Granule Lattice Protein 1 (Grllp), an Acidic, Calcium-binding Protein in *Tetrahymena thermophila* Dense-Core Secretory Granules, Influences Granule Size, Shape, Content Organization, and Release but Not Protein Sorting or Condensation

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**Abstract.** The electron-dense cores of regulated secretory granules in the ciliate *Tetrahymena thermophila* are crystal lattices composed of multiple proteins. Granule synthesis involves a series of steps beginning with protein sorting, followed by the condensation and precise geometric assembly of the granule cargo. These steps may to various degrees be determined by the cargo proteins themselves. A prominent group of granule proteins, in ciliates as well as in vertebrate neuronal and endocrine cells, are acidic, heat-stable, and bind calcium. We focused on a protein with these characteristics named granule lattice protein 1 (Grllp), which represents 16% of total granule contents, and we have now cloned the corresponding gene. Mutants in which the macronuclear copies of *GRL1* have been disrupted continue to synthesize dense-core granules but are nonetheless defective in regulated protein secretion. To understand the nature of this defect, we characterized mutant and wild-type granules. In the absence of Grllp, the sorting of the remaining granule proteins appears normal, and they condense to form a well-defined core. However, the condensed cores do not demonstrate a visible crystalline lattice, and are notably different from wild type in size and shape. The cellular secretion defect arises from failure of the aberrant granule cores to undergo rapid expansion and extrusion after exocytic fusion of the granule and plasma membranes. The results suggest that sorting, condensation, and precise granule assembly are distinct in their requirements for Grllp.

**Dense-core** granules are cytoplasmic storage vesicles for condensed secretory proteins (69). Their fusion with the plasma membrane with consequent secretion of the granule contents is triggered by extracellular stimuli (3, 16). This pathway has been most thoroughly described in vertebrate endocrine, exocrine, and neuronal cells, in which studies based chiefly on biochemical and cell biological approaches have demonstrated differences from the pathway of constitutive protein secretion at numerous steps (57). First, granule contents are efficiently sorted from other proteins in the secretory pathway (15, 48). A possible mechanism for sorting was initially proposed based on the appearance of large protein aggregates within the TGN of regulated secretory cells (29, 61, 85). Their formation suggested that granule proteins could coaggregate and thus segregate from other soluble proteins in transit. Neither the aggregate organization nor the molecular determinants that drive aggregation is understood. Various granule proteins show a tendency to aggregate in vitro under conditions believed to exist in the TGN (19, 38, 40, 46, 76, 90), namely mild acidity and high calcium (4, 20). In some cases, the aggregation of specific proteins is enhanced in the presence of others, leading to suggestions that specific coaggregation might be an important mechanism for sorting heterologous components to a developing granule (24, 39, 40). However, it should be noted that the physiological relevance of in vitro aggregation has been difficult to confirm. For example, the disruption of calcium gradients by ionophores did not interfere with granule protein sorting in a permeabilized cell system (17).

Granule protein aggregates are incorporated into vesicles budding from the TGN. The fusion of several such vesicles may form the initial immature dense-core granule, which subsequently undergoes a maturation process characterized by visible protein condensation and proteolytic processing (71, 82, 84, 85). This process is well illustrated by insulin storage granules. Insulin is synthesized and transported to the TGN as a prohormone (28, 60, 61). During granule formation, proinsulin is cleaved by at least
two enzymes in the prohormone convertase family (26, 79). The convertase activities are themselves regulated by proton and calcium concentrations, both of which increase during granule maturation (26, 62). The mature insulin product crystallizes to form a dense core. Crystals are compact and allow storage of macromolecules in an osmotically inactive state. They are also formed in some granules containing several distinct proteins (83), including those in ciliates (43).

Unicellular systems offer an additional approach to studying this pathway: the ciliated protozoa Tetrahymena and Paramecium, which contain well-characterized secretory granules, have been particularly useful for genetic analysis. The steps leading to granule formation in ciliates and vertebrate endocrine cells appear similar, including appearance of protein aggregates (64), coalescence of TGN-derived vesicles to form the immature granules (44), and the proteolytic maturation of granule proproteins (2, 23, 27, 87). After maturation, ciliate granules dock at defined sites at the cell cortex. Extracellular stimulation leads to membrane fusion, and secretion itself takes the form of rapid expansion of the condensed crystalline cores, which are thereby ejected from the cells in the form of large ordered aggregates (42, 44, 54, 65). In these features, ciliate granules resemble cortical granules in oocytes that undergo fusion and expansion after fertilization (75).

In ciliates, mutants defective in regulated secretion can be identified by eye, since the large released aggregates are visible at the single cell level (66, 80). By using biochemical and morphological markers, such mutants have been assigned to distinct phenotypic classes: defects in granule formation and/or accumulation, aberrant granule morphology, defective transport and/or docking, or insensitive membrane fusion upon stimulation (6, 9, 11, 36, 41, 52, 63, 73, 86, 87). A limitation is that the genetic lesions responsible for these defects have not yet been identified; a single exception is the link between calmodulin mutations and exocytosis in Paramecium (49). In that case, mutations in calmodulin were initially characterized based on motility defects and later shown to affect regulated exocytosis.

Using Tetrahymena thermophila, in which the granules are known as mucocysts, one can now use recently developed methods for mass transformation, gene disruption and replacement to evaluate the roles of specific granule proteins in vivo (32, 34, 47). This paper addresses unresolved issues in granule synthesis and function by using a combination of two novel mutants. In the first of these, identified after chemical mutagenesis, granules fail to dock at the plasma membrane but instead accumulate in the cytoplasm. While the molecular defect is not known, it appears not to be in the granules per se but in a cytosolic factor. The second mutant is the result of targeted disruption of the gene encoding an abundant, 40-kD granule core protein, which was identified as an acidic, calcium-binding, heat-soluble species (87). Also of interest was the fact that the Tetrahymena protein, originally called p40, appeared to undergo proteolytic maturation in a step potentially involved in dense core formation. This work, analysis of the individual and the double mutants allows us to investigate the roles of this core protein during protein sorting, granule maturation, and secretion.

Materials and Methods

All 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, Inc., Detroit, MI), with 0.003% ferric EDTA. Tetrahymena strains are designated by their micronuclear diploid genotype, followed by their macronuclear-determined phenotype in parentheses (14). The heterokaryon strain CU428.1, MPRR/MPRR(6-methylpurine-sensitive, VII) bears a dominant allele conferring 6-methylpurine resistance in the micronucleus and the corresponding sensitive allele in the macronucleus; strain B2086, mpr/mprr(6-methylpurine-sensitive, II) has the drug-sensitive allele in both nuclei. Both were kindly provided by Peter Bruns (Cornell University, Ithaca, NY) and are wild type with respect to exocytosis. The mutant strain MN173, MPRR/MPRR(6-methylpurine-resistant, V) was derived by Tim Soelter and Eric Cole (St. Olaf College, Northfield, MN), as a whole cell homoygote from strain CU428.1 after mutagenesis with nitrosoguanidine, using uniparental cytogamy (21). It was identified as exocytosis deficient because of its inability to produce visible pericellular capsules after stimulation with alcian blue; this assay has previously been used to identify a set of exocytosis mutants (12, 63, 80).

Nomenclature

Because no comprehensive genetic nomenclature for Tetrahymena thermophila has been uniformly adopted, this paper uses a nomenclature closely based on that used in Saccharomyces cerevisiae (77). GRL1 refers to the wild-type gene; GRL1::NEO2 designates the allele of that gene that has been disrupted by insertion of the NEO2 allele. Dominant and recessive alleles are indicated in upper- and lowercase, respectively. Granule lattice protein 1 (GrIlp) refers to the protein product of the wild-type gene.

Strain MN173 has not been genetically characterized and will be referred to in the text as doc- to indicate that its distinct phenotype is the accumulation of undocked granules, whereas the wild-type phenotype is Doc+. GRL1::NEO2 doc- designates disruption of the GRL1 locus in the MN173 background.

Protein Purification and Sequencing

Secreted protein was purified from a 200-ml exponential culture of cells stimulated with dibucaine as previously described (87) but with the following changes: the buffer was 10 mM Tris, pH 7.0, 1 mM CaCl2, and the protease inhibitors used were leupeptin (0.5 ,ug/ml), antipain (12.5 ,ug/ml), E-64 (10 ,ug/ml), and chymostatin (10 ,ug/ml). The purified protein was solubilized in SDS-containing sample buffer with 10 mM DTT at 65°C and fractionated by SDS-PAGE along with prestained molecular weight standards (BioRad Labs, Hercules, CA). After electrophoresis, the appropriate region of the gel was electrotransferred to a polyvinylidene difluoride membrane (BioRad Labs) and then stained with Coomassie blue R-250. The strong band at ~40 kD was excised. NH2-terminal sequencing was performed by the Protein Chemistry Laboratory, Washington University (St. Louis, MO). Protein concentrations were assayed using bicinchoninic acid (BCA) (Pierce, Rockford, IL). SDS-PAGE was performed according to Laemmli (51). Quantitation of Coomassie blue-stained gel bands was performed using a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Cloning of GRL1 cDNA and Genomic DNA

Based on known codon usage in Tetrahymena thermophila (55, with additional information provided by M. Gorovsky, University of Rochester, Rochester, NY), degenerate primers (Integrated DNA Technologies, Coralville, IA) were designed to amplify GRL1 via the PCR. The 5' primer 5'-GAATA (T/C)GT (T/C)AA (T/C)GC (T/C)GA (T/C)GA-3' corresponded to amino acid residues 189-195 of the precursor protein; the

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1. Abbreviations used in this paper: ECL, enhanced chemiluminescence; GrIlp, granule lattice protein 1; ORF, open reading frame; tmps, tri-chocyst matrix proteins.
3' primer 5'-CC (A/G)ATTTTC (A/G)GC (A/G)GTTGTCG-3' to residues 207-213. PCR was performed on a *Tetrahymena thermophila* Agt10 cDNA library generously provided by Tohru Takemasa (University of Tsukuba, Japan). The 30-μl reactions contained 10μl plaque-forming units in ~5μl SM buffer, 2.5 U Taq polymerase (Boehringer Mannheim Corp., Indianapolis, IN), 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 250 μM each dNTP, and 1 μl of each primer; 30 cycles each of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C. 0.5 min were performed. This amplification produced a faint band of the expected size, 75 bp, which was subsequently amplified using 1 μl of the primary product as template. This product was cloned into pCRII (Invitrogen, San Diego, CA).

A single intron was suggested by Southern blotting of restriction digests of genomic and cDNA using ClaI and EcoRI. The site of the insertion was mapped to an 80-bp region between (or within) the degenerate 3' primer and the EcoNI site. Using this information, primers were designed to border the intron: 5' primer: 5'-ACTATTAATAACTCGCTCTCCIT-3' and 3' primer: 5'-ATCCCTTTTCCTTACCTCTCT-3'. A fragment of the predicted size (718 bp) was amplified from total CU428.1 DNA (prepared as described above, 10 ng/μl) using the same conditions and program as above, and this product was subcloned into pCRII (Invitrogen, San Diego, CA).

Genomic sequences 5' of the cDNA were subcloned using inverse PCR (58) on genomic DNA digested with HindIII. The forward, 5'-CAGCACCAAACGCCAGGAA-3', corresponded to bases 52-34 relative to the start codon, and the reverse primer 5'-ACCAACCAAGCGTAAAGAAAGAAGAC-3' to bases 54-76. The PCR was performed on 200 ng of circularized genomic DNA using the same reagents and cycling times as above, but with 1.5 mM MgCl2 and 1.5 min of extension. These reactions produced a single product, which was the expected size (1.2 kb) by Southern analysis. This was cloned into pCRII.

A genomic subclone of GRL1 was obtained by amplifying total CU428.1 DNA (as above) using primers corresponding to the cDNA just 5' of the poly-A tail (5'-AAGTGAGAGGAGGAAACGGAAAGAC-3') and at the 3' end of the upstream sequence (5'-TCCACCTACATAGAACATCAGC-3'). An ~3 kb fragment was subcloned into pCRII as above, and the identity of the insert was confirmed by restriction analysis and partial sequencing.

The 75-bp PCR product was used to screen a cDNA phage library using standard techniques (72). A screen of 1.2 × 108 plaques identified 60 positives. 10 of these were analyzed and found to contain two insert classes of 1.2 and 1.5 kb, which had overlapping restriction maps. A 1.5-kb clone was cloned into pBlueScript SK (+) (Stratagene, La Jolla, CA).

**Southern and Northern Blotting Analysis**

The 1.5-kb cDNA was purified from vector and labeled with digoxigenin (Boehringer Mannheim Corp.) by randomly primed DNA synthesis. Genomic DNA was prepared as described (33). Southern blotting was conducted using the Genius system (Boehringer Mannheim), loading ~10 μg of DNA per lane.

RNA purification was performed according to Cathala et al. (18) with the following modifications: Pelleted cells were lysed with 5 M guanidine thiocyanate, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, 8 mM 2-mercaptoethanol, 1% (w/v) sodium dodecyl sulfate, and the supernatant was precipitated with 3 M LiCl and the pellet was washed once with 3 M LiCl and resuspended in 0.5% N-lauroyl sarcosine, 1 mM EDTA, 10 mM Tris, pH 7.5. After phenol/chloroform extraction, RNA was ethanol precipitated, washed, and suspended in water. RNA electrophoresis and Northern blotting were performed as described by Farrow (33). After electrophoresis on 1% agarose gels formaldehyde, RNA was transferred and UV-cross-linked to Nitrocellulose (SRL, Westboro, MA) nylon membranes. Blots were hybridized in 5x SSPE, 5x Denhardt’s, 50% formamide, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA, with 20 ng/ml probe concentration (~5 × 106 cpm/cm²), and visualized using a Phosphorimager (Molecular Dynamics).

**Mapping of RNA Ends**

The precise 3' end of the GRL1 transcript was mapped using primer extension, as described in the Promega Technical Bulletin (Promega, Madison, WI). Briefly, a 32P-labeled oligonucleotide primer corresponding to bases +15 through +11 (5'-TAATTCTTATATTCTGCGATTG-3') was annealed to 15 μg total RNA at 60°C for 20 min. The reactions were allowed to cool to room temperature for 10 min, at which point RNA-primer complexes were extended with avian myoblastosis virus reverse transcriptase (Boehringer Mannheim) for 30-90 min at 42°C and then ethanol precipitated and analyzed on a sequencing gel. Another primer corresponding to bases +52 through +33 (5'-CAGCACCAAACGCCAGGA-3') indicated the same 5' terminal. As a standard, sequencing using the same primers (but labeled with 32P) was conducted on a cloned genomic copy of GRL1.

**Sequencing and Sequence Analysis**

The initial 75-bp PCR product and the cDNA clone were sequenced using M13 primers. The complete cDNA sequence was obtained by constructing a deletion series using Exonuclease III and Mung Bean nuclease (72). Two deletion series, one from each end of the insert, were made so that both strands could be sequenced. The sequences of the intron and upstream region were obtained by primer walking. Sequencing was performed on plasmid or single-stranded DNA using the Applied Biosystems Taq DyeDeoxy Terminator Cycle Sequencing kit and an Applied Biosystem Model 373A automated sequencer (PerkinElmer Cetus, Foster City, CA). A sequencing primer set was generated with a 200 bp sequence (United States Biochemical, Cleveland, OH) and a manual sequencing apparatus. The cDNA sequence was determined with at least twofold redundancy over the entire open reading frame (ORF) and 5'UTR on each strand. The 12S bases directly upstream of the poly-A tail were sequenced on one strand with at least twofold redundancy. Three independently amplified clones of the intron and upstream region were sequenced on both strands with at least twofold redundancy. Protein secondary structure was predicted using the Lasergene software package (DNASTAR, Madison, WI). Alignment of the heptads between Grlp and Paramecium proteins was done by eye.

**Disruption of GRL1**

GRL1 was disrupted by replacement of the sequences −15 to +260 relative to the start codon with a construct comprised of the NEO gene (which endows resistance to neomycin/paromomycin) under the control of the histone H4 promoter and β-tubulin 3'UTR (34), for which the name NEO is proposed by J. Gaertig (University of Georgia, Athens, GA). Electroporation using the ECM 600 (BTX Inc., San Diego, CA) was conducted exactly as described on mating pairs of B2086 and CU428.1 (35). Initial drug selection was with 120 μg/ml paromomycin, added 6 h after transformation. Between 2 and 25 transformants were obtained per successful transformation. Because only some of the macronuclear GRL1 copies are replaced initially, cells were cultured with increasing concentrations of paromomycin for stringent selection of the GRL1::NEO allele. Several transformants were cloned and grown in increasing amounts of paromomycin (up to 1 mg/ml) for more than 80 generations. Three independent lines were analyzed further; they were phenotypically indistinguishable. Disruption of GRL1 in vegetative doc cells by the same construct was accomplished by biolistic transformation. Cells were starved overnight in 10 mM Tris, pH 7.5, and resuspended at 10 μg/ml cells in 10 μM Heps, pH 7.5. Gold particle microprojectiles (1 μM, BioRad Labs) were prepared according to Sanford et al. (72a). Rupture membranes, macro-carrier flying disks, and wire-stopping screens (Rumsey Loomis Machine Shop, Ithaca, NY) with a BioPac PDS-1000/0 Hz device (BioM Medics) (Courtesy of J. Narita, University of Illinois, Chicago, IL) were used, at settings of 27-28 Hz vacuum, 1/4 in gap distance, 8 mm macrocarrier travel, and a target distance of 9 cm, following an optimized protocol developed by Peter Bruns and colleagues (Cornell University, Ithaca, NY). Transformed cells were plated, and paromomycin was added to a final concentration of 120 μg/ml after 2 or 5 h. Resistant cells were isolated after 4 d. In two experiments, we obtained 527 and 685 transformants/shot.

**Isolation of Dense-Core Granules**

400-800 ml of GRL1 doc or GRL1::NEO2 doc cells were grown overnight to stationary phase and 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 Heps, pH 7.0, and the pellet was resuspended in 20 ml pad of 60% Percoll (Pharmacia LKB Biotechnology, Piscataway, NJ) in buffer A with gelatin. These tubes were centrifuged for 15 min at 27,000 rpm (model 7610; Beckman Instrs., Carlsbad, CA). Granules from GRL1 doc cells were harvested from a single distinct band near the tube bottom and were then diluted with buffer A without gelatin and pelleted.
for 5 min at 5,000 g. Granules from GRL1::NE02 doc" cells also appeared as a distinct though less intense band at a similar position.

Granules were also partially resolved from other cell fractions using Nycodenz gradients (GIBCO BRL, Gaithersburg, MD). 0.4 ml of cleared lysates in buffer A, prepared as above, were applied to 9 ml continuous Nycodenz gradients (top solution: buffer A with additional 50 mM sucrose; bottom solution: 45% Nycodenz in buffer A) and centrifuged for 90 min at 120,000 g. 0.5 ml fractions were collected.

**Electron Microscopy**

All reagents were from EM Sciences (Ply, 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. To visualize protein secretion, cells were starved and stimulated with alcin blue as described above and fixed 3 s later. 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 polypropylene, samples were embedded in EMBed 812. Sections were stained with uranyl acetate and lead citrate and viewed using an electron microscope (model CX100; JEOL USA Inc., Peabody, MA). Purified granules were fixed overnight as pellets coated grids and stained with 2% sodium phosphotungstate at pH 7.

**Western Blotting and Immunofluorescence**

The rabbit antiserum specific for Grlp and p80 and the mAb 4D11 against p80 have been previously described (86, 87). Antibody blotting was performed according to (87), and antibodies were visualized using the enhanced chemiluminescence (ECL) system (Amerham Corp., Arlington Heights, IL) and quantified using a computing densitometer (Molecular Dynamics). For immunofluorescence, cells were pelleted and fixed for 10 min in ice-cold 0.2% Triton X-100 in 50% ethanol. Blocking was for 30 min at room temperature in 0.5% BSA in Tris-buffered saline (0.9% NaCl in 20 mM Tris, pH 8.2), followed by pelleting and a 30-min incubation in 20% hybridoma supernatant in TBS. Cells were then washed three times for 5 min in BSA/TBS, followed by pelleting and a 30-min incubation in 0.1% rhodamine isothiocyanate-coupled rabbit anti-mouse IgG (Calbiochem-Novabiochem, La Jolla, CA). Purified granules were fixed overnight as pellets in 2% glutaraldehyde, 1% osmium tetroxide in buffer A, and processed as above. For negative staining, purified granules were adsorbed to carbon-coated grids and stained with 2% sodium phosphotungstate near pH 7.

**Results**

**Purification and Cloning of GRL1**

We stimulated *Tetrahymena thermophila* to induce exocytosis of dense-core granules and purified the proteins released into the cell supernatant. From these we isolated by SDS-PAGE a 40-kD protein, focusing on it because we had previously noted its in vitro calcium-binding activity (87). It is probably identical to a 34-kD protein noted by Maihle et al. (65). The purified protein was shown to be EYVNADDEYVNDPSTDGRTADEIGS, with the use of TAA to encode glutamine (45, 55). The proposed ORF is flanked by AT-rich sequences, and the 3' flank includes a sequence resembling a polyadenylation site (68) that is 73 bp downstream of the translation termination codon. Comparison with sequences obtained by amplification of genomic DNA revealed the presence of a single AT-rich 544-bp intron. The DNA and predicted amino acid sequences appear in Fig. 1.

**Sequence Analysis**

The sequence predicted by the ORF appeared to encode a precursor for Grlp in the form of a pre-proprotein. The predicted polypeptide begins with a canonical signal sequence for translocation into the endoplasmic reticulum. Cleavage of this precursor was predicted to occur after alanine 18 (88). The ORF then extends for 1,152 nucleotides, yielding a translation product of 384-amino acid residues with a calculated mass of 43.4 kD. The NH₂-terminal sequence, the intron sequence is underlined, a putative polyadenylation site (68) that is 73 bp downstream of the translation termination codon. Comparison with sequences obtained by amplification of genomic DNA revealed the presence of a single AT-rich 544-bp intron. The DNA and predicted amino acid sequences appear in Fig. 1.

**Figure 1.** Sequence of GRL1. The deduced amino acid sequence is shown below the corresponding oligonucleotide sequence. Features have been indicated as follows: For the nucleotide sequence, the intron sequence is underlined, and the transcription start sites are marked by asterisks. For the protein, the peptide sequence (obtained by Edman degradation) that identifies the amino terminus of mature Grlp is boxed; the predicted signal sequence is doubly underlined. These sequence data are available from GenBank/EMBL/DDB under accession number U72946.
peptide sequence obtained from the purified secreted protein appeared as an internal peptide beginning at glutamate 189, consistent with earlier evidence that the protein is derived proteolytically from a larger species (87). The calculated molecular mass of the mature protein is 24.3 kD. This was unexpected, since secreted Gr1lp migrates as a 40-kD protein by SDS-PAGE. Similarly, although the precursor has a calculated size of 43.4 kD, it migrates as a 60-kD species by SDS-PAGE. In theory, these disparities may be due to posttranslational glycosylation; however, no indication of this was found by endoglycosidase treatment or sugar-specific labeling (not shown). Moreover, glycosylation of *Tetrahymena* granule proteins appears to be very limited (5). Instead, the high negative charge on the protein is likely to explain the anomalous migration during SDS-PAGE, a phenomenon also noted in some acidic vertebrate granule proteins (89). Acidic residues make up 23% of the Gr1lp predicted sequence, leading to a predicted pI of 4.1 for the pro-protein and 4.0 for the mature protein. The pI of the mature protein has been estimated by isoelectric focusing at 4.0–4.2 (22); (Turkewitz, A.P., unpublished).

Structural predictions based on the translated sequence are shown in Fig. 2 A. Pre-pro-Gr1lp has no extended hydrophobic stretches except that found in the NH2-terminal presequence. Clusters of negatively charged residues are distributed throughout much of the protein, with notable exceptions in the signal sequence and two regions between amino acid residues 118–130 and 155–186. This second stretch contains a significant cluster of positively charged amino acids between residues 161–176. Interestingly, this cluster of single and paired basic residues is quite close to lysine 188, the site where proteolytic cleavage generates the mature protein. Most of the proprotein is predicted to adopt an α-helical conformation. Screening the protein data base for homologous sequences identified myosin family members and other proteins with coiled-coil domains (not shown). The homologies were confined to regions of Gr1lp containing coiled-forming heptads, as defined by the prevalence of apolar side chains at the a and d positions. Among these identified sequences was that of the T2-c protein present in the dense-core granule lattice of another ciliate, *Paramecium tetraurelia*. Granules in *Paramecium* are known as trichocysts, and T2-c is one of a family called trichynins, or trichocyst matrix proteins (tmps) (37, 78). Although the level of amino acid similarity between Gr1lp and T2-c was low (<20%), the alignment of the Gr1lp sequence with three tmps suggested significant structural similarity as indicated by the positions of the heptad motifs, particularly in the first half of the protein (Fig. 2 B and Discussion).

**Gene Structure and Transcription**

Northern blotting analysis of RNA indicated a single major mRNA transcript of 1.4 kb (Fig. 3 A). To determine

Figure 2. (A) The calculated translation product of the GRL1 gene was analyzed for charge distribution and for predicted structural features. (B) Sequence comparison of amino-terminal regions of Gr1lp and three *Paramecium* tmps. For Gr1lp, the sequence shown corresponds to the prorregion and ends 10 residues before the processing site that generates mature Gr1lp. Predicted signal sequences are darkly shaded. Four regions containing heptad motifs predicted to form coiled-coils are lightly shaded, and the expected positions for hydrophobic residues are indicated by dots. The alignment of tmps, and the labeling of heptad regions as A1, B1, C1, and D1, is based on Gautier et al. (37). A region rich in basic residues is also indicated, although the boundaries are not identical for the four proteins. Cysteine residues have been shaded or, if they are located within shaded areas, lightened.

Figure 3. Transcript analysis. (A) Northern blot of total RNA isolated from wild type (CU428.1), probed with *GRL1* cDNA as described in Materials and Methods, and detected using a Phosphorimager. (B) Mapping of the 5′ end of the *GRL1* transcript. The asterisk marks the lane containing products of a primer extension reaction, as described in Materials and Methods. The adjacent four lanes contain products of a sequencing reaction on cloned *GRL1* using the same primer. The three major bands, at positions -56, -53, and -49 relative to the start codon, are indicated at the right.
the 5' end of the mature transcript, primer extension was used to map the start sites to positions -49, -53, and -56, relative to the translation start site (Fig. 3 B). Such clusters of start sites have been observed for other *Tetrahymena* genes (70). Results of Southern blotting analysis using a GRL1 probe against genomic DNA were also consistent with a single macronuclear gene copy (Fig. 4 B).

**Targeted Disruption of GRL1**

We transformed wild-type cells with the construct shown in Fig. 4 A, which contains a copy of the neomycin-resistance gene in an independent transcription unit, replacing bases -15 to +260 of GRL1. Transformation with this vector should lead initially to disruption of one or more macronuclear copies of the GRL1 gene via homologous recombination (34). Growth of transformed clones in increasing concentrations of paromomycin resulted in selection of cells with progressively increasing replacement of GRL1 by GRL1::NE02 copies, through a process called phenotypic assortment (13). After ~50 generations, Southern blotting analysis suggested that all endogenous macromolecular copies of GRL1 had been replaced by the allele containing the disruption cassette (Fig. 4 B). The residual faint band probably corresponds to the intact micronuclear copy of GRL1, which is transcriptionally silent. Southern blotting analysis with a probe derived from NE02 demonstrated that recombination had occurred only within the endogenous GRL1 locus (not shown). Most importantly, no Gr11p accumulation was detected by Western blotting analysis of whole cell lysates, which would have detected <1% of normal protein levels (Fig. 4 C). A second disruption strategy was also used, in which the NE02 cassette was inserted at the EcoNI site. The results were identical. At least two independent clones were tested for each experiment, to assure that the results were not the consequence of unrelated secondary mutations induced during transformation. These GRL1::NE02 cells had no detectable defects except for those related to granule biosynthesis, described below.

**Granule Morphogenesis in the Absence of Gr11p**

The distribution of dense-core granules in wild-type *Tetrahymena* is highly characteristic and can be visualized using an mAb against a second identified granule protein, p80 (86). The p80 immunofluorescence pattern of GRL1::NE02 cells appeared identical to that of wild-type cells (Fig. 5 A, first row). Since this precise wild-type pattern of granule localization reflects the processes of granule synthesis, targeting, and docking, we infer that all three of these processes persist in the cells lacking Gr11p. The pattern also suggested that p80 accumulated only in a granule compartment, in the presence or absence of Gr11p. For further support, we sedimented postnuclear supernatants

![Figure 4](https://example.com/figure4.png)

*Figure 4.* Genomic structure of the *T. thermophila* GRL1 gene and Gr11p expression in control and transformed cells. (A) The gene conferring neomycin (and paromomycin) resistance, inserted between the histone H4 promoter region and the β-tubulin 3' region (34), was inserted into the 5' end of a genomic clone of GRL1 as shown. The orientation of the NEO gene is indicated by an arrow. (B) Genomic DNA was prepared from GRL1, GRL1::NE02, and GRL1::NE02 doc and digested with ClaI. Southern blotting using GRL1 cDNA as probe was performed as described in Materials and Methods. Successful targeting resulted in elimination of the bands at 1.1 and 5.9 kb, concurrent with the appearance of a band at 8.2 kb. (C) Western blot analysis of transformants. GRL1 and GRL1::NE02 cells (four independent transformants) were washed once and resuspended in 10 mM Hepes, pH 7. Whole cell lysates were prepared containing 1% SDS and 1 mM DTT, and protein concentrations were determined to permit equal loading for SDS-PAGE. 60 μg total protein was loaded for GRL1::NE02 samples and for a single sample of GRL1 cells. In addition, dilutions of the wild-type sample containing 30, 15, 12.5, 3, and 1 μg total protein, were loaded in the successive adjacent lanes. After electrophoresis, samples were transferred to nitrocellulose and separately blotted with polyclonal antisera recognizing Gr11p and p80 and then visualized using ECL. Film was exposed for roughly 20 s. At longer exposures, a signal for Gr11p could also be detected for the lane corresponding to 1 μg of GRL1 lysate, but not in GRL1::NE02 lanes.
Figure 5. Appearance of secretory granules in GRL1 and GRL1::NEO2 cells, in both the CU428.1 (wild-type Doc+) and MN173 (mutant doc-) backgrounds. (A) Indirect immunofluorescence. Cells were fixed and visualized by indirect immunofluorescence using an mAb recognizing the granule protein p80. Each pair shows two views of the same cell, focusing on tangential and sagittal sections. In the Doc+ background, both GRL1 and GRL1::NEO2 cells show similar patterns of punctate cortical immunofluorescence, corresponding to docked granules. In the doc background, both GRL1 and GRL1::NEO2 show little surface immunofluorescence but substantial punctate cytoplasmic immunofluorescence, corresponding to nondocked granules. (B) Thin section of docked granule in Doc+ GRL1. G, granule; PM, plasma membrane. (C) Thin section of docked granule in Doc+ GRL1::NEO2. Granules lacking Grllp are smaller, rounder, and do not contain a visible ordered matrix. Bars: (A) 10 μm; (B and C) 500 μm.

of GRL1 and GRL1::NEO2 lysates on Nycodenz gradients and compared the distributions of p80 by Western blotting analysis of gradient fractions. The distributions were superimposable and represented by a single peak (not shown). Thus, the absence of Grllp does not result in detectable missorting of p80 to a nongranule compartment or the accumulation of a granule population with altered density. This is in contrast with other characterized mutants in granule morphogenesis, which accumulate presumed granule intermediates of a buoyant density less than that of the wild-type mature granules (87, and Turkwitz, A.P., unpublished).
Although properly positioned, the docked granules present in the GRL1::NE02 cells were morphologically aberrant. Wild-type granule cores have a characteristic rod shape, with typical dimensions along the long and short axes of 0.90 ± 0.1 and 0.19 ± 0.02 μm (n = 9) (Fig. 5 B). Granule cores lacking Gr1lp resembled slightly deformed spheres with well-defined borders and had respective dimensions of 0.33 ± 0.02 and 0.27 ± 0.02 μm (n = 33) (Fig. 5 C). As in wild-type granules, the cores were highly condensed, and in many cases they did not fill the vesicle lumen. A notable difference in the organization of wild-type and mutant granule contents could be seen in electron microscope thin sections. Granules in wild-type cells contain an ordered protein lattice (Fig. 5 B) (43, 81). This could be seen even more clearly in purified granules that were negatively stained (see Fig. 7 C). In contrast, no lattices were ever seen in the spherical granules of GRL1::NE02 cells in thin sections (Fig. 5 C). Similarly, isolated spherical granules showed no internal lattice by negative staining (not shown).

**Effect of GRL1 Disruption on Granule Function**

When wild-type cells were stimulated with alcian blue, granule discharge resulted in formation within seconds of a thick flocculent coat surrounding the cells, composed of the released granule proteins (Fig. 6 A). Such rapid release is driven by the expansion of the granule lattice, which is thereby mechanically ejected from the cell (43). By optical microscopy, this protein coat has the appearance of a capsule in which the swimming cells are entrapped (12). In wild-type cultures, >95% of cells become encapsulated (80). When GRL1::NE02 cells were treated in an identical fashion, no capsules were formed, indicating that the absence of Gr1lp led to a defect in regulated secretion (not shown). This defect was also observed using a second secretagogue, dibucaine (not shown). The basis for this defect was investigated by electron microscopy. Thin sections prepared from cells fixed shortly after stimulation revealed that the spherical granules underwent fusion with the plasma membrane upon stimulation, but that release of the core proteins was clearly different from that of wild-type granules. No thick flocculent coat was present, but instead small clumps of loosely linked fibers were frequently seen surrounding cells (Fig. 6, B–D). A second feature of the stimulated mutant cells was the persistence of unreleased granule protein. Whereas lattice expansion drives the rapid extrusion of granule contents in wild-type cells, in the GRL1::NE02 cells a remnant of the granule core was frequently still visible within the profiles of fused vesicles (Fig. 6, B and C). This material appeared less electron dense relative to cores of unstimulated docked granules. This suggests that while some expansion of the granule contents had occurred, either its extent or its coordination was inadequate for normal secretion. Therefore, the secretary defect arising from disruption of GRL1 can be explained by a change in the pattern and/or timing of protein release from dense core granules.

**Effect of GRL1 Disruption on Granule Composition**

To determine the degree to which granule defects in GRL1::NE02 cells could be attributed to the absence of Gr1lp, we compared wild-type and mutant granules to see whether the single gene disruption had pleiotropic effects on granule composition. Granules had previously been prepared from wild-type cells (87), but the yield was limited by the attachment of most granules to plasma membrane docking sites. This problem was now circumvented by using strain doc−, one of a group of mutants that were recently selected based on the lack of a regulated exocytic response (Patterson, B., T. Soelter, E. Cole and A.P. Turkewitz, manuscript in preparation). In doc−, granules accumulate in the cytoplasm rather than at the plasma membrane (Fig. 5 A, second row). Evidence that the defect does not reside in the granules themselves was provided by experiments in which doc− cells were observed shortly after conjugation with wild type, when limited cytoplasmic mixing occurs (56). Under these conditions, dramatic phenotypic reversal occurred as the entire set of cytoplasmic granules was rapidly recruited to the normal membrane docking sites (Melia, S.M., and A.P. Turkewitz, unpublished results). Phenotypically similar mutants in Paramecium have been described (6, 9).

A highly enriched granule fraction was prepared from doc− homogenates (Fig. 7 B). The granule protein lattice was clearly visible by negative staining of isolated granules
**Figure 7.** Analysis of isolated secretory granules. (A) SDS-PAGE of granule proteins, visualized with Coomassie blue. A nearly identical set of proteins are found in granules (prepared as described in Materials and Methods) and in purified secreted protein. Lanes 1 and 2, two independent preparations of granules isolated from doc−, 60 μg protein/lane; lane 3, purified secreted protein from Doc+, 60 μg protein. (B) Thin section electron micrograph of enriched granule preparation from doc− cells. (C) Negatively stained isolated granule from doc−. A regular protein lattice is visible. Bars: (B and C) 500 nm.

(Fig. 7 C). We compared by SDS-PAGE the protein profile of this fraction to that of secreted granule proteins from wild-type cells (Fig. 7 A, lanes 1 and 2 vs 3). Major proteins in the two fractions appeared identical. We note that since the secreted granule fraction was isolated as a large multimolecular complex, the correspondence between stored and released species strongly suggests that all major granule proteins remained coassociated after exocytosis.

**Figure 8.** Comparison of protein content of granules from doc− and GRL1::NEO2 doc−. (A) Granules from GRL1 doc− and GRL1::NEO2 doc− cells were isolated and assayed for protein content. Aliquots containing 80 μg protein were electrophoresed on a 17.5% polyacrylamide gel, which was subsequently stained with Coomassie blue. Grl1p is indicated in the wild-type lane, and an asterisk marks a set of polypeptides that differ in the two samples. (B) Western blot analysis of enriched granule preparations from GRL1 doc− and GRL1::NEO2 doc−. Aliquots containing 10 μg protein were electrophoresed on a 12.5% polyacrylamide gel and Western blotted with polyclonal antisera against Grl1p and p80. Bound antibodies were visualized using ECL. Film was exposed for ~5 s; no Grl1p signal appeared in the GRL1::NEO2 lane at longer exposures. (C) Thin section electron micrograph of GRL1::NEO2 doc−, showing the cytoplasmic accumulation of undocked granules with no visible lattices. Bar, 500 nm.

To determine the composition of granules from cells lacking Grl1p, we created the double mutant GRL1::NEO2 doc− by transforming doc− cells with the disruption cassette shown in Fig. 4 A. As described for transformation of wild-type cells, we obtained several clonal lines with no detectable intact GRL1 macronuclear alleles (Fig. 4 B) or...
protein (not shown). Immunofluorescence suggested that undocked granules were located in the cytoplasm (Fig. 5 A, second row), and electron microscopy of cell thin sections confirmed that these had the same spherical appearance as granules in GRL1::NE02 cells (Fig. 8 C). The GRL1::NE02 doc− granules were gradient purified and the absence of GrIlp confirmed by protein staining as well as Western blotting analysis (Fig. 8, A and B). The gross composition was compared to that of the parallel fraction prepared from doc−, whose wild-type granules contain GrIlp (Fig. 8 A). Strikingly, nearly all of the other characteristic granule proteins were present at a relative abundance similar to those in wild-type granules. The exception was a difference between the wild-type and mutant granules in the cluster of low-Mr proteins (Fig. 8 A, asterisk). The mutant granules contained protein bands of similar, but not identical, mobility. These differences may reflect changes in granule-specific proteolytic cleavage, or alternatively may result from GrIlp-dependent sorting defects (see below). Differences in several minor bands were not consistent between preparations.

Discussion

The highly regular geometry of granule cores in wild-type cells reflects the orchestration of a set of proteins through a defined assembly program. It is easy to imagine that mutation or deletion of individual proteins in the ensemble could disrupt this process to various degrees, and granule maturation mutants that have been previously characterized in Paramecium (2) and Tetrahymena (87) may possibly involve defects in core proteins. Compared with these previously described cases, the GRL1::NE02 mutation is novel in two ways. First, it represents a defect in granule assembly for which the precise genetic lesion is defined. Secondly, this lesion appears to block completely the process of lattice formation without blocking granule accumulation, distinct from any previously characterized mutant strains. These include ten mutants in Paramecium (1, 70a) and six in Tetrahymena (Turkewitz, A.P., unpublished) that are marked by abnormal granule morphologies and/or partially ordered cores. However, it cannot be assumed that these mutants involve core proteins directly, since lattice assembly is implicitly dependent upon upstream steps in the secretory pathway.

A comparison between the pathways leading to granule exocytosis in wild-type and GRL1 cells is presented in Fig. 9. Granules lacking GrIlp, although containing no identifiable lattice, nevertheless have uniformly condensed cores with well-defined edges. This appearance is distinct from that of immature granules in wild-type cells, in which a more disperse aggregate fills the entire vesicle space (36). Protein condensation in granules lacking GrIlp is likely to proceed during maturation via local protein interactions that define a first level of granule organization and that are established independently of GrIlp-dependent lattice formation. Such local organization of granule proteins in the mutant granules is also suggested by the presence of sparse fibrillar elements in images of the expanded cores of GRL1::NE02 cells, which indicate that some polymer formation has occurred. These fibrils resemble subunits of the much denser meshwork of secreted granule cores from wild-type cells (42).

The appearance of short- but not long-range organization in granules in GRL1::NE02 cells suggests that GrIlp plays a specific role in lattice assembly. This role may involve a structural transition related to proprotein processing (2), as previously suggested by the finding that processing of pro-GrIlp correlates with a change in the physical...
state of the protein, from soluble to insoluble (87). We do not yet know with what other proteins Grllp interacts in the granule. If the protein lattice is built on multivalent interactions, a number of other granule constituents may also be essential to establish the characteristic architecture. One must then assume that none of the genes for such proteins is affected within the pool of \textit{Tetrahymena} or \textit{Paramecium} exocytosis mutants derived by chemical mutagenesis, since no comparable phenotypes have been observed. This issue can be addressed by targeted disruption of other \textit{Tetrahymena} granule protein genes.

Grllp is essential for normal granule function, as shown by the limited postexocytotic decondensation of granule cores in \textit{GRLI}::\textit{NEO2} cells. This may be explained as an indirect result of the failure to assemble a lattice, given that crystallloid granules have evolved in numerous ciliates as expandible mechanical devices involved in explosive protein secretion (43). However, preliminary results discussed below suggest that Grllp may play an active role in expansion. The various changes observed in granules of \textit{GRLI}::\textit{NEO2} cells cannot be due to gross missorting of proteins since most of the other wild-type granule components are present. The exceptions are several polypeptides that appear changed in granules from \textit{GRLI}::\textit{NEO2} cells, that could therefore also affect granule organization. It is noteworthy that these differences are restricted to the smallest identifiable granule proteins (<14 kD), which therefore appear likely to be proteolytic products. Preliminary results suggest that the major polypeptide that appears to be absent in \textit{GRLI}::\textit{NEO2} granules is disulfide-linked to Grllp in wild-type cells, and is likely to be derived from the Grllp proregion. The appearance of a small unique polypeptide in \textit{GRLI}::\textit{NEO2} granules may be due to aberrant processing of other granule proteins that occurs in the absence of lattice assembly.

Normal processing to generate mature Grllp occurs following lysine 188, and there is a roughly 16-residue basic region slightly amino-terminal to it. Similarly, in \textit{Paramecium}, a block of basic amino acids is located slightly NH$_2$-terminal to an identified cleavage site within a tmp precursor, but no basic residues are found at the site itself (37, 53). The proximity of these basic regions to the cleavage sites is suggestive, since prohormone convertases in endocrine granules recognize dibasic and possibly monobasic targets (7, 8, 28). If the ciliate processing enzymes are related to characterized endocrine prohormone convertases, they are likely to be regulated quite differently. In endocrine granules, low pH and high calcium are important in regulating convertase activity (26). In contrast, granules in \textit{Paramecium} do not appear to be acidified (36, 52a) and contain little calcium (10, 74).

Indeed, cytoplasmic granules may need to be actively maintained at low calcium concentrations, since calcium from the extracellular milieu can trigger granule decondensation after exocytosis (10). The targets for calcium binding and mechanism of expansion are unknown. However, calcium binds to Grllp in vitro, and recent data suggest that this triggers an irreversible conformational transition (Turkewitz, A.P., manuscript in preparation). Thus, calcium binding to Grllp in wild-type granules may be an important step in lattice expansion, which is similarly an irreversible process.

Homology searches of the protein database using the \textit{GRLI} sequence identified a \textit{Paramecium} dense-core granule lattice protein, T2-c, as well as other proteins with known coiled-coil structures, e.g., myosins. T2-c belongs to the tmp family of granule proteins in \textit{Paramecium}, which are synthesized as precursors encoded by a large number of closely related genes (53). \textit{GRLI} does not appear to belong to a gene family, based on DNA and RNA hybridization. In addition, several polyclonal anti-Grllp antisera did not cross-react with other granule proteins (Turkewitz, A.P., unpublished observations). The sequence similarity to \textit{Paramecium} TC-2 is too weak to provide firm evidence for evolutionary relatedness between Grllp and tmps, but we note that diversity among ciliates is enormous; the genetic distance between the two organisms is similar to that between animals and plants (67). Nonetheless, physico-chemical similarities may suggest similar roles for Grllp and tmps in formation of extensible lattices. Tmp precursors are roughly the same size as pro-Grllp and are similarly hydrophilic and $\alpha$-helical (37). A folded structure for tmp precursors has been proposed based on sequence analysis. It consists of two bundles of four-stranded anti-parallel helices in which each short helix is identified by its heptad repeat motifs (37). Such motifs are also a feature of the predicted Grllp sequence, leading us to compare the distribution of heptads with those in tmps. The introduction of a single 28-residue gap in the Grllp sequence allowed close alignment of four heptad-defined regions with those in tmps, over a total length of 120 residues. This alignment also brings into near register the basic regions of the proteins, which may be functionally related as targets for recognition by converting enzymes. This comparison suggests structural similarity between amino-terminal domains of granule protein precursors in \textit{Paramecium} and \textit{Tetrahymena}.

Analysis of the carboxy-terminal halves of the proteins, however, was less conclusive. Again, Grllp is strongly predicted to form coiled coils, but only by the introduction of multiple gaps in all sequences could we align heptads (not shown). There is also a significant difference between Grllp and tmps in the ratio of charged to apolar residues, which in some cases may be diagnostic of short vs. extended helix formation. For three tmps, the ratios are 0.55, 0.73, and 0.74 respectively (37). That ratio for the proregion of Grllp is 0.75, but for mature Grllp it is 1.16, a ratio similar to that of extended $\alpha$-fibrous proteins (25). The position of the intron at the boundary between the proregion and the mature protein may suggest an origin for Grllp as a fusion between a tmp-like protein, now represented in the proregion, and a more extended $\alpha$-helical protein.

Specific sorting of granule proteins is believed to depend upon aggregation intermediates in the TGN. One way to assess the interactions within these aggregates is to ask how well-defined perturbations might change the composition of the sorted product. This study showed the deletion of a single protein constituting 16% of normal granule content appeared to have relatively minor effects on the allocation of other granule proteins but a dramatic effect on granule function. We infer that the set of protein interactions involved in sorting and condensation is not identical to that present in the mature granule. Although Grllp is a major lattice element, specific coassociation with Grllp
does not appear to be an essential sorting step for granule proteins in general. If Grl1p in wild-type cells does play an important role in sorting/aggregation, that role must be compensated for by another core protein in the GRL1::NE02 cells. Several of the small (<25 kDa) polypeptides in Tetrahymena DCGs are acidic and bind calcium in vitro and are thus candidates for such a role (Turkwitz, A.P., unpublished). None of these, however, appears to be over-expressed in the GRL1::NE02 cells.

Several models have been considered to account for sorting in the absence of specific heterotypic interactions: (a) Primary sorting could depend upon homoaggregation, with extensive reorganization in the maturing granule. This could account for the two distinct classes of immature granule vesicles seen in another ciliate, Pseudomicrothorax (64), and also appears consistent with formation of distinct classes of secretory granules in Aplysia neurons (31). (b) If the determinants for heterointeractions were common to multiple granule components, sorting would proceed in the absence of Grl1p based on equivalent interactions. (c) The majority of secretory protein traffic may be nonselectively directed to a post-TGN immature granule-like compartment, from which nongranule proteins would then be removed. This negative sorting could involve specific receptors or be the consequence of passive exclusion from a condensing core. This last model has been proposed for insulin-secreting cells and would appear efficient for such a system, in which granule proteins represent the bulk of secretory traffic (50). This issue has not been examined quantitatively in ciliates. Extension of the current approach in Tetrahymena, a biochemically and genetically manipulable system, should help to define individual vs. collective roles of proteins in the regulated secretory pathway.

We thank William Szychinski for technical assistance, John Perrino and Michael Lewis for EM, Paul Gardner for DNA sequencing, and Julie Feder for assistance with confocal microscopy. We gratefully acknowledge the help and advice of Alan Schwartz (Washington University) for protein sequencing, and of Steve Wiekert for RNA isolation. Our thanks also go to the help and advice of Alan Schwartz (Washington University) for protein sequencing, and of Steve Wiekert for RNA isolation. Our thanks also go to Jacek Gaertig and to Jody Bowen and Martin Gorovsky. Condi-

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