Proprotein Processing within Secretory Dense Core Granules of *Tetrahymena thermophila*

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In the ciliate *Tetrahymena thermophila*, the polypeptides stored in secretory dense core granules (DCGs) are generated by proteolytic processing of precursors, and the mature products assemble as a crystal. Previous observations suggested that this maturation involves precise cleavage at distinct motifs by a small number of enzymes. To test these inferences, we analyzed the determinants for site-specific processing of pro-Grl1p (Granule lattice protein 1) by complete gene replacement with modified alleles. Contrary to the predictions of previous models, none of the component amino acids in a putative processing motif was necessary for targeted cleavage. Indeed, cleavage at a range of alternative positions near the native site was consistent with normal DCG assembly. Furthermore, substitution of other classes of processing site motifs did not perturb DCG structure or function. These results suggest that processing can be catalyzed by multiple proteases, for which substrate accessibility may be the prime determinant of site specificity. Consistent with this, inhibition of either subtilisin or cathepsin family proteases resulted in delayed processing of pro-Grl1p.

In some eukaryotic cells, a subset of proteins destined for exocytosis can segregate, condense, and then crystallize within vesicles called dense core granules (DCGs).1 Core assembly occurs during granule maturation, in which the best characterized feature is site-specific proteolytic processing of DCG cargo proteins (1). In pancreatic β-cells, pro-insulin is cleaved by subtilisin-related enzymes called prohormone convertases (PCs), to generate mature active insulin (see Ref. 2 and reviewed in Ref. 3). This is also linked to structural transitions; whereas proinsulin will dimerize in the trans-Golgi network and immature granule compartments, only processed insulin forms the crystal core of mature DCGs (4, 5). The PCs recognize and cleave substrates following paired basic residues, which may be presented in the context of defined local structures (6–8). The enzyme activities are sensitive to both pH and calcium concentration, with the result that processing occurs in specific sub-compartments of the secretory pathway (9). PCs are not the only class of enzymes involved in proprotein processing in secretory granules. A variety of less well characterized enzymes appear to play roles in metazoans (10, 11). Similarly, recent results (12–14) from studies of protozoa indicate that both serine and cysteine proteases may be involved in regulated secretory vesicle maturation.

The ciliate *Tetrahymena thermophila* has DCGs, termed mucocysts, whose cargo includes a family of polypeptides derived proteolytically from Grl (Granule lattice protein) precursors (15–17). An orthologous set of granule cargo proteins in *Paramecium tetraurelia* are the trichocyst matrix proteins (tmps) (18–21). In both organisms, the granule cores consist of highly organized protein crystals that undergo spring-like expansion upon exocytosis. This mechanism propels the granule cargo into the environs of the cells. Each *T. thermophila* cell contains several thousand DCGs, virtually all of which are docked at the plasma membrane, so cargo release upon exocytic stimulation is massive and synchronous (22). Proprotein processing within immature DCGs in ciliates appears closely linked with organization and function of the granule cores. First, only processed proteins can be detected within the crystallizing cores (23, 24). Second, defects in proprotein processing and core assembly are strictly correlated in mutants (25–28). Third, mutants defective in core assembly are also defective in rapid extrusion of DCG cargo upon exocytosis (29). Finally, while the precise structural basis for the spring mechanism is unknown, a conformational transition in processed Grl1p is correlated with expansion but does not occur in the proprotein (17). These observations suggest that, as in neuroendocrine cells, proprotein processing is an essential regulatory mechanism for assembly of functional DCGs in ciliates. However, neither the processing enzymes themselves nor the processing determinants within the proproteins are known. Candidates for the latter have been deduced by comparing Grl and tmp sequences.

As a family the Grls/tmps share less than 20% amino acid identity, but regions of limited sequence similarity flank the known proteolytic processing sites and have therefore been tentatively identified as targets for processing proteases (17, 28). At least some of the Grls/tmps are cleaved twice to yield two mature peptides, and a single proprotein can contain a different motif at each of two processing sites. The simplest model is that, as for proinsulin, each of the motifs is recognized by a distinct endopeptidase whose activity is regulated by substrate availability and compartmental conditions. Alternatively, a single enzyme may recognize multiple motifs but with different affinities (30).

We have now characterized the activities responsible for Grl1p processing *in vivo* by generating Grl1p variants with substitutions at residues surrounding the known cleavage site. By using complete replacement of the wild type gene with these modified alleles, we could test the relevance of the putative recognition motifs for processing site specificity and granule
assembly. To extend this analysis we asked whether the cleavage of different putative motifs was dependent on their position within the polypeptide. We demonstrate that cleavage does not depend on any residues in the putative processing motifs. The precise locus of cleavage can be subtly shifted in these variants, but this does not compromise the assembly or function of the resulting cores. These results, supported by protease inhibition experiments, strongly suggest that this system depends on multiple proteases that can collectively cleave at a wide variety of peptide bonds.

**EXPERIMENTAL PROCEDURES**

All reagents were from Sigma unless otherwise indicated.

**Cell Culture**—Cell cultures were gently swirled at 30 °C in SPP (2% proteose peptone, 0.2% yeast extract (both from Difco) with 0.009% ferric EDTA). T. thermophila strain CU428.1 (mp1 arrest) was provided by Peter Bruns (Cornell University, Ithaca, NY). The strain is wild type with respect to regulated exocytosis. Genetic nomenclature for T. thermophila can be found in Ref. 31. Site-directed Mutagenesis—In general, variants of Gr1p were generated by inverse PCR using as template pBluescript SK+ (Stratagene, La Jolla, CA) containing the KpnI-BglII fragment of the GRL1 genomic sequence. Expand High Fidelity Polymerase was used (Roche Molecular Biochemicals) followed by a 5-min polish with T4 DNA polymerase (New England Biolabs, Beverly, MA) to remove 3′ As. Several variants (details on primers accessible at home.uc.edu/~nelseide) were generated using the Quick Change Mutagenesis kit (Stratagene). Clones were sequenced across the entire insert using M13.F and M13.R primers and the Big Dye sequencing kit (Amersham Biosciences). Inserts were then shuttled into a vector containing GRL1 cDNA sequence carrying a silent mutation (C249G, T250C, A252G) that introduces a PstI site and 1100 bases of 3′- and 500 bases of 5′-genomic flank. The NEO2 cassette, which confers resistance to paromycin sulfate in T. thermophila, was inserted in the Xhol site 500 bases downstream. In cases where novel sequences were inserted, we chose the codons used most frequently in highly expressed T. thermophila genes (32). In the case of the exchange of the GRL1 sequence with those from GRL3, GRL5, and GRL7, we used the exact coding sequence from the respective genes.

**Transformation and Gene Replacement**—Particle-mediated transformation of T. thermophila was as described (33). Cells were bombarded with 15 μg of DNA digested with BamHI and ScaI and were recovered in SPP containing 100 μg/ml paromycin sulfate. Cell lines were transferred every 2nd day to media of increasing drug concentration with additional 100 μg/ml paromycin sulfate. Cell lines were recovered 15 min with 0.1 μCi/ml [3H]lysine (PerkinElmer Life Sciences) in DMC. Cells were then separated from the labeling medium by pelleting through a pad of 5% Ficoll in DMC with 2 mg/ml lysine and resuspended in DMC with 2 mg/ml lysine at 106 cells/ml. At each time point, 106 cells were withdrawn and lysed by addition of 1/4th volume of 5× NDET (NDET is 1% Nonidet P-40, 0.4% sodium deoxycholate, 66 mM EDTA, 10 mM Tris-HCl, pH 7.0) with protease inhibitors (0.5 μg/ml leupeptin, 12.5 μg/ml antipain, 10 μg/ml E-64, 10 μg/ml chymostatin). Samples were incubated for 1 h at 4 °C, after which the insoluble fraction was pelleted at 17,000 × g for 10 min. Supernatants were pre-cleared with Sepharose CL4B and then incubated with protein A-conjugated Sepharose that had been preincubated with anti-p40 antibody. For experiments done in the presence of protease inhibitors, cells were treated as above with the following variations. Following stimulation cells were allowed to settle to remove the small fraction of cells trapped in capsules. Cells were incubated for 1 h following stimulation in DMC supplemented with protease inhibitors as follows: 20 μM subtilisin inhibitor III (Calbiochem), 10 μM cathepsin inhibitor III (Calbiochem), 50 μM benzoyloxycarbonyl-Phe-Ala-CH2F (gift of Jim Miller, University of Chicago), and all subsequent steps prior to detergent lysis were done in the presence of inhibitors.

**Microscopy**—For electron microscopy, cells were fixed in 1.5% glutaraldehyde, 1% osmium tetroxide, as described (26). Indirect immunofluorescence images of regranulating cells were obtained by stimulating 50 ml of a 2-h starved culture with Alcian blue, as described above. Cells were pelleted and resuspended in 0.25% protease peptone, 0.5 mM CaCl2. The cells were allowed to settle for 10 min until a clear separation was visible between the settled blue-stained secreted material and free swimming cells that concentrated at the meniscus. These were collected and diluted to 20 ml with DMC. At each time point, 4 ml were pelleted and fixed in ice-cold 50% EtOH, 0.2% Triton X-100 for 10 min, pelleted, and resuspended in 0.5% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl. DCGs were detected using monoclonal antibody 4D11 (30), a gift of Marlo Nielsen and Joe Frankel, University of Iowa.

**RESULTS**

From earlier studies, pro-Gr1p is known to be cleaved following Lys329, and the surrounding region can be aligned with sequences flanking cleavage sites in two other Grl proproteins (17, 29). First, each cleavage site lies downstream of a small zone of basic residues (scheme in Table II). Short positively charged regions upstream of cleavage sites are conspicuous in proteins that are otherwise highly acidic. Close upstream of each cleavage site lies a cluster of hydrophobic residues at moderately conserved positions (Table I). The P’3 position (the residue 3 positions downstream of the cleaved peptide bond) is generally occupied by a hydrophobic residue, and there is always at least one hydrophobic residue in the first three positions following the cleavage site. Most positions significantly, the P1 position (that immediately preceding the cleaved peptide bond) is always occupied by a basic residue. Some or all of these shared features are potentially important for cleavage site specificity and are therefore indirect determinants of lattice assembly during granule biosynthesis. Alternatively, the role played by these features may be secondary to specificity based...
The N-terminal residue of the mature processed peptides, as determined by Edman degradation, are shown in boldface in A–C. Amino acids that are relatively conserved are boxed. A, alignment of sequences in which cleavage occurs following a single basic residue. B, alignment of sequences in which cleavage occurs following a glycine residue. C, alignment of sequences in which cleavage occurs following a single asparagine residue.

on accessibility in an otherwise tightly folded polypeptide. Only in the first instance would modifying these putative motifs be likely to interfere with pro-Grl1p processing and DCG formation.

To test the physiological importance of these features, we replaced all expressed (macronuclear) copies of GRL1 with variants altered in sequence at or around the cleavage site. In general, these variants of Grl1p, shown in Table II, fall into four classes as follows: 1) deletion of large regions around the general, these variants of Grl1p, shown in Table II, fall into groups altered in sequence at or around the cleavage site. In second instance would modifying these putative motifs be likely to interfere with pro-Grl1p processing and DCG formation.

The N-terminal residue of the mature polypeptide remained identical to that in the wild type, Glu189. This implied that processing takes place following Lys188, the identical residue targeted in the wild type protein. This result suggested that target site selection is not designated in any straightforward manner by the amino acid sequence upstream of the P1 residue and, in particular, that the basic region is not an essential determinant.

We therefore focused on the potential role of the single basic residue at the P1 position, by replacing Lys188 with a variety of other amino acids (Table II, lines 3–9). The conservative substitution of Lys188 with Arg had no effect, but replacement with either negatively charged or uncharged residues led to small shifts in the processing site. Substitutions by Ala (K188A) or Pro (K188P) shifted the processing site one residue downstream, to Glu189. Substitution with either Asp (K188D), Glu (K188E), or Trp (K188W) led to cleavage following Glu187. A basic residue in the wild type protein may therefore be part of the preferred substrate for the processing protease, but the locus of cleavage, as defined within the range of a single residue, must be determined by other features. A striking outcome of these results was the implication that processing, which takes place after a basic residue in the wild type protein, can also occur following negatively charged residues. In addition, a hydrophobic residue at the P1 position is acceptable.

One possibility was that charge density rather than the particular charges was important for site determination, since the wild type sequence is Glu187–Lys188–Glu189 and the variants above maintain at least two charged residues. Accordingly, we deleted both Lys188 and Glu189 (ΔLys188, Glu189) (Table II, line 10). Processing was also seen in this variant, producing a doublet of products whose mobility indicated that the cleavages had almost certainly occurred within the first three or four residues, none of which is charged. While the precise N termini were not determined for these products, results below directly demonstrate that a variety of downstream residues can be accommodated and confirm the premise that no flanking charged residues are required.

Replacement of Adjacent Hydrophobic Residues Leads to a Change in Site Selection.—Since the residues at, or immediately adjoining, the cleavage site are apparently not essential for specificity, we focused on potential structural cues. In particular, we asked whether flanking residues somewhat more distal to the cleavage site were involved in creating a 2° element, such as a flexible exposed loop, which could be targeted by a protease. As noted above, the cleavage site is flanked by nearby hydrophobic residues that could anchor and thereby define a short loop. We therefore replaced these residues both upstream and downstream of Lys188.

We substituted the downstream residues Tyr190 and Val191 with acidic residues to generate Y190D, V191E (Table II, line 12). This resulted in a minor change in processing specificity. We observed two products, which were generated by alternative cleavage following Glu187 and Lys189. To be certain that this change did not result from the presence of charged residues as opposed to the absence of hydrophobic residues, we...
replaced Tyr^{191}, Val^{192} with alanines (Y191A, V192A) (Table II, line 11). Similar cleavage products were obtained. These results suggest that if local 2° structure orients cleavage specificity, it does not depend on these downstream residues. However, the presence of two alternatively processed products hinted that these residues might stabilize a local structure.

The upstream hydrophobic residues Leu^{185} and Leu^{186} were similarly replaced with Ala (L185A, L186A) (Table II, line 13). In this variant, processing occurred following Ala^{184}. Cleavage after an uncharged amino acid confirms that the range of acceptable P1 residues is quite broad. That the cleavage site was shifted four positions upstream suggests a role for these upstream hydrophobic residues in site selection. This is also consistent with the fact that cleavage after Lys^{188} was maintained in A161–187, as described above. In this protein, Lys^{188} follows immediately after Phe^{158}–Leu^{159}–Gln^{160}. As a result, hydrophobic residues occupy the P3 and P4 positions. This is equivalent to the wild type but unlike the L185A,L186A construct. In summary, these results indicated that most of the conserved features are not critical for processing at or near the wild type site. The largest deviation seen was a shift 4 residues upstream from the wild type site, resulting from substitutions at Leu^{185} and Leu^{186}.

In light of the finding that local modifications had little or no effect on site specificity, we considered the possibility that the signal for processing is distal in the primary sequence. Such a determinant could still lie nearby in the folded polypeptide. A signal of this type might be detected as an additional feature shared among the Grls/tmps. The most obvious candidate was a 3-residue motif, FLQ, that is found immediately upstream of the basic regions discussed above and that is the most highly conserved sequence element in these proteins (17). We therefore expressed Phe^{158}, Leu^{159}, Glu^{160}, in which these three residues were deleted (Table II, line 14). However, cells expressing this variant were indistinguishable from wild type with regard to DCG functions, and Grl1p processing occurred after Lys^{188} as in wild type.

### Table II

**GRL1 mutant alleles**

Modified sequences are aligned vertically with respect to the wild type sequence shown at the bottom. The arrow indicates the position of the peptide bond between Lys^{188} and Glu^{189} that is cleaved in the wild type. Sequenced N termini of the mutant proteins are shown in boldface. In cases where alternative cleavages generated two mature polypeptides, the sequence determined from the smaller product is underlined. N termini that were inferred based on gel mobility shifts are indicated in italics. The single exception is construct 10 (ΔLys^{188},Glu^{189}), for which we concluded that the cleavages occur somewhere within the 4 downstream residues following Glu^{181}, based on the mobility of the products. Names of constructs are based on the single letter code for amino acids.

<table>
<thead>
<tr>
<th>#</th>
<th>Construct</th>
<th>Sequence and N-Termini</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Δ161-187</td>
<td>Q---------------------D</td>
</tr>
<tr>
<td>2</td>
<td>Δ189-200</td>
<td>E---------------------D</td>
</tr>
<tr>
<td>3</td>
<td>K188R</td>
<td>REYVNA</td>
</tr>
<tr>
<td>4</td>
<td>K188A</td>
<td>AEYVNA</td>
</tr>
<tr>
<td>5</td>
<td>K188N</td>
<td>NEYVNA</td>
</tr>
<tr>
<td>6</td>
<td>K188P</td>
<td>PEYVNA</td>
</tr>
<tr>
<td>7</td>
<td>K188D</td>
<td>EDREYN</td>
</tr>
<tr>
<td>8</td>
<td>K188W</td>
<td>EWEYV</td>
</tr>
<tr>
<td>9</td>
<td>K188E</td>
<td>E==YVNA</td>
</tr>
<tr>
<td>10</td>
<td>ΔK188,E189</td>
<td>EKADN</td>
</tr>
<tr>
<td>11</td>
<td>Y190A,V191A</td>
<td>LTEMASITNEVQDA</td>
</tr>
<tr>
<td>12</td>
<td>Y190D,V191E</td>
<td>LEOVVTAYLA</td>
</tr>
<tr>
<td>13</td>
<td>L185A,L186A</td>
<td>LSSNRNDDI</td>
</tr>
<tr>
<td>14</td>
<td>ΔF158,L159,Q160</td>
<td>S---K</td>
</tr>
<tr>
<td>15</td>
<td>–4:E187,K188,E189</td>
<td>QEKELAL</td>
</tr>
<tr>
<td>16</td>
<td>+6:E187,K188,E189</td>
<td>LYNVADDEKEY</td>
</tr>
<tr>
<td>17</td>
<td>+6:L185,L186,E187,K188,E189</td>
<td>LYNVNLLEEKEYSVNP</td>
</tr>
<tr>
<td>18</td>
<td>GRL1-GRL3</td>
<td>SFLQKKNFN</td>
</tr>
<tr>
<td>19</td>
<td>GRL1-GRL5</td>
<td>TRLILYKTNYYQTLQ</td>
</tr>
<tr>
<td>20</td>
<td>GRL1-GRL7</td>
<td>ALLEKEYVNADYSVNP</td>
</tr>
</tbody>
</table>

The Processing Site Can Be Shifted by Translocation of Lys^{188} and Flanking Residues—An alternative strategy for asking whether the residues flanking Lys^{188} specify the cleavage site, or whether the site is determined largely by the position within the polypeptide, was to determine whether the site could be translocated to another position within the 1° sequence (Table II, lines 15–17). For this we deleted Glu^{187}–Lys^{188}–Glu^{189} and inserted the tripeptide both upstream and downstream. Guided by the hint that the downstream hydrophobic residue might help to stabilize a cleavage site structure, we translocated Glu^{187}–Lys^{188}–Glu^{189}, either four positions upstream (−4;Glu^{187}, Lys^{188}, Glu^{189}) or six positions downstream (+6;Glu^{187}, Lys^{188}, Glu^{189}), so that a hydrophobic amino acid
Sites Show Surprising Interchangeability—Identification of three classes of putative processing motifs suggested previously that the differential cleavage at these sites was a regulatory mechanism for core maturation. Because the results reported above, following extensive modifications at the Grp1p cleavage site, were not easily reconciled with this hypothesis, we tested the model directly by substituting the Lys188 motif in Grp1p with the other classes of motifs that are present within the Grl and tmp protein family. We replaced the region around the known cleavage site in Grp1p with the putative cleavage motifs from Grlp(Aa to Val189), Grlp(Val217 to Ser231), and Grlp(Val46 to Tyr500) (Table II, lines 18–20). In each case, the polypeptide transplanted from the donor corresponded to the sequence previously identified as conserved between at least two family members. The GRL1 gene was completely replaced by these constructs, and the chimeric proteins were stably expressed as determined by Western blotting. In all cases processing took place, yielding a single product that was packaged into DCGs (Fig. 2C). We conclude that such motifs were accessible to proteases when transplanted into a heterologous Grp1p context.

was present as the next residue. In these variants the sequence of amino acids remaining at the original cleavage zone is Leu-Ala-Leu-Leu-Tyr-Val, very dissimilar to the wild type. In cells expressing either −4:Glu187, Lys188, Glu189 or +6:Glu187, Lys188, Glu189, the mature polypeptide appeared as a doublet (Fig. 1B). In the former, N-terminal sequencing revealed that the predominant product resulted from cleavage following new amino acid 189. The absolute position of the cleaved bond within the polypeptide was therefore reasonably well conserved with respect to wild type. This conservation was striking in light of the fact that residue 189 in the variant is Leu, indicating that processing can also occur following a large hydrophobic residue. We conclude that the processing of Glu-Lys-Glu, with a downstream hydrophobic residue, is sensitive to the position of this peptide within the 1° sequence. It is clear that Glu-Lys-Glu is not necessary to specify the site of processing.

Consistent with this conclusion, downstream translocation of the same motif (+6:Glu187, Lys188, Glu189) also generated processed products whose mobility was very similar to the wild type. Although we did not determine the precise N termini of the bands in this closely spaced doublet, it is clear that cleavage did not occur within the EKE motif. These results led us to conclude that Glu-Lys-Glu is also not sufficient to specify the cleavage site. We therefore considered whether Leu185 and Leu186 might be part of a minimal site-determining motif, since their replacement with Ala induced a large shift in the size of the processed product. To examine this, we included these two residues in the sequence that was translocated. The sequence Leu185-Leu186-Glu187-Lys188-Glu189 was deleted from its native locus and re-inserted at position +6 following Asp189. Processing of this protein generated a doublet of products that corresponded to cleavage following Tyr189 and Val189. In this case, therefore, translocation of the putative target sequence led to a downstream shift in the cleavage site by 8–9 residues. However, cleavage did not occur at the peptide bond between Lys and Glu that is targeted in the wild type, even though this was close to the peptide bond that was hydrolyzed in the variant. This is consistent with the evidence that Glu-Lys-Glu is not sufficient to determine the cleavage site.

Because cleavage can occur for all the variants discussed above, we conclude that the precise cleavage of the wild type protein following Lys188 is not accounted for either by its ab-
The rates of processing were indistinguishable (Fig. 2, pulse labeling and immunoprecipitation to compare the rate of processing sites might be cleaved with different kinetics (30)). To examine this directly in our system, we used biosynthetic inhibitors. Band intensities are shown below B. These are displayed relative to the earliest "no inhibitor" sample, arbitrarily set at 100. The units are arbitrary.

The processed products of these chimeras were functionally indistinguishable from the wild type, in that neither DCG morphology (Fig. 4) nor exocytosis competence was affected. This strongly suggests that if the distinct sites present within the proproteins are indeed differentially cleaved in a manner that is important for DCG biosynthesis, the regulation of processing is unlikely to be based on the class of sequence at each site or on the specific proteases that are involved. The latter conclusion assumes that a processing motif, even in a novel context, is still targeted by the same enzyme. Although this was impossible for us to test directly, we could ask whether cleavage occurred at the same peptide bond. The motif identified in Gr1p and Gr15p, in which processing occurs between Gly and Thr, is the most clearly distinguishable from that in Gr1p. We found that processing occurred at the identical peptide bond when the 11-residue peptide from pro-Gr15p was translated to replace the region surrounding Lys8–98 in pro-Gr1p (Table II, line 19). In this case, the non-native cleavage motif appears sufficient to direct processing site selection. On the basis of these data, it is unlikely that processing by specific enzymes at functionally distinct sites is essential for DCG biogenesis.

In P. tetraurelia, it has been suggested that different classes of processing sites might be cleaved with different kinetics (30). To examine this directly in our system, we used biosynthetic pulse labeling and immunoprecipitation to compare the rate of processing of pro-Gr1p with that of the Gr1p-Gr15p chimera. The rates of processing were indistinguishable (Fig. 2, B and C), as judged by the disappearance of the proprotein. We cannot detect the processed products by immunoprecipitation due to their insolubility, making it impossible for us to quantify the processing efficiency. Nonetheless, the similarity in the steady-state accumulation of the mature products in wild type and variants suggests that processing, rather than degradation, is responsible for proprotein disappearance. These results suggested that the differences in primary sequence surrounding the cleavage sites do not play a major regulatory role in kinetics of T. thermophila DCG cargo processing. It should be noted that the core lattice structure in T. thermophila is simpler than that in P. tetraurelia, and it is possible that an additional level of regulation exists for processing of the P. tetraurelia orthologs. In addition, the time resolution of the assay is limited by the 5-min sampling time intervals and the 15-min labeling period.

These results demonstrated that an enzyme or enzymes were capable of processing a very wide range of substrates but did not rule out the possibility that some targets were relatively disfavored. Such differences could underlie the precise cleavage seen in wild type cells. Specifically, the kinetics of processing might be altered if a processing enzyme were being forced to cleave at a site less favorable for binding or cleavage relative to the wild type. We therefore estimated the rate of processing of a set of the pro-Gr1p variants, as above. By this measure, processing proceeded at a rate indistinguishable from the wild type in all cases. The extent of processing also appeared to be normal, since there was no substantial accumulation of precursor in any of the mutant strains (not shown).

**Inhibitors Specific to Both Subtilisin and Cathepsin Proteases Delay Gr1p Processing—**Shaw et al. (13) recently reported that regulated secretory vesicle (rhopt) formation in Toxoplasma gondii, an organism that like Ciliates belongs to the order Alveolata, was disrupted in the presence of inhibitors targeting subtilisin or cathepsin family proteases. To ask if related proteases might be involved in processing of pro-Gr1p during the formation of DCGs in T. thermophila, we biosynthetically pulse-labeled cells undergoing regranulation in the absence or presence of such inhibitors. As shown in Fig. 3A, inhibitors against both subtilisin and cathepsin family proteases caused a delay in the processing of pro-Gr1p. Combination of the inhibitors produced additive effects (Fig. 3B) demonstrating that it is likely that both subtilisin and cathepsin family proteases are involved in DCG maturation.

**Sequences Surrounding Processing Sites Are Important for Lattice Assembly—**As discussed earlier, proprotein processing and core assembly are tightly linked in T. thermophila DCGs, and correct assembly is essential to rapid extrusion of the granule cargo upon exocytosis. The similarity in the extent and apparent kinetics of processing between the variant and wild type Gr11 proproteins suggested that core assembly and extrusion in the variants should be identical to wild type. However, it was also possible that core assembly depended on features of the processing site other than its proteolytic cleavage. To address the issue of assembly, we examined core structures by electron microscopy. Function in bulk cultures was implicitly addressed via preparation of DCG cargo protein following dibucaine stimulation, as described above. To extend this to the level of individual cells, we evaluated cargo extrusion based on a visual assay. When wild type cells are exposed to the secretagogue Alcian blue, each cell rapidly discharges the contents of its several thousand docked DCGs (37). As a result, each cell becomes trapped in a robust proteinaceous capsule formed of those contents, easily seen by light microscopy (Fig. 5B). Capsule formation indicates extensive synchronous extrusion of DCG cargo but does not demonstrate that 100% of the DCGs have undergone exocytosis.

All but two of the mutants accumulated DCGs that were indistinguishable from the wild type in both shape and organization of contents (Fig. 4). The exceptions were A161–187 and +6:Leu185, Leu186, Glu187, Lys188, Glu189. The former contained cores with a variety of shapes and electron densities, in which no organized protein lattices were detectable. The defect in cells expressing +6:Leu185, Leu186, Glu187, Lys188, Glu189 was more subtle. The mutants contained DCGs with an elliptical or egg shape rather than the extended wild type rods. Nonetheless, the contents were organized in protein crystals as in the wild type.

In strong confirmation of predictions based on previous correlative studies, we found that defects in DCG morphology were associated with functional abnormalities. As judged by the Alcian blue assay, all variants with wild type DCGs were reproducibly equivalent to wild type in capsule formation (wild type = 91%; mutants = 88–98%, not shown). The two mutants

**Fig. 3. Processing of pro-Gr1p in the presence of protease inhibitors. A,** newly synthesized proteins were pulse-labeled with [3H]lysine for 15 min in the presence or absence of 20 μM subtilisin (Subt.) inhibitor III, 10 μM cathepsin (Cath.) inhibitor III, 50 μM benzoylxyloxybenzyl-Phe-Ala-CH₂F. Gr1p was immunoprecipitated from detergent cell extracts, resolved by SDS-PAGE, and visualized by autoradiography. Under these conditions only the pre-processed form is soluble and therefore detectable. Time 0 corresponds to the beginning of the pulse. B, cells were treated as in A, except combinations of protease inhibitors were used. Band intensities are shown below B. These are displayed relative to the earliest "no inhibitor" sample, arbitrarily set at 100. The units are arbitrary.
with aberrant DCG morphologies had exocytic defects. Cells expressing Δ161–187 showed no encapsulation following stimulation with Alcian blue. In contrast, cells expressing Δ116:Leu185, Leu186, Glu187, Lys188, Glu189 were initially encapsulated to the same level as wild type. However, within minutes ~75% of the mutant cells had escaped from their capsules, which appeared as empty shells, while virtually all wild type cells remained imprisoned. We hypothesize that rapid escape from capsules is due to a structural weakness in the capsules themselves and is related to the aberrant core structure in these mutants.

However, the quick-escape phenomenon might also be expected if the mutants contained fewer secretory granules or if exocytic release following stimulation was incomplete or delayed. To rule out these possibilities, we visualized DCGs in cells expressing +6:Leu185, Leu186, Glu187, Lys188, Glu189 by indirect immunofluorescence, both before and after stimulation, using a monoclonal antibody directed against a granule cargo protein unrelated to Grl1p. The cells showed an extensive array of docked DCGs at the cell surface (Fig. 5A), comparable to wild type. Furthermore, after stimulation the cells showed virtually complete degranulation, indicating that the extent of exocytosis was not decreased. We note that a subtle delay in exocytic release would not be detected by this assay, since the wild type response may occur in milliseconds while the assay measures exocytosis over a period of ~30 s.

Finally, we asked whether the assembly of abnormal lattices in Δ116:Leu185, Leu186, Glu187, Lys188, Glu189 proceeded at a reduced rate. Wild type cells, following degranulation, can synthesize a replacement set of DCGs within several hours (Fig. 5) (37). We made side-by-side comparisons of wild type and a variety of mutant strains by fixing cells at intervals following degranulation and detecting new DCGs by indirect immunofluorescence. Among the mutants, only the strain expressing Δ116:Leu185, Leu186, Glu187, Lys188, Glu189 was distinct from wild type, demonstrating a reproducible delay in regranulation (Fig. 5A). Since we detected no delay in proprotein processing in this mutant, the result suggests that assembly itself is delayed in cells expressing a Grl1p variant with a subtle shift in the cleavage site.

The rate of de novo DCG biosynthesis could not be determined for cells expressing Δ161–187. These cells demonstrated a pattern of immunofluorescence strikingly different from all other strains. The cell cytoplasm showed abundant punctate labeling unlike the wild type pattern (Fig. 5B). These puncta appear to be vesicles distinct from DCGs, since there was no hint of non-docked dense core bodies when cell sections were analyzed by electron microscopy (not shown). These cells therefore appear to accumulate DCG protein in at least two compartments, since they also contain the docked dense-core struc-

**Fig. 4. Morphology of DCGs in wild type (wt) and mutant cells.** Thin sections were imaged by transmission electron microscopy. With the exception of strains expressing +6:Leu185, Leu186, Glu187, Lys188, Glu189 and Δ161–187, mutant strains make DCGs that are indistinguishable from the wild type. Bar, 200 nm.
Fig. 5. Degranulation and regranulation of wild type and mutant cells. A and C, DCGs were visualized by indirect immunofluorescence using a monoclonal antibody directed against a DCG cargo protein p80. Cells were visualized before stimulation (t = 0 min) and at various intervals following the triggering of massive exocytosis by Alcian blue. A, wild type (wt) and 17 (17) (6:Leu185, Leu186, Glu187, Lys188, Glu189). The extent of degranulation of cells expressing the mutant protein is similar to the wild type, but the cells show a reproducible delay in the rate of regranulation. B, capsule formation resulting from Alcian blue stimulation, visualized by phase contrast microscopy. The upper image shows a wild type cell entrapped in a capsule formed from extruded DCG cargo. More than 90% of cells remain trapped at 5 min post-stimulation. After the same interval, the large majority of mutants expressing 17(6:Leu185, Leu186, Glu187, Lys188, Glu189), have escaped from their now-empty capsules, an example of which is shown in the lower image. C, wild type and 1(Δ161–187). Upper panel, wild type cells, as in A, show extensive degranulation followed by DCG replacement. Lower panel, in the mutant expressing Δ161–187, DCG cargo localizes chiefly to cytoplasmic puncta that are not docked at the plasma membrane and that do not respond to an exocytic stimulus. Bar, 10 μm.

DISCUSSION

There are two possible interpretations of the results presented here. The first is that a single enzyme is responsible for processing both wild type and variant Grl1 proteins. This would imply that the enzyme is highly promiscuous and also that the mechanism of site selection is relatively independent of the local sequence. Although protease inhibition suggests that more than one family of processing enzymes may be active in pro-Grl1p cleavage, one of these inhibitors could be acting on a regulatory protease involved in zymogen activation and therefore indirectly on processing. The second explanation is that the number of processing enzymes in this system is greater than previously suspected. While each protease may have a limited range of substrates, the collection is capable of cleaving a wide range of exposed peptide bonds. Cleavage following Lys188 might be catalyzed by a single enzyme, but other enzymes can act at nearby accessible residues. In this scenario, accessibility could sharply delimit the stretches of amino acids that are targets for a variety of enzymes. This is consistent with previous results from in vitro digestion of pro-Grl1p with chymotrypsin or elastase, each of which generated a species very similar in size to the in vivo product (17). This scenario is also consistent with a proposed model of the proprotein 3° structure based on sequence comparisons and 2° structure predictions (41), in which at least one of the processing sites lies within a linker between two tightly folded domains. The relative precision of processing site selection within a limited accessible zone, both in wild type and variants, may arise from competition between DCG endoproteases, each of which recognizes a narrow range of substrates. A simple hypothesis to explain these findings is that paralogous proteases, similar in their activity and regulation, have diverged at the active site pocket to collectively accommodate many substrates. Alternatively, more than one protease family may be directly involved.

In either case, the presence of multiple processing enzymes in T. thermophila DCGs could serve to generate small peptides that would not have been identified by the approaches used to date. Similarly, new DCG peptides continue to be detected even in well studied mammalian neuroendocrine cells (39). There is indirect evidence in ciliates that small peptide products may be generated, since we have never detected all the products of the GRL genes that are predicted based on known processing sites. Of the nine predicted products of the known GRL genes, we have detected only six (15). This is consistent with a significant fraction of pro-Grl proteins, including residues 19–188 of pro-Grl1p (1–18 composes the signal sequence; (29)), being degraded to small polypeptides. Such degradation may occur in part via amino- and carboxypeptidases, and indeed we cannot eliminate the possibility that the N termini of mature wild type Grl1p and/or variants thereof are generated by endoproteolysis and subsequent N-terminal trimming. This uncertainty does not compromise our conclusions that processing does not depend on a conserved motif and can occur following a wide range of residues.

We previously identified a gene encoding a cysteine protease, targeted to the secretory pathway, whose transcript abundance

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3 N. Bradshaw, unpublished data.
increases dramatically during massive regranulation. 4 This was a promising candidate for a DCG-processing enzyme, but disruption of the gene had no obvious consequences for lattice assembly. 5 However, the results reported here hint that the absence of any single processing enzyme would be unlikely to show a defect in DCG assembly, although it might lead to the generation of alternate polypeptides during granule maturation.

Our results demonstrate that cleavage at precise sites is not required for DCG biogenesis. We are left with the question of why the motifs at the cleavage sites in Grls and tmps have apparently been conserved. We found that gross changes in processing site selection can impair granule lattice formation and exocytic capacity, but cannot rule out the possibility that less dramatic alteration produces defects not detected by our assays. Alternatively, conserved sequences may reflect biological activities following exocytosis, rather than requirements during assembly or expansion. It is tempting to speculate that a collection of small peptides may play important roles in this organism.

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