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Analysis of a Mutant Exhibiting Conditional Sorting to Dense Core Secretory Granules in *Tetrahymena thermophila*

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ABSTRACT

The formation of dense core granules (DCGs) requires both the sorting of granule contents from other secretory proteins and a postsorting maturation process. The *Tetrahymena thermophila* strain SB281 fails to synthesize DCGs, and previous analysis suggested that the defect lay at or near the sorting step. Because this strain represents one of the very few mutants in this pathway, we have undertaken a more complete study of the phenotype. Genetic epistasis analysis places the defect upstream of those in two other characterized Tetrahymena mutants. Using immunofluorescent detection of granule content proteins, as well as GFP tagging, we describe a novel cytoplasmic compartment to which granule contents can be sorted in growing SB281 cells. Cell fusion experiments indicate that this compartment is not a biosynthetic intermediate in DCG synthesis. Sorting in SB281 is strongly conditional with respect to growth. When cells are starved, the storage compartment is degraded and *de novo* synthesized granule proteins are rapidly secreted. The mutation in SB281 therefore appears to affect DCG synthesis at the level of both sorting and maturation.

EUKARYOTIC cells secrete proteins by a route that begins at the endoplasmic reticulum, continues through the Golgi, and is then completed in membrane-bound transport vesicles that link the trans-Golgi network (TGN) to the plasma membrane. Fusion of vesicles with the plasma membrane, the event that actually results in protein secretion, is constitutive in the sense that it does not require any specific extracellular signals. A second route, called regulated secretion, is distinguished by the fact that proteins undergo release from distinct secretory vesicles [called dense core granules (DCGs)] in a fashion that depends on extracellular stimulation (Almers 1990). Regulated secretion is employed in a wide range of processes. In vertebrates, it underlies the long-range transmission of nerve impulses and serum glucose homeostasis, among numerous other functions. Cell types with a capacity for regulated exocytosis also demonstrate constitutive secretion (Kelly 1985). Therefore, at least two different populations of vesicles, with different protein compositions and activities, must be maintained in the same cytoplasm. The pathways leading to constitutive *vs.* regulated secretory vesicles diverge at the level of the TGN where proteins bound for constitutive vesicles *vs.* DCGs must begin sorting from one another (Arvan and Castle 1998).

DCGs may be the only major cellular organelle for which the mechanism of sorting, either for lumenal (cargo) or membrane proteins, has not yet been clearly determined (Tooze 1998). No sorting receptor has been unambiguously identified, and the only consensus is that selective aggregation of DCG cargo proteins in the TGN is likely to play a critical role (Glombik *et al.* 1999). Some cargo proteins may also have important interactions with specific lipid domains in the TGN (Tooze *et al.* 2001). It is not clear how aggregation of cargo proteins leads to the formation of a specific vesicle with characteristic morphological features and, presumably, a specific complement of membrane proteins. Moreover, it is an open question whether specific cytosolic machinery at the level of the TGN is required for DCG formation.

Ciliates as a group, and *Tetrahymena thermophila* and *Paramecium tetraurelia* in particular, offer unique model systems for examining these phenomena because of the possibilities for genetic analysis (Orias and Bruns 1976; Ruiz *et al.* 1976; Orias *et al.* 1983). Tetrahymena cells contain an impressive array of DCGs docked at the plasma membrane (Allen 1967a). DCG synthesis and exocytosis are dispensable for laboratory growth, which has made it possible to isolate cells with nonconditional mutations in this pathway that leave the essential constitutive pathway unaffected. Such mutants have been generated by chemical mutagenesis (Orias *et al.* 1983; Melia *et al.* 1998), gene disruption (Chilcoat *et al.* 1996), and antisense gene silencing (Chilcoat *et al.* 2001). Among the most mysterious and therefore potentially informative mutant strains are those that appear to be entirely defective in DCG formation, as the analysis of such mutants may present an opportunity for understanding the mechanisms involved in DCG biogenesis. Currently, two nonallelic mutations in Tetrahyema strains named SB281 and UC1, respectively, result in

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failure to produce DCGs (Orias *et al.* 1983; Melia *et al.* 1998). It will be essential to identify the affected genes and to understand the phenotypes themselves. Cloning by complementation is not yet available in Tetrahymena, but this article provides a detailed analysis of the SB281 phenotype. With the exception of the ciliates, the number of mutants in this pathway that have been characterized is very limited, the most notable being a pheochromocytoma cell culture line (PC12) that fails to synthesize DCG proteins (Corradi *et al.* 1996).

We and others have previously analyzed SB281 using a variety of criteria. The nitrosoguanidine-induced mutation is recessive and segregates as a single locus (Gutierrez and Orias 1992). SB281 cells do not process DCG proproteins to their mature forms (Ding *et al.* 1991; Turkewitz *et al.* 1991) although specific proteolytic processing is a distinctive feature of DCG formation in ciliates as well as in many other systems (Docherty and Steiner 1982). Since proprotein processing occurs shortly after exit from the TGN, this defect suggested that the primary defect in SB281 lies in a very early step in granule biogenesis. Significantly, somatic complementation assays implied that the defective factor was diffusible (Sauer and Kelly 1995; Melia *et al.* 1998), an important result because it provided the only evidence in any system that unique cytosolic factors might be necessary for DCG formation.

The most telling aspect of the SB281 phenotype was that a newly synthesized DCG proprotein was rapidly secreted rather than being stored as in the wild type (Turkewitz *et al.* 1991). Since pathways leading to regulated *vs.* constitutive secretory vesicles are considered to be alternative streams diverging from a common pool, the rapid release of proteins normally stored in DCGs appeared to likely reflect their shunting from the TGN to this parallel pathway. However, there is no direct evidence to rule out the possibility that DCG proteins are secreted via a DCG-like pathway that has, as a result of the SB281 mutation, taken on constitutive features. The formation of a DCG-like pathway is consistent with the observation that SB281, under some conditions, appears to retain rather than secrete newly synthesized cargo proteins (Turkewitz *et al.* 1991). The organelle in which these proteins accumulate had eluded characterization by previous methods, including the important question of whether it represents a stable compartment and, if so, whether it is an intermediate in DCG synthesis.

In experiments reported in this article, we have reexamined several key issues. Analysis of double mutants, with an expanded set of markers, provides independent confirmation of several earlier conclusions that were based on cell biological criteria. We have also used direct cell fusion to test conclusions that were based on cytosolic complementation between conjugating cells. Our results provide important details regarding the precise phenotypic defects in SB281 and shed light on the nature of the causative mutation.

MATERIALS AND METHODS

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

Cells and cell culture: *T. thermophila* strains (see Table 1) CU428.1 and A*III were kindly provided by Peter Bruns (Cornell University). SB281, SB210, and SB283 were provided by Eduardo Orias (University of California, Santa Barbara). Strains MN173, MN175, and MN171 have been described (Melia *et al.* 1998). Cells were grown at 30° with agitation in either SPP (1% proteose peptone, 0.2% dextrose, 0.1% yeast extract, 0.003% ferric EDTA) or PPYS (2% proteose peptone, 0.2% yeast extract, 0.009% ferric EDTA) unless otherwise indicated.

Strategy for the creation of double-mutant strains: The numbers refer to the steps illustrated in Figure 1:

1. Individual clones from a MN173 × SB281 cross were screened for the encapsulation [caps(+)] phenotype when challenged with the secretagogue Alcian blue (Turkewitz *et al.* 2000).
2. After 3 weeks of continuous growth the clones had reached sexual maturity, and cell lines with nonparental mating types were identified. A proportion of these clones lost the caps(+) phenotype during this period due to the phenomenon called phenotypic assortment (Doerder *et al.* 1992), which refers to the tendency of mitotically dividing cells to become homozygous at all loci.
3. The caps(−) clones were then individually crossed to the A*III strain in a genomic exclusion mating (Allen 1967b).
4. The clones with F_1 macronuclei were then crossed to both of the parental strains. Those that did not produce any caps(+) progeny when crossed to either of the parental strains were identified as the lines possessing double-mutant micronuclei. As a control, the heterokaryons were crossed to a nonparental exocytosis deficient mutant (MN171) to demonstrate that the cell lines were fertile and capable of complementing other mutations.
5. The double-mutant micronuclei were then brought into macronuclear expression by mating the F_1 macronucleus heterokaryons with their corresponding A*III macronucleus siblings from the genomic exclusion mating. The double-mutant genotype of these strains was then verified by doing another round of backcrosses. Consistent with the expected results of genomic exclusion, approximately one-fourth of the heterokaryons contained double-mutant micronuclei.

The strategy to obtain the MN173,SB281 double mutant was an improvement over the strategy used to obtain the MN175,SB281 double mutant, which was more labor intensive. The major difference was that the F_1 clones from the MN175 × SB281 mating were not screened to isolate caps(−) cells following phenotypic assortment. As a result, the progeny arising from the backcrossing of the heterokaryon strains to the parentals had to be isolated into single drops and tested for mating-type immaturity before performing the Alcian blue stimulation test. Macronuclear expression of the double-mutant genotype was accomplished by two rounds of mating to an A* strain and the genotype was verified by another round of backcrosses.

Screening techniques and other genetic methods: Unless otherwise stated, incubations were at 30°. Large-scale matings were done according to Hamilton and Orias (2000), who also describe single-pair and single-cell isolation. In the first

TABLE 1

Tetrahymena strains

Strain	Phenotype	Mutagen	Mutation	Drug resistance	Source
Cu428.1	Caps+	—	—	Mpr/Mpr (mp-s)	P. J. Bruns
SB210	Caps+	—	—	2-dgal/2-dgal (dg-s)	E. Orias
A*	Caps+	—	—	Wild type	S. L. Allen
MN171	Caps−	Nitrosoguanidine	Recessive	Mpr/Mpr (mp-r)	Our laboratory
MN173	Caps−	Nitrosoguanidine	Recessive	Mpr/Mpr (mp-r)	Our laboratory
MN175	Caps−	Nitrosoguanidine	Recessive	Mpr/Mpr (mp-r)	Our laboratory
SB281	Caps−	Nitrosoguanidine	Recessive	2-dgal/2-dgal (dg-r)	E. Orias
SB283	Caps−	Nitrosoguanidine	Recessive	2-dgal/2-dgal (dg-r)	E. Orias

Strains that are wild type for regulated exocytosis as determined by Alcian blue stimulation are indicated as caps+ while mutant strains that do not secrete after stimulation by Alcian blue are indicated as caps−. Mpr and mp, 6-methylpurine; 2-dgal and dg, 2-deoxygalactose.

round of screening for double-mutant micronuclei, the clones were transferred to 96-well plates and grown to stationary-phase cell density in 100 μl SPP and then replicated using a screw-type replicator into another 96-well plate that contained 100 μl/well DMC [a one-tenth dilution of Dryl's media (1.7 mM sodium citrate, 1 mM NaH_2PO_4, 1 mM Na_2HPO_4, 1.5 mM $CaCl_2$) with an additional 100 μM $MgCl_2$ and 0.5 mM $CaCl_2$]. After 24 hr, an approximately equal number of SB281 or MN173 cells that had been starved overnight (at room temperature) in DMC were distributed in the 96-well plate. Eight hours later the plate was scored for mating pairs, and then 100 μl of PPYS was added to allow the conjugating cells to grow. On the next day, the plate was replicated into a second 96-well plate with 100 μl/well of DMC. After 24 hr, the plates were screened for capsule formation by adding Alcian blue (Turkewitz *et al.* 2000). Those clones that tested positive for F_1 macronuclei and double-mutant micronuclei in the first screen were subjected to a second more rigorous screen, which also served to verify the genotype of the double-mutant strains after they had been brought into macronuclear expression. The cell lines to be mated were grown to log phase and then starved overnight in DMC at room temperature. Equal cell numbers were then mixed for mating. Each clone was individually mated with SB281, MN173, and MN171. After 8 hr, an equal volume of SPP was added; 24 hr later, 50 μl of cells from each well was transferred to 1 ml DMC. After overnight starvation, 50 μl of cells from each well was transferred to a 96-well plate containing 50 μl/well of Dryl's and then tested for capsule formation.

Immunofluorescence: Cells were grown to log-phase density ($0.5–1.5 \times 10^5$/ml) in SPP, and then 2 ml were centrifuged for 30 sec in a clinical centrifuge and washed once in 10 mM HEPES, pH 7.0, at room temperature. The tubes were gently tapped to loosen the pellet, placed on ice for 1 min, and then diluted with 3 ml of ice-cold 4% paraformaldehyde in 50 mM HEPES, pH 7.0. After 20 min, cells were pelleted and washed twice in ice-cold 50 mM HEPES, pH 7.0, and then permeabilized in 3 ml of ice-cold 0.1% Triton X-100 in 50 mM HEPES for 8 min. The cells were then washed twice in ice-cold 50 mM HEPES, pelleted, and then warmed to room temperature for all subsequent steps. Cells were incubated in 3 ml blocking solution [1% bovine serum albumin (BSA) in TBS: 10 mM Tris, pH 7.5, 154 mM NaCl] for 15 min and then pelleted and resuspended in 50 μl of a 20% v/v solution of 4D11 hybridoma supernatant in blocking solution for 20 min. 4D11 was the kind gift of Marlo Nelson (University of Iowa) and is specific for the DCG protein p80, as previously described (Turkewitz and Kelly 1992). Following three 3-ml washes with 0.1% BSA in TBS, cells were resuspended in 100 μl of a 1% solution of Texas-red coupled goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for 30 min, washed, and suspended in 5–10 μl of antifade reagent consisting of 5% n-propylgallate, 10 mM HEPES, pH 7.0, and 90% glycerol. Approximately 6 μl of cells were immobilized on a slide with a coverslip and viewed under a Zeiss Axiovert microscope interfaced with a Zeiss LSM 510 confocal laser system and software or with a Zeiss Axiovert scope and standard fluorescent optics.

Density gradients and Western blots: Homogenates of cells grown to log phase ($1–2 \times 10^5$ cells/ml) in SPP and then starved for 24 hr in DMC were prepared in a ball-bearing homogenizer using buffer A (20 mM HEPES, pH 6.9, 36 mM KCl, 300 mM sucrose, 2 mM $MgCl_2$, 2 mM EGTA) in the presence of protease inhibitors (leupeptin, 1 μg/ml; antipain, 12.5 μg/ml; chymostatin, 10 μg/ml; E64, 10 μg/ml) as previously described (Turkewitz *et al.* 2000). To fractionate the homogenates, 800 μl was overlaid on 11.2 ml of a continuous Nycodenz gradient [bottom solution: 58.5% nycodenz A.G. (GIBCO BRL, Gaithersburg, MD), 10 mM HEPES, pH 7.0, 1 mM EGTA, pH 7.0, 1 mM $MgCl_2$, 0.02% gelatin; top solution: same as bottom with 280 mM sucrose in place of Nycodenz]. The gradients were spun overnight (14–18 hr) at 4° in a Beckman SW41Ti rotor at $105{,}000 \times g$ and harvested as 12 1-ml fractions. A sample of each fraction was dissolved in sodium dodecyl sulfate (SDS)-containing sample buffer [final concentration 62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 0.005% bromophenol blue, 50 mM dithiothreitol (DTT)] for 8 min at 100°. The starvation period was found useful for decreasing occasional degradation of proGrlp during sample preparation. In similarly fractionated homogenates of growing cells, the distribution of proGrl1p was also identical in the double mutants and the SB281 single mutant. This experiment was performed three times on growing cells and once on starved cells (shown in Figure 3), and each trial gave the same result.

To prepare whole cell lysates, cells were pelleted and washed twice with 10 mM HEPES, pH 7.4. Protease inhibitors (as above) were added prior to addition of SDS-containing sample buffer. Protein concentrations were determined with bicinchonic acid (Pierce, Rockford, IL); for such samples, cells were dissolved in 100° 1% SDS and aliquots withdrawn for protein assay before addition of SDS-PAGE sample buffer. All samples were resolved by SDS-PAGE along with molecular weight standards (GIBCO BRL). Antibody blotting with antiserum to dense core granule marker Grl1p was as previously described

(Turkewitz *et al.* 1991), and the blots were developed on a phosphor screen (Molecular Dynamics, Sunnyvale, CA).

Pulse chase and immunoprecipitation: To look at total proteins secreted in logarithmic cultures, cells were pelleted and resuspended in DMC at 10^6/ml. Labeling was for 5 min with 0.2 mCi/ml of [^{35}S]methionine + cysteine (*trans*-^{35}S-label; ICN Biomedicals, Irvine, CA). Cells (1 ml) were then pelleted through an underlaid pad of 3% Ficoll in DMC and resuspended in 1 ml of growth medium supplemented with 2 mg/ml of cysteine and methionine. At 20-min intervals, 250-μl aliquots were withdrawn, and the cells were pelleted into an underlaid Ficoll pad. Solution (200 μl) above the interface was withdrawn, and 80 μl were combined with 20 μl 5× SDS-PAGE sample buffer containing DTT and incubated at 100° for 3 min. Samples were then analyzed by SDS-PAGE followed by autoradiography.

For immunoprecipitation of secreted Grl1p, cells from log-phase cultures were resuspended and starved for 2–3 hr in DMC at room temperature. A total of 2.5×10^6 starved cells were labeled for 20 min with 0.35 mCi of [^{3}H]lysine (NEN Life Science Products, Boston) in a volume of 2.5 ml. Cells were then pelleted through a Ficoll pad (5% Ficoll in DMC + 2 mg/ml lysine) and resuspended in 2.5 ml DMC + 2 mg/ml lysine and protease inhibitors. At specified time points, 0.5×10^6 cells were withdrawn per time point and the cells separated from the culture supernatant using a Ficoll pad, as above. The supernatant and cell fraction samples were then processed for immunoprecipitation (Turkewitz *et al.* 1991).

Cell fusions: Cells were grown to a density of $2–4 \times 10^5$/ml in SPP (immunofluorescent images) or to early log phase $0.5–1.0 \times 10^5$/ml [green fluorescent protein (GFP) images)] and fused by a procedure similar to that of Gaertig and Cole (2000) and Cole *et al.* (2001). Cells were washed twice in 50 mm sucrose and 0.1 mm $CaCl_2$ and resuspended at 10^6/ml. Equal cell numbers were mixed and 20 μl was placed in a BTX (San Diego) Microslide Electrode P/N 450 (0.5-mm gap) fusion chamber. Fusion was in the BTX ElectroCellManipulator 2001 with the following settings: AC voltage, 17 V; AC duration, 5 sec; DC voltage, 70 V; DC pulse length, 0.50 msec. This cycle was repeated two to three times. Thirty seconds after fusion, cells were transferred to 1 ml SPP +/−25 μg/ml cycloheximide. After 2 hr at room temperature, cells were processed for immunofluorescence as above or visualized using a Zeiss Axiovert microscope with standard fluorescent and Nomarski optics. GFP labeling of the DCG content protein Igr1p was accomplished by transforming SB281 with a replicative vector (Gaertig *et al.* 1994) that contains the *IGR1* gene fused to GFP at its C terminus (A. Haddad and A. P. Turkewitz, unpublished results).

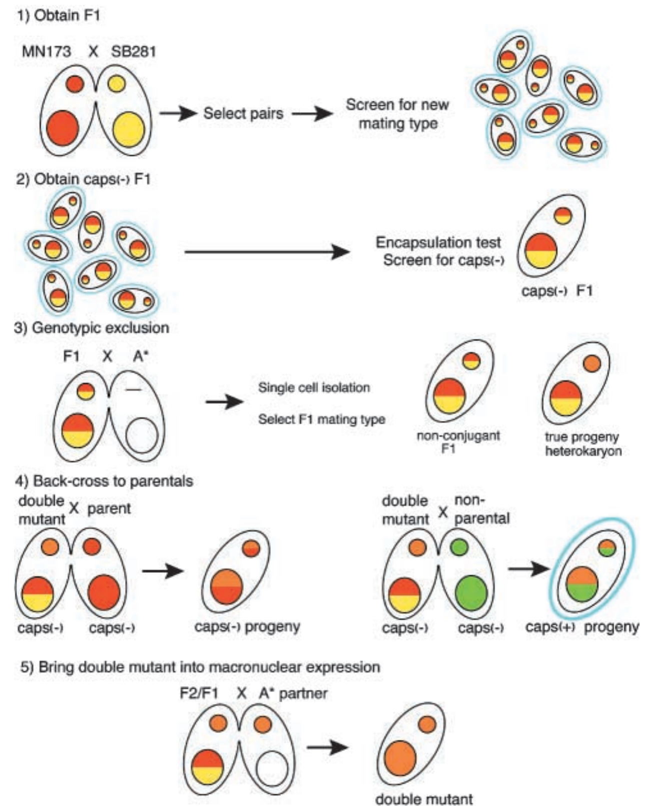

Figure 1.—Strategy for the creation of double-mutant strains, as described in materials and methods. Individual cells are represented by an oval shape, and a mating pair is shown as two ovals fused together. Cells that are capable of DCG exocytosis are surrounded by a blue capsule and are labeled "caps(+)," while cells with an exocytosis-deficient phenotype are labeled "caps(−)." Normal cells contain two nuclei, which are represented by colored circles in this diagram. The macronucleus is represented by the larger circle at the bottom of the cell, and the micronucleus is represented by the smaller circle. Homozygous nuclei are solid colored, while heterozygous nuclei have multiple colors. Note that heterozygous macronuclei will eventually become homozygous because of phenotypic assortment.

RESULTS

Wild-type Tetrahymena show dramatic release of DCG contents in response to exocytic stimulation with the polyanionic dye Alcian blue. SB281, MN173, and MN175 were initially isolated as defective in the secretion of DCG contents by this assay. On the basis of the specific defects, as judged by biochemical and morphological characteristics, we inferred that the mutants fell in an ordered pathway, with SB281 upstream of MN173, which was in turn upstream of MN175 (Melia *et al.* 1998). This ordering was based in large part on the observations that SB281 failed to synthesize recognizable DCGs, while MN173 made apparently normal DCGs, which were nonetheless defective in localization to the plasma membrane. DCGs in MN175 showed correct localization but were nonetheless unresponsive to stimulation by Alcian blue. In order to use such mutants to map a pathway it was important to confirm these assignments using genetic criteria by establishing the epistatic relationships in double-mutant strains.

The procedure used to obtain the MN173, SB281 and MN175,SB281 double mutants is described in materials and methods and outlined in Figure 1. We used several criteria to compare the double mutants with the parental single-mutant strains to ascertain whether the SB281 mutation is epistatic to those in MN173 and MN175.

Phenotypic comparison of double *vs.* single mutants: *Proprotein processing:* In wild-type cells, the abundant protein Grl1p undergoes processing from an ∼40-kD proprotein to an ∼20-kD mature product that is stored in DCGs (Turkewitz *et al.* 1991). (Note that, probably due to the strongly acidic nature of this protein, these two polypeptides appear as ∼60 and ∼40 kD when ana-

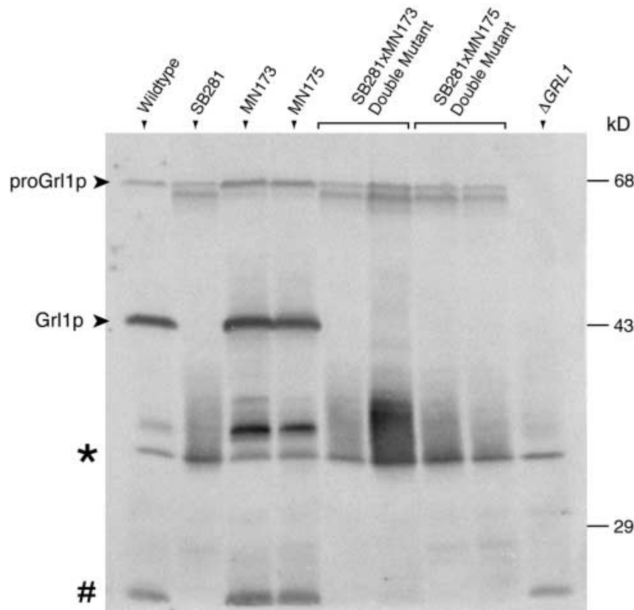

Figure 2.—proGrl1p processing in wild-type and mutant strains. Western blots of whole-cell lysates (60 μg of protein loaded in each lane), using the αGrl1p antibody that recognizes Grl1p at 40 kD and its precursor, proGrl1p, at 60 kD. The processing defect in SB281 is also apparent in the double-mutant strains. Cross-reactive bands are indicated (*, #) in the lysate (ΔGRL1) taken from cells in which *GRL1* gene expression has been eliminated by the removal of the start codon along with a large portion of the coding sequence (Chilcoat *et al.* 1996). The double-mutant lanes contain lysates from independently isolated cell lines. Molecular weight markers are indicated to the right.

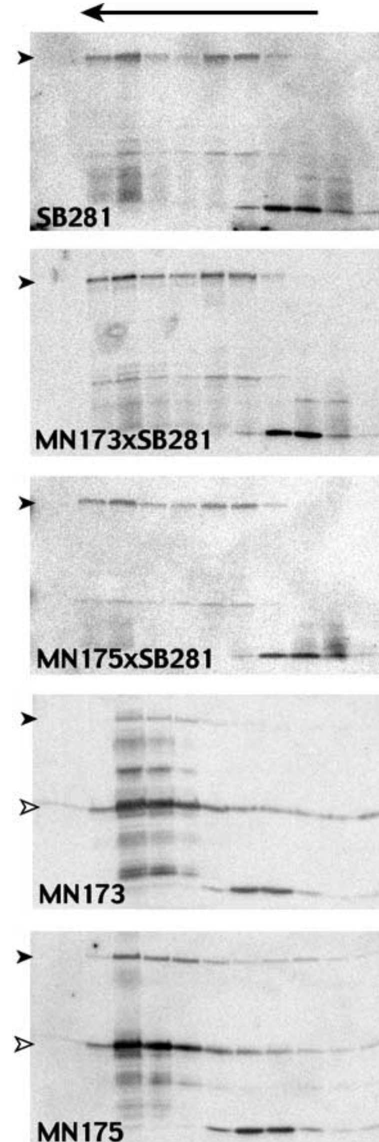

Figure 3.—Equilibrium density distribution of proGrl1-containing fractions in single-mutant and double-mutant strains. Cell homogenates were fractionated by centrifugation in continuous Nycodenz gradients, as described in materials and methods. The top arrow indicates the direction of increasing density within the gradient. A total of 100 μl of each gradient fraction was loaded. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with the αGrl1p antibody. The band corresponding to proGrl1p is marked by a solid arrowhead, and the band corresponding to mature Grl1p (absent in the SB281 and double-mutant strains) is marked by an open arrowhead. The bimodal proGrl1p distribution in SB281 is identical to that in the MN173,SB281 and MN175,SB281 double mutants, but clearly distinct from the distributions in the single-mutant (MN173 and MN175) strains.

lyzed by SDS-PAGE.) As previously demonstrated, MN173 and MN175 both synthesize DCGs and the processing of Grl1p in these lines is similar to that in wild-type cells (Figure 2). Grl1p in SB281, in contrast, exists only as the proprotein (Figure 2). We compared processing of proGrl1p in the double mutants to that in wild type and the single mutants. As expected if the SB281 mutation is farthest upstream, the double mutants like SB281 show no visible processing of Grl1p.

Subcellular distribution of Grl1p as a granule cargo marker: To evaluate further the relationship of the double mutants to the parental strains, we analyzed the subcellular distribution of Grl1p. Postnuclear supernatants of cell homogenates were fractionated on the basis of equilibrium density, and the fractions were analyzed by immunoblotting using the anti-Grl1p antibody (Figure 3). In fractionated SB281 homogenates, proGrl1p shows a bimodal distribution. In contrast, the distribution of proGrl1p in MN173 and MN175 homogenates is primarily a single peak with a shoulder that extends toward lower-density fractions; this is identical to the distribution in wild-type cells. The distribution of proGrl1p in the double-mutant strains is identical to the distribution of proGrl1p in SB281. This implies that the subcellular distribution of proGrl1p in SB281 cells, like the degree of processing, is not dependent on those gene products whose functions are compromised in MN173 and MN175.

Characterization of a storage compartment in SB281 and the double mutants: SB281 cells in exponentially growing cultures do not secrete newly synthesized Grl1p in pulse-

chase-labeling experiments, implying that the proprotein is stored in some vesicular compartment. A second DCG cargo protein, p80, might be similarly stored in SB281, and the monoclonal anti-p80 antibody, 4D11, has provided a useful reagent for immunofluorescent visualization of compartments containing DCG cargo in wild-type and mutant cell lines. [p80 is unrelated to proteins of the *GRL* family (N. Elde, J. Coralic and A. Turkewitz, unpublished results).] Nonetheless, previous attempts at immunolocalization in SB281 had failed to identify a convincing p80-containing compartment. On the other hand, a polyclonal antibody prepared against a DCG cargo protein of 34 kD was seen to label heterogeneous vesicles by immuno-electron microscopy (Maihle and Satir 1985). The molecular identity of the p30 antigen and the nature of the vesicles containing them were unclear, but they bore no resemblance to either immature or mature DCGs.

By improving fixation conditions to maintain better subcellular structure, we were now able to visualize p80 labeling in SB281 cells as a bright, punctate cytoplasmic pattern (Figure 4A). This vesicular pattern appears likely to represent the compartment previously inferred from pulse-chase experiments. Importantly, the labeling pattern persists in cells that have been treated with cycloheximide for 2 hr, behavior expected for a relatively stable compartment (Figure 4B). The pattern is clearly different from that seen in either wild type or MN175 (Figure 4, C–F), both of which show only cortical labeling, and in MN173 in which abundant cytoplasmic mature DCGs are labeled (Figure 4G). Using this antibody, we characterized p80 in the double mutants. Confirming the subcellular fractionation results, its localization in the double mutants was indistinguishable from that in SB281 (Figure 4, H and I).

We interpret these experiments to indicate that SB281 can accumulate DCG cargo proteins in a stable compartment. This implies that the DCG cargo protein is being sorted from a pathway of rapid constitutive protein secretion. However, similar results might be obtained if p80 were entering a constitutive secretory pathway, if that pathway were itself compromised by the mutation in SB281. Such a defect in constitutive secretion seems unlikely because mutations that affect constitutive secretion in the budding yeast *Saccharomyces cerevisiae* are associated with strong growth inhibition, while SB281, in contrast, grows at wild-type rates (Rothblatt and Schekman 1989). To examine this, we briefly incubated wild type and SB281 with [^{35}S]methionine to tag newly synthesized polypeptides and analyzed cell culture supernatants after brief chase intervals. The results in Figure 5 indicate that SB281 has no gross deficiency in constitutive protein secretion. Indeed, SB281 appears to release several polypeptides that are not secreted from wild-type cells. These may represent a subset of DCG cargo proteins that are released from SB281 even under growth

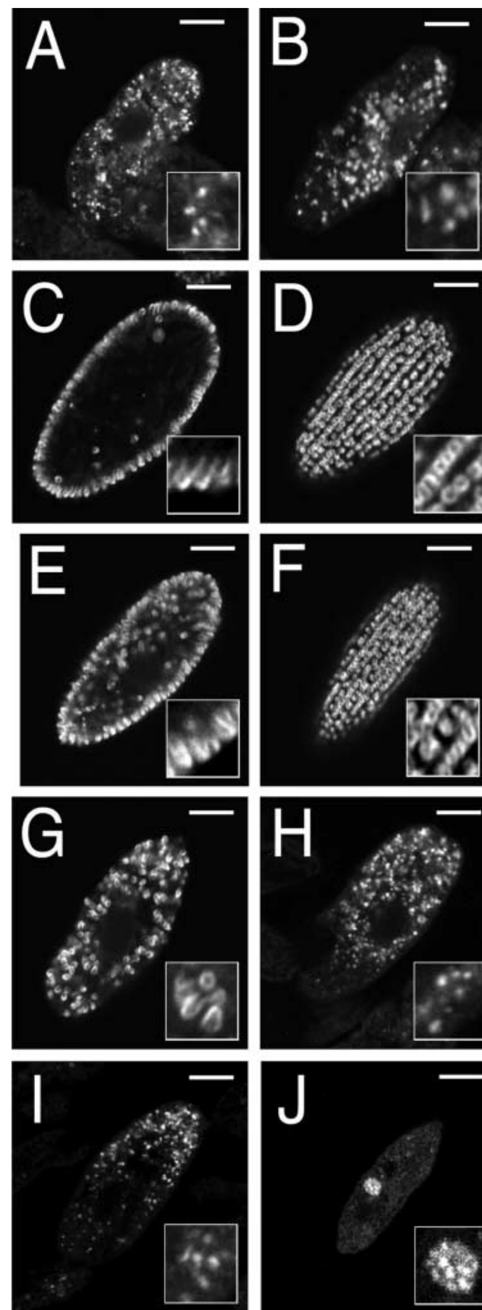

Figure 4.—Localization of DCG protein p80. p80 was visualized by indirect immunofluorescence using the 4D11 Mab. (A) SB281 cell midsection. (B) SB281, after 2 hr with 25 μg/ml cycloheximide. The persistence of the pattern indicates that p80 accumulates in a stable compartment. (C and D) Distribution of p80 in wild-type cells: the cell midsection (C) and the cell surface (D). (E and F) Corresponding images of p80 distribution in the secretion mutant MN175. (G) MN173 mutant cell midsection exhibiting nondocked DCGs. (H and I) The localization and morphology of the p80-containing compartment in the double-mutant strains is indistinguishable from that observed in SB281. (H) MN173,SB281 double mutant. (I) MN175,SB281 double mutant. (J) SB281 cell midsection after 3 hr of starvation in DMC. The majority of p80 appears to be localized to large multivesicular bodies. Bar, 10 μm; insets are a part of the image magnified, 3 μm per side.

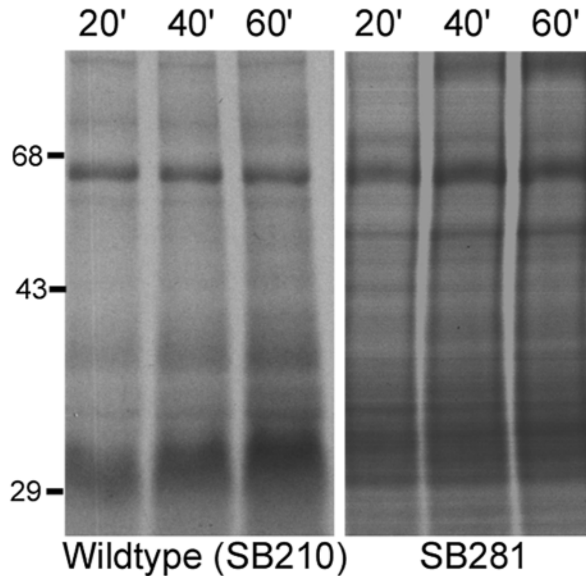

Figure 5.—Pulse-chase analysis of protein secretion from growing cells. Wild-type (SB210) and SB281 cells were pulse labeled, and aliquots of the cell culture supernatant were sampled at times shown. Proteins secreted into the medium were analyzed by SDS-PAGE and autoradiography. Most of the labeled cohort was secreted by 20 min. The pattern of bands is similar, but not identical, in SB210 and SB281. Molecular weight markers are indicated to the left.

conditions. In addition, the mutation may directly or indirectly affect other aspects of membrane traffic, leading to aberrant secretion.

Functional characterization of the 4D11 immunoreactive compartment in SB281: The results above, particularly in combination with previously reported findings, suggest that both p80 and Grl1p accumulate in a stable vesicular compartment in SB281. One attractive possibility was that this compartment represents a normal intermediate in DCG biosynthesis, the further maturation of which is blocked due to the genetic lesion. This hypothesis was consistent with previous cytosol mixing experiments that suggested that the defect in SB281 could be repaired by a diffusible, cytosolic factor (Melia *et al.* 1998). These cytosol mixing experiments were done by setting up highly synchronized matings between the mutant strain and a wild-type partner. Such mating pairs are joined by a membrane, perforations in which allow exchange of cytoplasmic factors for several hours before any nuclear exchange. Biochemical data, also based on conjugation complementation, also supported the view that a diffusible factor could repair the defect in SB281 and, it was argued, allow maturation of a DCG intermediate (Sauer and Kelly 1995). However, an unavoidable weakness of this experimental design is that *de novo* protein synthesis occurs during the several-hour time course of the assay because inhibition of protein synthesis would cause separation of the mated pairs. A second weakness is that conjugation can be performed only with starved cells, and SB281 accumulates only very low levels of known DCG proteins in that state. To clarify this important issue, we performed cytosol mixing experiments using an approach to circumvent these problems. SB281 and wild-type cells were fused in an electric field, allowing for instantaneous cytosol mixing. Since this could be done in the presence of cycloheximide, the protocol allowed us to focus on the pool of granule proteins preexisting in the SB281 vesicular compartment. To demonstrate the validity of this approach we first fused MN173 with strain SB283, since previous results indicated that the MN173 strain, with morphologically mature but undocked DCGs, should be rapidly cured by cytosolic mixing with a strain in a different complementation group (Melia *et al.* 1998). SB283 was used for these experiments because the strain contains DCGs that, for an unknown reason, are not recognized by the p80 monoclonal antibody (Turkewitz and Kelly 1992). As a result, all of the DCGs visualized in experiments that include the addition of cycloheximide must be at least partially derived from material that was produced in the non-SB283 partner prior to fusion. These results are shown in Figure 6A, in which fusion between nonimmunoreactive SB283 and immunoreactive MN173 leads to rapid docking of the immunoreactive MN173 DCGs. The identical result was obtained in the presence (as shown) or absence of cycloheximide.

If the p80-containing vesicles in SB281 represent functional DCG intermediates, or if the material that they contain can be directed onto the DCG synthesis pathway after cytoplasmic complementation, then the mixing of SB281 and SB283 cytosol in the presence of cycloheximide should result in the appearance of docked DCGs. Since the SB283 partner has no immunoreactive p80, any fluorescently labeled DCGs that appear after such a fusion must derive at least in part from material contained in the SB281 partner prior to fusion. Even if these are few in number, one can reliably detect even a very small number of DCGs because of their predictable positions at the cell periphery. Nonetheless, we failed to detect any DCGs in such experiments; an example is shown in Figure 6B. In contrast, when cells were fused in the absence of cycloheximide we saw the appearance of DCGs, which therefore arose from *de novo* DCG protein synthesis.

As a second approach to the same question, we followed the localization of a different DCG protein in live cells by the use of a GFP tag (Figure 6C). These experiments made use of an SB281 mutant strain that was transformed with a vector expressing the DCG content protein Igr1p fused at the C terminus to GFP (A. Haddad and A. Turkewitz, unpublished results). Prior to fusion, these cells exhibited punctate cytoplasmic fluorescence like that observed for immunolocalized p80 (not shown). When these cells were fused to wild-type Tetrahymena in the absence of cycloheximide, docked DCGs were easily visible at the cell periphery within 2 hr. In contrast, when the same fusion was per-

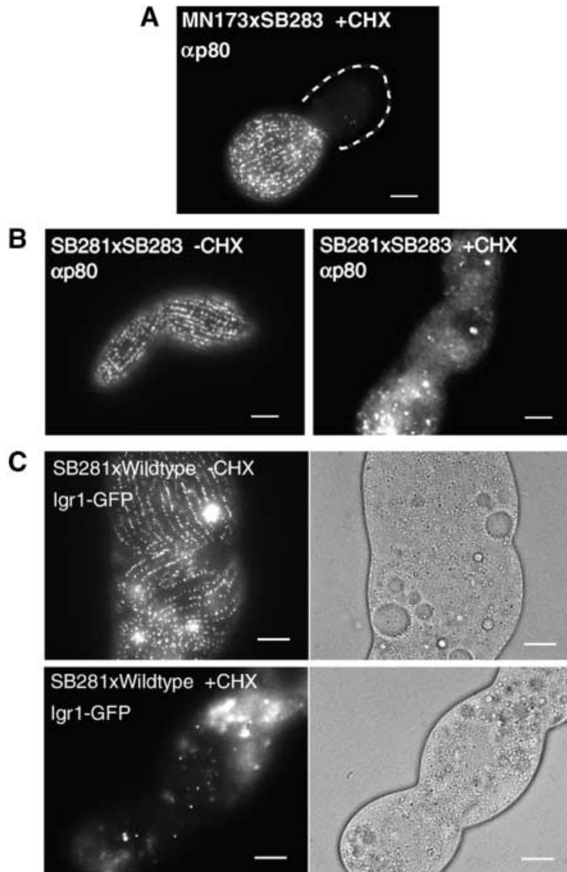

Figure 6.—Complementation of SB281 following cell-cell fusion requires new protein synthesis. (A) MN173 cells were fused with SB283 cells in the presence of cycloheximide (CHX) at 25 μg/ml, as indicated. After 1 hr, the cells were fixed and prepared for immunofluorescence visualization of p80. The SB283 member of the fused pair exhibited only faint background staining, and a dotted line is drawn around the perimeter of this cell to more clearly indicate its position. (B) SB281 cells were fused with SB283 cells in the presence or absence of cycloheximide, and after 2 hr, the cells were fixed and prepared for immunofluorescence visualization of p80. (Left) A SB281 × SB283 fusion incubated without cycloheximide, showing parallel rows of docked DCGs at the cell surface. (Right) A SB281 × SB283 fusion incubated in cycloheximide and focused through the middle of the fused cells. In the absence of new protein synthesis, docked granules are not present in fused cells. (C) SB281 cells expressing Igr1-GFP were fused with wild-type cells in the presence or absence of cycloheximide, as indicated. The images shown were obtained 2 hr after cell fusion. For each pair of images, the left shows GFP fluorescence and the right is a Nomarski image of the same fused cells. For the untreated cells, a cell surface image shows that parallel rows of GFP-labeled DCGs are docked at the plasma membrane. For the cycloheximide-treated cells, an image of the cell interiors shows that the GFP-labeled bodies remain throughout the cytoplasm. Bar, 10 μm.

formed in the presence of cycloheximide, there was no evidence of DCGs at the cell periphery after a similar lag. In those cells, the cytoplasmic vesicles containing Igr1p-GFP appeared unchanged in appearance or distribution from those in unfused cells. The inhibitory effect of cycloheximide on complementation was observed up to 5 hr after the fusion of SB281 with wild-type cells (not shown).

These results contradict two previous inferences: that the SB281 defect lies in a diffusible cytoplasmic factor and that the bodies that accumulate in SB281 represent biosynthetic intermediates. From these experiments we conclude that at least a subset of DCG cargo proteins in SB281 does not enter the constitutive secretory pathway, instead accumulating in stable vesicles that are not demonstrable intermediates in DCG biosynthesis but rather are likely to represent a cell biological "dead end."

Conditional proprotein secretion in SB281 and the double mutants: Starved SB281 cells secrete newly synthesized Grl1p in its proprotein form. We note that wild-type cells are equally efficient at DCG biosynthesis under either growth or starvation conditions. By using the same improved fixation conditions applied to growing cells, we were able to now document this conditional phenotype by morphological criteria. Under conditions in which proGrl1p was rapidly released, the cells were dramatically depleted of immunoreactive p80 (not shown). Thus two different cargo proteins that are stored in growing cells are apparently secreted under starvation conditions.

The secretion of newly synthesized proGrl1p is rapid (Figure 7). The simplest explanation is that a sorting step in the TGN, normally responsible for selective targeting of DCG proteins from the constitutive secretory pathway, is absent in starved SB281 cells. A second possibility is that proGrl1p is first targeted to a DCG-like vesicle, but that these vesicles undergo constitutive exocytosis due to the SB281 defect. This second possibility would also be consistent with the persistence of p80-containing vesicles in growing cells. If the second model is correct, rapid secretion under starvation conditions might rely on steps that are blocked in MN173 or MN175. For example, the efficient transport of a DCG-like vesicle to the cell surface might well depend on the transport step that is blocked in MN173.

We examined these alternate possibilities by comparing the secretion kinetics of proGrl1p from the single and double mutants, all under starvation conditions, using a pulse-chase protocol. Newly synthesized protein was pulse labeled with [^{3}H]lysine, and proGrl1p was immunoprecipitated from both the cell culture medium and cell lysates at time points thereafter. In MN173 and MN175 cells, proGrlp is processed into mature Grl1p within 30 min of the pulse labeling (Figure 7). Processing in these cells, as in wild type, coincides with the incorporation of the mature protein into a highly insoluble protein lattice (Turkewitz *et al.* 1991); for that reason, the mature protein cannot generally be recovered by immunoprecipitation. In SB281, by contrast, the unprocessed proGrl1p is secreted into the media. The double-mutant strains, which also exhibit the proGrl1p processing defect (Figure 2), also secrete

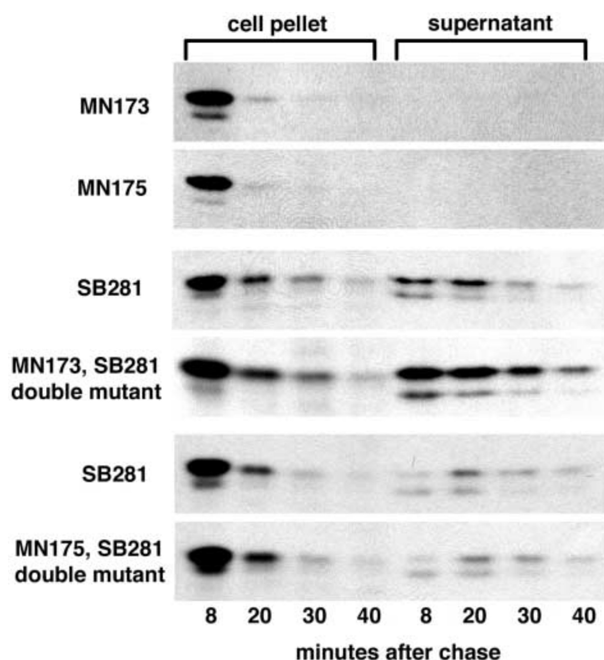

Figure 7.—Pulse-chase analysis of proGrl1p secretion in single- and double-mutant strains. Cells were starved for 2–3 hr and then metabolically labeled with ^{3}H-lysine for 20 min. At the indicated times after the beginning of the chase period the cells were separated from cell supernatants, and these were processed independently for immunoprecipitation with the αGrl1p antibody. Samples were analyzed by SDS-PAGE and fluorography. Variable degradation of proGrl1p by proteases in the culture supernatant led to variable yield of proGrl1p in that fraction. The bottom two panels are data from a single experiment in which the two strains were processed in parallel. The middle two panels represent experiments on different days. The kinetics of proGrl1p secretion in SB281 is unchanged in the double-mutant strains.

proGrl1p into the media (Figure 7) with kinetics that are indistinguishable from those of SB281. These results show that the nonregulated secretion of proGrl1p in SB281 does not depend on activities required downstream in the regulated secretory pathway and are therefore consistent with the idea that secretion occurs through a *bona fide* constitutive pathway.

The rapid secretion kinetics are also consistent with our conclusion that the p80-immunoreactive vesicles in growing cells are not intermediates in any secretory pathway, since secretion via such an intermediate might be relatively slower. To pursue this issue, we looked at p80 localization in cells during the time period following a transition from growth-to-starvation conditions. If the p80-containing vesicles were part of a secretory pathway whose kinetics were altered during starvation, one might expect to see a rapid reduction in observable vesicles. Instead, we observed the persistence of immunofluorescent bodies, which, however, were distinct from those seen during growth. In these cells, p80 was largely localized to vacuolar bodies, some of which appeared multivesicular (Figure 4J). These images strongly suggest that the p80-containing vesicles are degraded, perhaps by autophagy, when cells are shifted from growth into starvation conditions, while newly synthesized proteins are secreted via a rapid constitutive route.

DISCUSSION

The analysis of a collection of mutants affecting a physiological process offers a relatively unbiased approach to resolving that process into a series of steps. The collection of Tetrahymena mutants with deficiencies in regulated exocytosis is a valuable resource for understanding the biogenesis and exocytosis of DCGs. The pathway inferred by analysis of these mutants previously was based on morphological and biochemical characteristics (Orias *et al.* 1983; Melia *et al.* 1998). Among the most interesting of the mutants is SB281, whose phenotype suggested a defect in granule biogenesis at the level of sorting or storage of cargo proteins. A phenotypically similar mutant, *Trichless*, has been described in Paramecium (Gautier *et al.* 1994). However, the Paramecium mutant is slow growing while SB281 grows at wild-type rates, suggesting there may be fundamental differences between the defects. Because our understanding of the mechanisms involved in DCG biogenesis is incomplete in any system, we have chosen to revisit SB281. Our results significantly extend the characterization of the defects and reveal a compartment in which protein storage is uncoupled from proprotein processing.

The issues addressed in this study are the following. First, using genetic criteria, where does SB281 fit in the pathway of granule biogenesis? Second, what is the nature of the pathway by which SB281 proproteins can be secreted rapidly from cells? Finally, what is the nature of the conditional defect in the storage of granule content proteins and what might account for the differences between growing and starved cells?

At the core of the experiments designed to address these points was the construction of strains that were homozygous for mutations at pairs of three different loci. The phenotypes of these strains, relative to those of the single mutants, first confirmed that the defect in SB281 lay upstream of that in MN173, which is lacking a factor required for transport of mature cytoplasmic DCGs to the plasma membrane (Melia *et al.* 1998). Similarly, the SB281 defect lies upstream of that in MN175, a mutant that is defective in a factor required for the stimulated exocytosis of docked DCGs with the plasma membrane. We analyzed MN173,SB281 and MN175,SB281 double mutants using criteria established in earlier studies: DCG proprotein processing, subcellular fractionation, and proprotein secretion. By all these measures SB281 is epistatic to the other mutants.

The results of the double-mutant analysis were also central to the second question addressed: by what route does unstimulated protein release occur in SB281? On

the basis of current models of post-TGN secretory pathways, there appeared to be two possibilities for the route taken from the TGN to the cell surface for DCG cargo proproteins in SB281. First, in the absence of formation of immature DCGs, cargo proteins may be shunted into a parallel pathway leading to constitutive release from the cell surface. The second possibility is that cargo proteins are localized to vesicles that have some but not all features of authentic DCGs: sorting, but not stimulus-dependent fusion. Stimulus-regulated fusion, in the case of synaptic vesicles, results from the presence of a fusion clamp on an otherwise constitutive fusion apparatus (Brose *et al.* 1992; Lao *et al.* 2000). Exocytosis in the absence of stimulation would therefore be permitted if the fusion clamp failed to become associated with, or active in, the vesicles that are formed in SB281. A phenomenon that may be related, called constitutive-like secretion, has been noted in some endocrine cells as a by-product of DCG formation (Kuliawat and Arvan 1992).

If this is the case for SB281, one might imagine that secretion from such DCG-like vesicles would still depend upon a set of the gene products required in the wild-type pathway. For example, the active transport of such vesicles to the plasma membrane might require the same transport factors, *e.g.*, microtubule-based motors, that are used by wild-type DCGs. Nonetheless, the double-mutant studies indicated that no discernible features of the SB281 phenotype depend on steps that are interrupted in MN173; in particular, proprotein secretion in SB281 does not depend on gene products that are essential for wild-type DCG transport. This lends support to the model that proproteins in SB281 are being shunted to the constitutive secretory pathway and that the constitutive and regulated secretory pathways can indeed be considered alternate, parallel routes.

An intriguing aspect of the SB281 phenotype is its conditional nature with respect to the storage of proGrl1p. Previous experiments had established that rapid secretion of proproteins was a property of starved cells; the situation in growing cells was more ambiguous since proproteins did not appear to be secreted, but neither did they accumulate in any discernible subcellular compartment. This paradox has now been resolved by the improved fixation conditions for light microscopy, which allowed us to visualize a compartment that could previously only be inferred. At least three unrelated DCG cargo proteins (p80, Igr1p, and Grl1p) are stored within intracellular vesicles in SB281 under growth conditions. This DCG cargo has apparently been diverted, or excluded, from a robust pathway of constitutive secretion. These storage vesicles are stable, persisting for hours following treatment of cells to block protein synthesis. By biochemical and immunological criteria, the stability of the polypeptides also indicates that the compartment is not hydrolytic. Since there is no detectable defect in constitutive secretion in SB281, the accumulation of these vesicles does not represent a general block in exocytic membrane traffic. Nonetheless, these vesicles do not appear to represent viable intermediates on the route to DCG formation. Instead, evidence from direct cell fusion experiments and from visualization of the p80 compartment in newly starved cells indicates that these are a dead-end compartment that can undergo autophagic-like degradation when cells are starved, although they are apparently stable under growth conditions. Such a transition mirrors other changes in the physiology of unicellular organisms progressing from growth to starvation; in Tetrahymena, these include a drastic change in cell shape, remodeling of the oral apparatus, and growth of a singular elongated caudal cilium (Frankel 2000).

We do not yet know the genetic lesion that is responsible for the SB281 phenotype; this will depend upon development of cloning by complementation in this organism, a current aim of our and other laboratories. Results of earlier experiments suggested that the defective factor was cytosolic and diffusible, an important result because it implied the existence of cytosolic factors unique to this pathway. However, because these conclusions were derived essentially from somatic complementation experiments of the kind discussed earlier, they are superceded by the results reported here that rely on direct cell fusion in the presence of cycloheximide. Most importantly, the granule cargo-containing bodies that accumulate in SB281 are not converted into DCGs when exposed to wild-type cytoplasm. Labeled DCGs do appear after such fusions when cycloheximide is omitted, but we cannot distinguish between the wide variety of roles that newly synthesized proteins may be playing. For example, a labeled granule protein expressed from the SB281 nucleus may be post-translationally transported into the complementing cell's functional secretory pathway. Alternatively, granule synthesis could require the cotransport of labeled DCG cargo, expressed from the SB281 nucleus, with a lumenal or membrane protein that is expressed from the wild-type nucleus. Because of the range of possibilities, our results do not make any predictions about the nature or location of the activity that is defective in SB281.

The SB281 phenotype is more complex than previously appreciated, and it is challenging to provide a satisfactory model for the defect. Our results limit the possible models for its nature. It is important to note that while the mutation is conditional for growth conditions, it still produces clear defects in both growing and starved cells. First, the mutation does not appear to produce a global defect in protein sorting since cargo proteins can still be diverted from a constitutive secretory pathway in growing cells. This could occur as positive sorting at the TGN or as retention in immature DCGs. Second, the defect does not appear to be in the transition between immature and mature DCGs, since under starvation conditions there is no evidence for

immature forms as judged by immunolocalization, ultrastructure, and the kinetics of proprotein secretion. Third, the primary defect seems equally unlikely to be at the level of proprotein processing, because starved cells have a sorting defect, and previous data argue that sorting precedes processing. Finally, the defect seems unlikely to result from mis-sorting of any single DCG component, because the difference in sorting between growing and starved cells is remarkably clear: no rapid secretion in the former and no storage in the latter.

We posit that the mutated protein in SB281 may be responsible for the establishment or maintenance of compartment-specific conditions both in the TGN and in immature DCGs. An example of such a protein could be an ion pump that existed in both compartments. Under starvation conditions, the defective activity could lead to a change in the TGN environment that prevented the normal aggregation of DCG proproteins. This is particularly easy to imagine if aggregation is initially nucleated by assembly of a specific protein complex. In the absence of aggregation, proteins would not be sorted away from inclusion in constitutive secretory vesicles. Under growth conditions the pump activity could still be adequate to maintain an aggregation-promoting TGN environment, but that same pump when included in immature secretory vesicles might be inadequate to maintain conditions needed for enzymes involved in proprotein processing. The result would be vesicles resembling immature DCGs that accumulate unprocessed cargo. In electron-microscope thin sections of wild-type cells one never observes immature DCGs docked at the plasma membrane, suggesting that granules prior to maturation may be incompetent for transport or docking. A secondary processing defect in SB281 could therefore explain the absence of secretion from growing cells. Finally, our hypothesis is consistent with the observation that the defects in either growth or starvation cannot be complemented by a cytosolic factor.

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