same level of sequence identity with the Tmps as they do with one another. In addition to the pattern of structural similarity without sequence conservation, there are also small regions of sequence identity, described below, that are shared between *Paramecium* and *Tetrahymena* proteins. The significance of this conservation is underlined by the fact that the two ciliates diverged at least several hundred million years ago (Wright and Lynn, 1997), and suggests that the proteins are anciently derived from common precursors.

Granule Proproteins Are Processed at Multiple Conserved Sites

Many metazoan granule polypeptides are derived by processing of precursors into nonoverlapping fragments by cleavage at multiple sites. This also occurs during granule synthesis in *Tetrahymena*. As shown in Table 1 and Figure 4, the N-termini of two abundant polypeptides were both located within the same precursor, proGrl4p. N-Grl4p begins at Gly63, and C-Grl4p began at Phe226 (see terminology in MATERIALS AND METHODS). Although we have not identified the C-termini of the fragments, the MW_a of N-Grl4p (12.3 kDa) suggests that these are nonoverlapping fragments, because the predicted molecular mass for a polypeptide extending from residue 63 to 225 would be 18.4 kDa. The generation of the two fragments from proGrl4p can be explained by two distinct endoproteolytic processing events.

To characterize proprotein processing, we looked for conserved sequence motifs near the known amino termini. Metazoan prohormone convertases cleave at

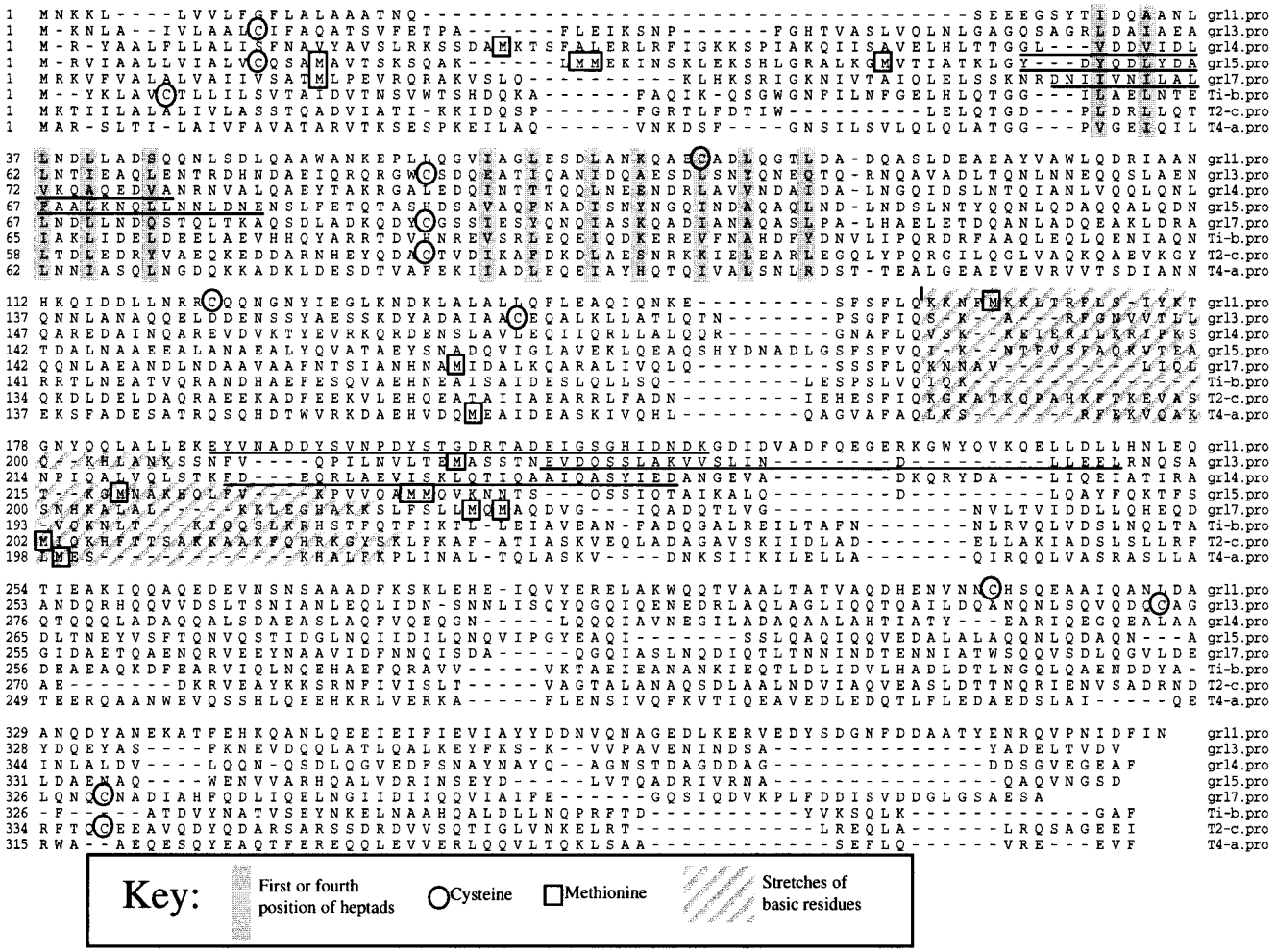

Figure 4. Deduced amino acid sequences from *GRL3*, *GRL4*, *GRL5* and *GRL7*. For the purpose of comparison, *GRL1* and *Paramecium TMPs* (called Ti-b, T2-c, and T4-a) have been included. Sequences were aligned to illustrate alignment of several heptad repeats. The majority of the heptad repeats are not labeled since it was not possible to select a single best alignment. In each of the deduced Grlp sequences, the experimentally determined polypeptide sequence is underlined. Cysteines, methionines, and the basic stretches diagrammed in Figure 3 are indicated. Both nucleotide and amino acid sequences are deposited under the following GenBank acquisition numbers: *GRL3*: AF031319; *GRL4*: AF031320; *GRL5*: AF031321; *GRL7*: AF031322.

specific positions in their prohormone substrates (Rouille *et al.*, 1995). Important residues at these positions include both a target residue, after which cleavage occurs, and surrounding residues that are likely to form specific secondary structures. Sequence comparison between the proGrlps suggested at least three kinds of processing sites. A first putative processing site was identified in proGrl4p (generating N-Grl4p) and proGrl5p. In these, the residue before the amino-terminus of the processed polypeptide is glycine, which is also present at an equivalent position in three of the Tmps. Comparison of all five sequences suggests the consensus shown in Figure 6A. We note that the spacing of conserved hydrophobic residues within this consensus does not conform to a standard heptad motif.

Figure 5. Evidence for an internal repeat in proGrl5p. Sequences from the amino- and carboxyl-terminal halves of proGrl5p have been aligned to show amino acid conservation.

A second kind of site occurs in proGrl4p and proGrl7p, as well as in the previously cloned Grl1p. Cleavage occurs following a single basic residue (Lys188 in proGrl1p, Lys225 in proGrl4p, Arg56 in proGrl7p) (Figure 6B). In the first two, this site is associated with a fragment generated from the carboxyl-terminal half of the proprotein, whereas in the

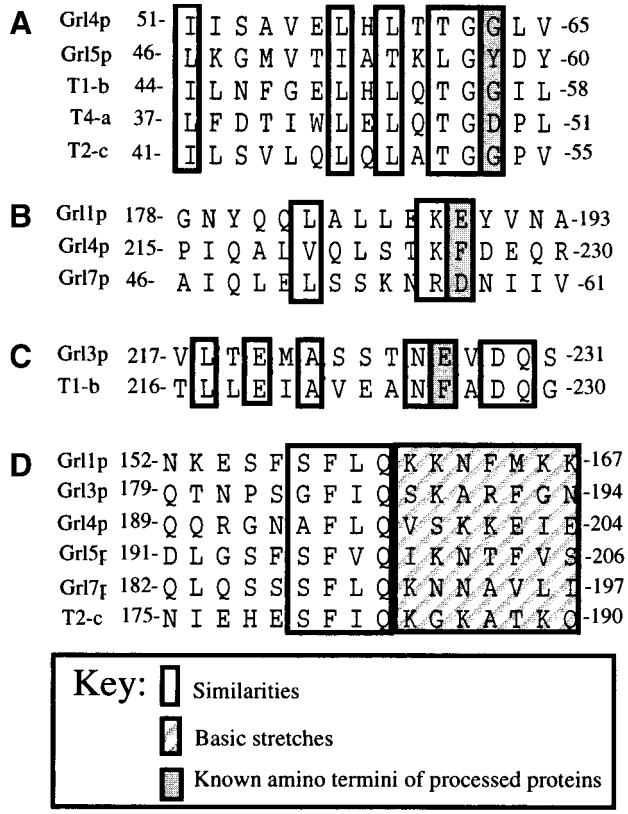

Figure 6. Conserved motifs in proGrlps and Tmps. (A) Alignment of sequences at which cleavage occurs following a Gly. The amino-terminal residue of the processed peptide is shaded in A–C. (B) Alignment of sequences at which cleavage occurs following a single basic residue. (C) Alignment of sequences at which cleavage occurs following a single asparagine residue. (D) Alignment of sequences illustrating a tetrapeptide motif that lies amino-terminal to the central basic stretch.

last, the fragment comes from the amino-terminal half.

A third type of site is noted in proGrl3p, associated with the carboxyl-terminal fragment. Here the residue after which cleavage occurs appears to be Asn226 rather than a basic residue. Cleavage following Asn has also been deduced for a *Paramecium* Tmp. Several nearby residues are also common to both proteins (Figure 6C).

In addition to the sequences associated with cleavage sites, a conserved sequence that is not overtly associated with a cleavage site is located near the prominent central stretch of basic residues discussed above. Sequence conservation was not detected within the basic region itself but is evident in the faithful maintenance of a tetrapeptide at what may be considered the amino-terminal junction of the basic region (Figure 6D). The identical motif is also found in the corresponding position in *Paramecium* Tmps.

The implication of sequence conservation at cleavage sites is that two to three distinct endoproteases are involved in processing of the proGrlps. In addition to these endoproteases, the size of the processed polypeptides indicates that exoproteases may also contribute to granule protein maturation. Many of the isolated polypeptides appear to be smaller than the sizes predicted by simple endoproteolytic cleavage of precursors at the recognized motifs. For example, the predicted size of C-Grl4p is 18.4 kDa, but the size estimated by SDS-PAGE is approximately 12.5 kDa. More direct evidence for a carboxypeptidase activity comes from sequencing data. Two well-resolved polypeptides differing in MW_a by ~1 kDa were found to have the identical amino terminus (9/9 residues), corresponding to Phe226 in proGrl4p. The difference between the two species, which appear to be of roughly equal abundance on the basis of Coomassie Blue staining, was unlikely to be attributable to non-physiological proteolysis, since the pattern of polypeptides appears identical whether isolated from secreted protein or from isolated granules (Chilcoat *et al.*, 1996). Another possible cause for such a mobility shift, post-translational glycosylation, appears to be extremely limited, if it occurs at all, in *Tetrahymena* granule proteins (Attanoos and Allen, 1987). Since Southern blotting also indicated that *GRL4* is a unique gene (our unpublished results), these two species may be accounted for by alternative carboxyl-terminal trimming of the same initial fragment.

In summary, the results of peptide sequencing and gene cloning indicate that the polypeptides comprising the bulk of *Tetrahymena* dense-cores are derived from five structurally related proproteins. These polypeptides form the basis for the ordered expandable lattice, and may therefore be expected to undergo rearrangements during assembly and disassembly. Since calcium binding and/or proprotein processing may contribute to regulation of these events, we designed experiments to detect their influence at the level of the polypeptides.

Assessment of Grlp Conformation by Proteolytic Digestion

Isolated demembranated granules were incubated in buffers of defined calcium concentration, and then exposed to protease. Products were analyzed by Coomassie staining after SDS-PAGE. We looked for changes in proteolytic patterns that might indicate altered accessibility of protease-sensitive sites. Since the electrophoretically separated Grlps can be individually recognized, this approach was well suited for assessing the mixture of proteins in the physiological lattice. In addition, we found that this assay could also be applied to crude cell lysates, followed by detection with specific antibodies on Western blots.

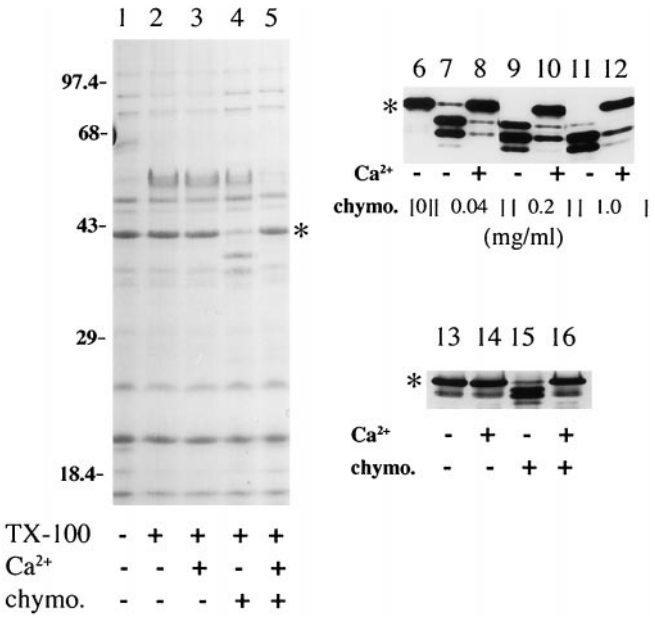

Figure 7. Chymotryptic digestion of Grl1p in isolated granules and cell homogenates. In all three panels, * indicates the position of mature Grl1p. Several of the smaller Grlps cannot be seen on this gel; their chymotrypsin insensitivity was noted in other experiments using higher percentage gels. Lanes 1–5: Aliquots of isolated granules were treated as described in the table beneath the lanes; ~40 μg of granule protein was loaded per lane of a 15% polyacrylamide gel. In the presence of Triton X-100, chymotrypsin (1 mg/ml) generated distinct fragments of Grl1p (lane 4). Incubation with calcium (1 mM) before addition of chymotrypsin resulted in protection of Grl1p (lane 5). Samples were visualized with Coomassie blue. Lanes 6–12. Isolated granules were incubated with 1% Triton X-100, and aliquots containing 8 μg of protein, were treated as indicated in the table beneath the lanes. After SDS-PAGE, proteins were transferred to nitrocellulose and antibody blotted with antiGrl1p. A ladder of proteolytic products was generated with increasing concentrations of chymotrypsin (lanes 7, 9, 11). At all chymotrypsin concentrations, preincubation with 1 mM calcium provided extensive protection (lanes 8, 10, 12). Lanes 13–16. Aliquots of a crude particulate fraction from MN173 cells were treated as indicated. One hundred and fifty micrograms of total protein was loaded per lane; the chymotrypsin concentration was 0.5 mg/ml. Proteins were visualized by antibody blotting with anti-Grl1p.

First, a variety of proteases were incubated with granule samples in the absence of calcium. The majority had no discernible effect on any of the proteins visualized by SDS-PAGE, indicating that the core proteins in general were highly protease resistant (our unpublished results). However, addition of chymotrypsin resulted in rather specific cleavage of Grl1p, with the appearance of 2 smaller products (Figure 7 lane 4). Higher concentrations of chymotrypsin produced 3 major proteolytic products, as visualized by antibody blotting with an antiserum against Grl1p (Figure 7 lanes 7, 9, 11). Similar results were obtained using hypotonically lysed particulate fractions of MN173 homogenates (Figure 7 lane 15). MN173 cells, in which the granules accumulate in a nondocked state, were used for these experiments to avoid the inevitable low level of exocytosis that occurs during preparation of wild-type lysates.

Effect of Calcium

Having establishing a baseline digestion pattern, we asked whether addition of calcium would alter the chymotrypsin sensitivity of any Grlps. In the presence of calcium, Grl1p became highly resistant to chymotryptic digestion (Figure 7 lanes 5, 8, 10, 12). Other Grlps did not show altered proteolytic sensitivity. The same result with Grl1p was obtained using a crude particulate fraction (Figure 7 lane 16). The calcium-dependent decrease in protease sensitivity of Grl1p suggested the polypeptide had adopted a more stable conformation. Alternatively, it could indicate masking of an accessible site by bound calcium or by another lattice component during expansion. Results presented below argue strongly in favor of the first explanation. Chymotrypsin itself is not inhibited by calcium (Fioretti *et al.*, 1994); this was directly confirmed for these experimental conditions in Figure 12.

Characterization of the Response to Calcium

To see whether the apparent change in Grl1p conformation might be related to calcium-dependent expansion after exocytosis, we tested three predictions. These also served to distinguish the role played by calcium in this system from its known activity in regulation of many cytosolic proteins, which occurs at low micromolar concentrations. First, the calcium concentration required to promote the putative conformational transition should be consistent with extracellular levels. *T. thermophila* inhabits a variety of fresh water bodies in which the range of calcium concentrations is roughly 0.1 to several millimolar (Wetzel, 1983). We exposed isolated granules to chymotrypsin in calcium-buffered solutions, and found that the transition to chymotrypsin resistance took place in the relevant range of 0.1 to 1 mM calcium (Figure 8), where in vitro granule expansion also occurs.

Granule expansion is a unidirectional process that is not reversed if calcium is subsequently removed (Hayashi, 1974). This suggests that individual steps driving the reaction may also be unidirectional, and we therefore examined the reversibility of the Grl1p transition. Detergent-permeabilized granules were incubated with millimolar calcium, and EGTA was subsequently added to reduce free calcium to less than micromolar levels. Once exposed to calcium, Grl1p remained in a chymotrypsin-resistant state (Figure 9 lanes 2, 3, 4). As a control, the addition of EGTA before calcium prevented Grl1p from acquiring chymotrypsin resistance (Figure 9 lane 5). This also supports the expectation that calcium binding by Grl1p is of low

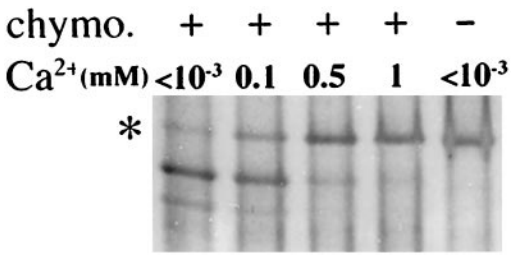

Figure 8. Calcium titration. Aliquots of isolated granules in 1% Triton X-100 were incubated with the indicated calcium concentrations, followed by addition of chymotrypsin. 60 μg of protein were loaded per lane, and samples were visualized with Coomassie blue. Grl1p is marked by *.

affinity and therefore not competitive with high affinity binding by EGTA.

Since the transition required exposure to calcium but not sustained binding, these results could be explained by a model in which calcium acts to lower a folding barrier, permitting a transition from a metastable to a stable conformation. Such a folding barrier might also be overcome by thermal energy. We accordingly tested whether a similar transition could be promoted by heat-treatment. A small fraction of Grl1p can be solubilized from secreted (expanded) lattices by incubating them at elevated temperature (Turkewitz *et al.*, 1991). We repeated this procedure, but substituted isolated condensed granules as substrate. Heat treatment of condensed lattices results in extensive and preferential release of Grl1p and a set of smaller Grlps (Figure 10). Effective solubilization of Grl1p was inhibited by preexposure to calcium (Figure 10 lane 3), explaining the much smaller yield we previously observed from expanded lattices. This may be a result of a change in lattice contacts upon expansion. In the absence of calcium, soluble Grl1p was obtained from the heat-treated supernatant after a 2 h, 150,000 $\times$ *g* spin.

The ability to isolate soluble Grl1p by heat treatment also made it possible to study its chymotrypsin sensitivity in the absence of an extended lattice, an issue discussed above. This soluble fraction was found to be strikingly resistant to chymotryptic digestion, similar to Grl1p in calcium-treated granules (Figure 11). In fact, no additional change in protease sensitivity was observed after addition of calcium to this preparation. In summary, these data indicate that chymotrypsin resistance does not depend on stabilization within an expanded lattice. Instead, protease resistance may reflect a stable conformation of Grl1p alone or in a soluble complex that does not depend on extensive lattice connections. Furthermore, both transient heating and transient exposure to calcium appear to produce a similar effect on Grl1p conformation. These data can be explained by a transition that occurs on

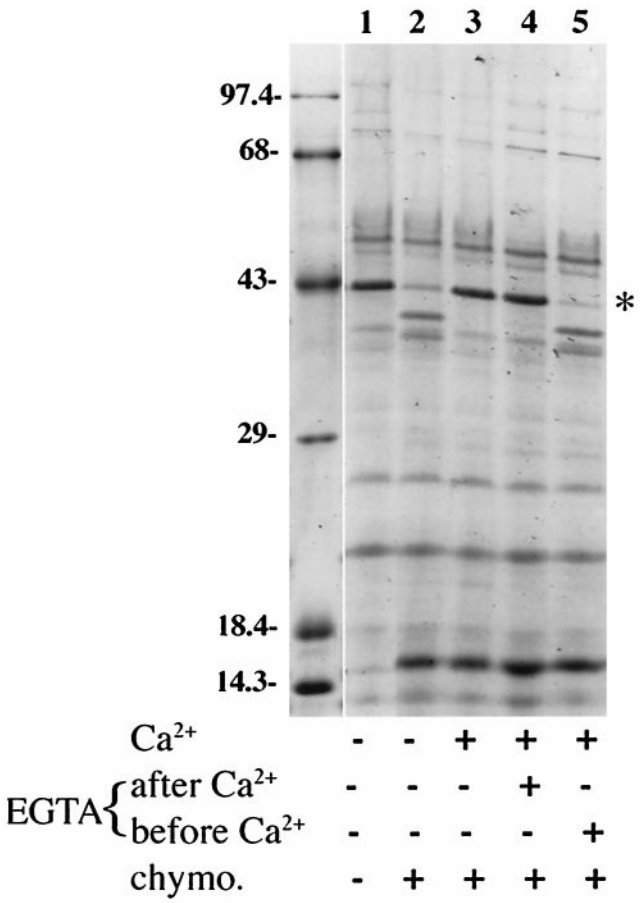

Figure 9. Nonreversibility of calcium effect. Lanes 1–5: Isolated granules were treated as in Figure 7, with two additional variables. In lane 4, 10 mM EGTA was added after the calcium incubation, to reduce free Ca^{2+} to <1 μM. In lane 5, 10 mM EGTA was added before calcium. Samples were then incubated with chymotrypsin. Gel samples contained ~40 μg of protein, and were visualized with Coomassie blue. Grl1p is marked by *; the prominent band migrating near 15 kDa in lanes 2–5 is chymotrypsin. Molecular weight standards are indicated.

discharge, involving the relaxation of a kinetically trapped intermediate.

Protein Folding during Granule Synthesis

Core assembly involves conversion of proteins from a soluble to insoluble form. In addition, the experiments described above hint at functional changes during this process; for example, Grl1p may be converted to calcium sensitivity. The correlation between proprotein processing and core assembly, in these as well as in neuroendocrine granules, suggested that processing might play a regulatory role at this stage.

The hypothesis that processing might alter the calcium sensitivity of the granule protein was tested by examining chymotryptic sensitivity in the presence

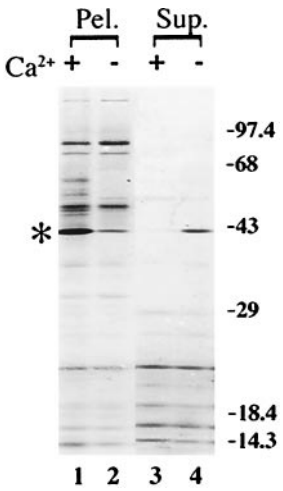

Figure 10. Heat solubilization of Grl1p. Isolated granules (30 μg protein/aliquot) in 1% TX-100 were incubated at 95°C for 30 min, with or without previous exposure to 1 mM calcium. Samples were then centrifuged at 17,000 $\times$ *g* for 30 min. Fifty percent of the supernatant fractions and 100% of the pellets were analyzed by SDS-PAGE (17.5% polyacrylamide) and visualized by Coomassie blue.

and absence of calcium. We made use of the exocytosis-defective *Tetrahymena* strain SB281 in which proGrl1p is not processed to mature Grl1p, so that only the former is detected by antibody blotting of a cell lysate (Figure 12 lane 1). Use of SB281 allowed unambiguous identification of the chymotryptic products of proGrl1p. In the absence of calcium, incubation of an SB281 crude membrane fraction with low concentrations of chymotrypsin generated discrete proGrl1p fragments with mobilities similar to that of in vivo-processed Grl1p and the major chymotryptic fragment thereof (Figure 12 lane 2). In marked contrast to the results seen with processed Grl1p, addition of

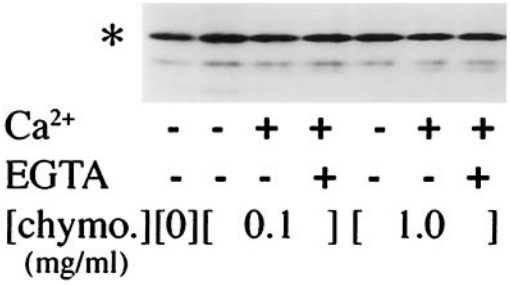

Figure 11. Chymotrypsin resistance of soluble Grl1p. Isolated granules were incubated at 95°C for 30 min, and then sedimented at 150,000 $\times$ *g* for 2 h. Aliquots of the supernatant, containing ~7 μg of protein, were incubated with or without 1 mM calcium, followed by 10 mM EGTA, as indicated. Chymotrypsin was then added at the indicated concentrations. Samples were visualized by antibody blotting after SDS-PAGE. Grl1p is marked by *.

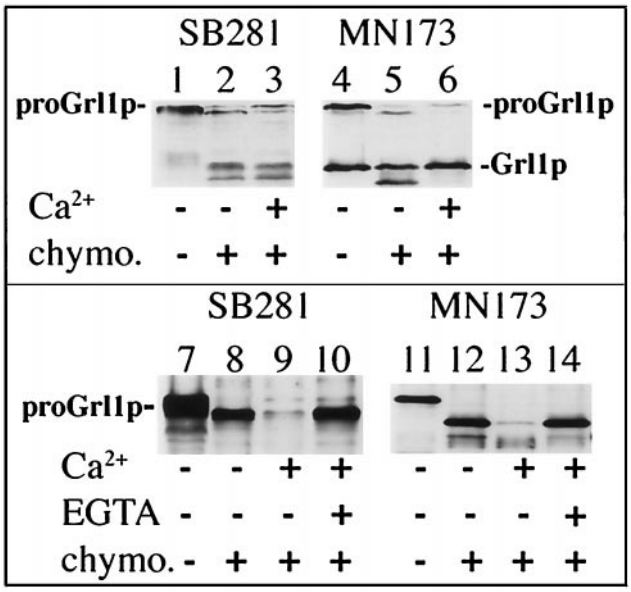

Figure 12. Effect of calcium on proGrl1p. Crude membrane fractions of either SB281 or MN173 were prepared, incubated with calcium as noted, and digested with 1 mg/ml chymotrypsin. Approximately 100 μg of protein were loaded per lane. In lanes 1–3, chymotrypsin digestion resulted in disappearance of proGrl1p and appearance of Grl1p-sized fragments. Comparison of lanes 2 and 3 with 5 and 6 indicates that exposure to calcium inhibited digestion of Grl1p, but not proGrl1p. At lower chymotrypsin concentrations (0.1 mg/ml), intermediate digestion products of proGrl1p are visible in membrane fractions from both SB281 (lane 8) and MN173 (lane 12). These appeared to be reversibly destabilized by addition of calcium (lanes 9, 10 and 13, 14). The difference between the sizes of the proteolytic fragments (lanes 8 and 12) was variable; both MN173 and SB281 showed fragments of both sizes in other experiments. Seventy-five micrograms of protein per lane was loaded. Samples in Figure 7 were visualized by antibody blotting.

calcium before chymotrypsin did not reduce the degree of digestion (Figure 12 compare lanes 3 and 6), although the proteolytic products formed were slightly shifted in mobility. This difference suggested there was no comparable stabilization by calcium of a folded proGrl1p structure.

The relative chymotryptic sensitivities of proGrl1p vs. Grl1p were examined by repeating this experiment using lysates from MN173 cells, which contain both precursor and processed forms (Figure 12 lanes 5, 6). ProGrl1p underwent extensive digestion at chymotrypsin concentrations that caused only minor digestion of the processed protein (Figure 12 lane 5). To identify intermediate digestion products of proGrl1p, we incubated SB281 lysates with a lower concentration of chymotrypsin (Figure 12 lane 8). The addition of calcium to these reactions appeared to destabilize these products, since they accumulated only in the absence of calcium (Figure 12 lanes 8, 9). Similar results were obtained with MN173 cells (Figure 12 lanes 12, 13). The precise intermediates formed were some-

what variable between experiments, and may reflect variability in the ratio of chymotrypsin to substrate. In all cases these intermediates appeared to be destabilized in the presence of calcium.

We examined the reversibility of the apparent destabilization, using sequential additions of calcium and EGTA followed by chymotrypsin at a concentration that generated intermediate digestion products. In contrast to the irreversible calcium stabilization of mature Grl1p, the destabilization of precursor-derived intermediates was quantitatively reversed by addition of EGTA after calcium, and before chymotrypsin (Figure 12 lanes 10, 14). Taken together, these data indicate that proGrl1p differs from processed Grl1p both in its response to calcium exposure and in the reversibility of that response. Combined with evidence that the two forms also differ in their chymotrypsin sensitivity, these data are consistent with a model in which proGrl1p undergoes a change after processing, in a manner that affects its interaction with calcium.

DISCUSSION

The contents of vesicles along most membrane traffic pathways are generally considered as passive cargo. In contrast, the morphological changes during core formation of secretory granules have focused attention on the physical and biochemical conversion of cargo proteins. Numerous factors have been described that may influence granule protein condensation in the TGN and immature granule, including the regulated action of prohormone convertases. What is less clear is the actual mechanism of core assembly. Both assembly and dispersal reflect the coordination of a group of proteins in which individual species may have unique roles, entailing interactions with one another and with membrane proteins.

One approach is to identify a system in which a simple set of granule proteins can be individually analyzed. On the basis of results reported here, a substantially complete molecular catalog of the abundant proteinaceous granule contents in *T. thermophila* may be easily compiled. More than 80% of granule contents are derived by proteolytic processing of five ~40 kDa *GRL* gene products to produce polypeptides of approximately 12–25 kDa. Supporting this conclusion, a study of transcripts induced during periods of massive granule synthesis identified gene products already cloned by the reverse genetic methods described in this article, but no additional *GRL*-related transcripts (Haddad and Turkewitz, 1997)(Haddad, unpublished data). On the basis of the pattern of Coomassie Blue-stained bands after SDS-PAGE, three larger species of approximately 50 kDa, 80 kDa and 90 kDa constitute a large fraction of the remainder of the major granule contents. These appear less acidic and do not show in vitro calcium binding, suggesting that they are unrelated to the *GRL* products described here.

The *GRL* genes encode five preproproteins that are, to our knowledge, the only secreted proteins in *T. thermophila* for which sequences have been reported. The signal sequences conform closely to the established eukaryotic consensus (Von Heijne, 1985). The proteins are similar in a number of predicted features, including an abundance of aspartate and glutamate residues. Similar densities of acidic residues have been correlated with calcium binding in many proteins (Huttner *et al.*, 1991). This is specifically true for proteins in the lumen of the secretory pathway or exposed to the extracellular environment, where the high calcium concentrations are appropriate to the low-affinity high-capacity binding that is mediated by grouped acidic side chains. As expected, the processed Grl polypeptides show in vitro calcium binding. Abundant acidic proteins have been well-characterized in neuroendocrine dense-core granules and in the ER, where they can function as calcium buffers (Bastianutto *et al.*, 1995; Winkler and Fischer-Colbrie, 1992). In contrast, the calcium content of ciliate granules (measured in *Paramecium*) appears to be low (Schmitz *et al.*, 1985). Thus, calcium binding may occur only when the core proteins are exposed to extracellular calcium.

Three granule core proteins have been sequenced in a distantly related ciliate *Paramecium tetraurelia* (Gautier *et al.*, 1996). The amino acid sequences at sites of proteolytic processing have been highly conserved, strongly suggesting that the proteases themselves are also shared and were present in an early ciliate ancestor. A conserved tetrapeptide motif, if not involved in processing, must be associated with another common function during biogenesis or after secretion. In both organisms, the sequences predict formation of multiple α-helical coiled-coils over large stretches of the protein. Extensive coiled-coils may underlie the interactions in the condensed lattice and/or the extended filamentous structure of expanded lattices. This points out a specific ambiguity in structural predictions of core polypeptides. Since these may exist in more than one conformation, a 2° structure prediction may contain features of two differently folded structures.

Although the core proteins in the two ciliates have suggestive similarities, they are used to construct markedly different structures. The *Tetrahymena* core can be approximated by a 1 μm-long cylinder with rounded ends; the *Paramecium* equivalent is much larger (3 μm) tapered cone with a distinct "tip" extension from the wide end that specifies the docking site. What the structures have in common is that they are well ordered, and that they expand rapidly and in an ordered manner upon exocytosis. The simplicity of the *Tetrahymena* core suggests that a complex dynamic lattice can be largely determined by a small number of

proteins, whose individual functions can now be explored by gene disruption.

The lattice is constructed of processed fragments that may represent only a fraction of the original proGrlp structures. For proGrl4p, both amino and carboxyl-terminal fragments were identified, but for four of the five proGrlps, only single fragments were recovered. It seems unlikely that we are overlooking the additional products because of comigration of multiple polypeptides. First, 2-D gel analysis revealed only a single spots at any given molecular weight, with the exception of the smallest identifiable band in the preparations (Turkewitz, unpublished data)(Cole and Stuart, 1991; Maihle and Satir, 1986). Secondly, N-terminal sequencing produced unique sequences, except for that same band. The current data suggest that granule biosynthesis may involve either extensive degradation of some polypeptides or their sorting away from the maturing granule, both of which occur in neuroendocrine granules (Halban and Irminger, 1994). The first possibility suggests that *Tetrahymena* granules, like neuroendocrine granules, might contain small products as bioactive peptides, an issue that has not been examined.

The only demonstrated role for granule proteins in *Paramecium* or *Tetrahymena* is mechanical, in driving ordered postexocytic expansion. Rapid expansion is also a feature of metazoan granules, for example those in the oocyte cortex, which undergo exocytosis after sperm penetration. In *Paramecium*, exocytosis may be a defensive response to predation (Harumoto and Miyake, 1991). The speed of this expansion, occurring in tens of milliseconds (Knoll *et al.*, 1991), makes global protein rearrangements seem an unlikely mechanism. Grl1p and its precursor, for which previous genetic evidence suggested a role in expansion, are now shown to exist in at least three distinct states with regard to chymotrypsin sensitivity. We interpret these as alternative conformations. Other potentially informative assays, such as circular dichroism, are difficult to apply to a multicomponent lattice. Proprotein processing converts the protein from a protease-hypersensitive to a sensitive state, which is then converted to a protease-resistant state by exposure to calcium. Since chymotrypsin shows no activity against other granule proteins under these conditions, the data do not address the possibility of parallel transitions. It is possible that the difference in chymotrypsin sensitivity between Grl1p and the other Grlps reflects a genuine structural distinction, since both proGrl1p and the processed polypeptide are significantly longer than the others. Granules in cells lacking Grl1p showed some limited core expansion (Chilcoat *et al.*, 1996), indicating that Grl1p is an important but not unique element in this process.

Calcium and heat treatment had the same apparent effect on Grl1p structure, suggesting that calcium acts to promote a transition rather than to maintain the final state. This transition may be connected with granule expansion, since both are irreversible and triggered by high calcium. We imagine that Grl1p, along with other polypeptides in the granules, are essentially loaded springs, the stored energy of which is released by specific ionic changes after exocytic membrane fusion. An interesting mechanistic question is how such proteins are constrained to fold into metastable, "loaded" conformations during granule assembly. Folding pathways accessible to the proproteins may not permit folding into the most stable conformations accessible to the processed polypeptides. Although the compartments in which the transitions occur are different, the folding pathway of Grl1p may bear comparison with that of influenza hemagglutinin, which includes a long-lived intermediate that is kinetically determined (Baker and Agard, 1994). The transition of the viral protein to its final folded state is triggered as the virus enters a low pH endosome of an infected cell ((Bullough *et al.*, 1994), and references therein).

The assembly of an intracellular spring to be triggered by extracellular calcium presented a curious paradox, since all the core proteins in their proprotein forms are first required to pass through the ER and the TGN, which themselves contain high Ca^{2+} concentrations (Pezzati *et al.*, 1997). The concentration in mammalian ER appears to reach roughly 400 μM (Miyawaki *et al.*, 1997). The nature of such a spring, however, is that it can be released only once. One trivial explanation could be that ciliates are aberrant in that the entire secretory pathway contains only low levels of calcium. However, the role of the ER in cytosolic calcium homeostasis suggests that its capacity to store calcium is an essential eukaryotic feature (Montero *et al.*, 1995). In addition, *Tetrahymena* microsomes, like those of mammalian cells, will take up calcium in vitro in an ATP-dependent manner (Muto and Nozawa, 1985).

Part of the resolution to this paradox may lie in the difference between proGrl1p and Grl1p, in that the former cannot adopt a stable conformation in the presence of calcium. The proregion may prevent folding of the proprotein into the specific conformation favored by processed Grl1p in the granule lattice. This inhibition may be direct, or mediated by interaction with a second protein. Detergent-solubilized proGrl1p sediments as a soluble 8.5S complex, which may be a homo-oligomer (Turkewitz *et al.*, 1991). A function of proproteins in this system may therefore be to allow transport through the calcium-rich early secretory pathway. Once in the maturing granule, protein processing results in generation of the mature polypeptides that are incorporated into the core lattice. How then is processing regulated to avoid premature expansion? In the case of the conversion of proGrl1p to

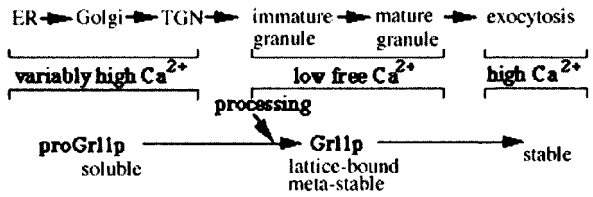

Figure 13. Model for compartmental regulation of Grl1p and core dynamics. The activity of proteases in maturing dense-core secretory granules cleaves the protein proregion. Low calcium may contribute to their activation. At least one domain of the processed protein, Grl1p, undergoes refolding to form a metastable structure that is an essential element of the granule lattice. Upon exocytosis, Grl1p is exposed to high extracellular calcium, inducing refolding to a fully stable structure.

Grl1p, one solution would be to make processing itself a calcium-regulated step: the protease(s) could be inhibited at calcium concentrations high enough to trigger expansion. Although pH might also be a regulatory factor, ciliate granules do not appear to be acidified (Garreau de Loubresse *et al.*, 1994; Lumpert *et al.*, 1992). A working hypothesis, presented in Figure 13, illustrates a putative mechanism for step-wise coordination of a set of interactions. More broadly, our results underscore the potential for programmed regulation for proteins encountering successive compartments of the secretory pathway.

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