

Mutational analysis of regulated exocytosis in *Tetrahymena*

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SUMMARY

Genetic analysis of regulated exocytosis can be accomplished in ciliates, since mutants defective in stimulus-dependent secretion of dense-core vesicles can be identified. In *Tetrahymena thermophila*, secretion in wild-type cells can result in their encapsulation by the proteins released from vesicle cores. Cells with defects in secretion were isolated from mutagenized homozygous cells that were generated using a highly efficient method. Screening was based both on a visual assay for encapsulation, and on a novel panning step using differential centrifugation to take advantage of the selective mobility of mutants that fail to encapsulate upon stimulation. 18 mutants with defects

in several ordered steps have been identified. Defects in a set of these could be localized to three stages: granule formation, transport to cell surface docking sites, and exocytosis itself. Mutants with defects in this last stage can be ordered into successive steps based on several criteria, including their responsiveness to multiple secretagogues and Ca²⁺ ionophores. The results of both somatic and genetic complementation on selected pairs also help to characterize the defective factors.

Key words: Dense-core secretory vesicle, Granule, Ciliate, Membrane fusion

INTRODUCTION

The pathway leading to stimulus-dependent exocytosis of secretory dense-core vesicles (DCVs), also called secretory granules, is one of several routes diverging at the *trans*-Golgi network, and is responsible for release of many hormones, neuropeptides and other macromolecules from endocrine, exocrine and neuronal cells (Halban and Irminger, 1994). Several of the alternative routes, most notably constitutive secretion and protein delivery to the vacuole, are being effectively dissected by genetic analysis in yeast, based on efficient screening techniques for pathway-specific mutations. Since regulated exocytosis has not been detected in yeast, genetic analysis of this pathway relies on other accessible organisms. Relevant genes have been identified by screening for behavioral mutants in fruit flies (van der Bliek and Meyerowitz, 1991) and nematodes (Hall and Hedgecock, 1991), but such screens are not focused on a single pathway.

Beginning with characterization of spontaneously arising exocytosis mutants in *Paramecium tetraurelia* (Pollack, 1974), this organism became a tool for pursuing focused mutational analysis of regulated secretion (see Skouri and Cohen, 1997, and references therein). DCVs in these cells are docked at the plasma membrane and undergo synchronous exocytosis within milliseconds of stimulation (Knoll et al., 1991). This event can be directly observed by eye, forming the basis for detection of secretory mutants. The DCV pathway is non-essential for laboratory cultures, and approx. 30 non-conditional and conditional mutants have been isolated whose defects illuminate several steps in the pathway (see Bonnemain et al.,

1992, and references therein). Ten exocytosis (exo⁻) mutants in the distantly related ciliate *Tetrahymena thermophila* have similarly been identified by visual screening (Orias et al., 1983). These have been described both genetically and phenotypically (see Chilcoat et al., 1996, and references therein), providing evidence that granule biogenesis occurs via distinct intermediates (Turkewitz et al., 1991). A first exocytosis-related gene has recently been characterized (Chilcoat et al., 1996), taking advantage of some of the powerful tools for analysis of gene function in this organism (Cassidy-Hanley et al., 1997; Gaertig and Gorovsky, 1995; Sweeney et al., 1996). In addition, strategies for cloning by complementation of mutant phenotypes are being developed.

The work reported here was aimed at analyzing additional steps in granule synthesis and exocytosis by isolating novel mutants. To facilitate both this and the projected direct cloning of genes associated with exo⁻ phenotypes, we sought improved screening techniques. First, a recently developed technique was used to generate homozygous cells following mutagenesis and mating. *Tetrahymena* contain a somatically active macronucleus and a silent (genetic) micronucleus. Mating normally results in fusion of haploid meiotic micronuclear products derived from the two partners to form a heterozygous diploid product, which becomes the macronucleus (Bruns, 1986). A method called uniparental cytogamy induces a developmental program that mimics the normal sequence, except that the newly formed nuclei are derived from a single parent and are homozygous, expressing recessive traits (Cole and Bruns, 1992). Second, we developed a panning step for screening, taking advantage of the fact that exocytosis results

in transient encapsulation. Analysis of a set of the mutants obtained contributes to a finer mapping of several steps.

MATERIALS AND METHODS

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

Cells

Cells were grown at 30°C with agitation in 2% proteose peptone, 0.2% yeast extract (both from Difco), with 0.003% ferric EDTA. For mutagenesis and drug selection, the medium was 0.25% proteose peptone, 0.25% yeast extract, 0.5% glucose and 1 mM ferric chloride. *T. thermophila* strains used, from the inbred B strain, are shown in Table 1; they are described by their micronuclear diploid genotype (Mic), followed by their macronuclear-determined phenotype in parentheses (Mac) (Bruns, 1986). CU428.1, B2086, A*III and A*V were provided by Peter Bruns (Cornell University, NY). A*III and A*V are genetically useful 'star' strains that pair normally, but do not contribute genetic information to the progeny (Doerder and Berkowitz, 1987). In addition, microtiter plates of homozygous strains derived from mutagenized CU438 were provided by Donna Cassidy-Hanley and Peter Bruns (Cornell University, NY). SB210 and SB281 were provided by Ed Orias (UC Santa Barbara). *Tetrahymena thermophila* strains are frequently designated by a two-letter abbreviation for an institution, followed by a number. In the strains described in this paper, MN refers to Minnesota (St Olaf College), and UC to the University of Chicago.

Mutagenesis and screening

Mutagenesis and uniparental cytogamy for screening of individual clones

CU428.1 was grown to 200,000 cells/ml, and incubated for 3 hours with nitrosoguanidine (10 µg/ml). Cells were sedimented, washed with 10 mM Tris, pH 7.4, and starved overnight in the same buffer. A*III cells were similarly starved, as a mating partner. After 18 hours, cells were mixed at a final concentration of 200,000 cells/ml; 5 hours later, cells were fed with growth medium to prevent re-pairing. Pairs were manually isolated into drop plates containing growth medium and transferred to microtiter plates (Orias and Bruns, 1976). These plates were later replicated into microtiter plates containing pre-starved A*V partners in Dryl's medium (1 mM Na₂HPO₄, 1 mM NaH₂PO₄, 1.5 mM CaCl₂, 2 mM Na citrate, pH 7.1) (Orias et al., 1983) and allowed to mate again. A hyperosmotic shock was delivered to cells 6 hours after mixing by adding glucose to a final concentration of 1.4% in growth medium (Cole and Bruns, 1992; Orias and Hamilton, 1979). Homozygotes were selected during 4 days of incubation in 6-methyl purine (15 µg/ml). Survivors were brought to sexual maturity by serial replication.

For screening, microtiter plates containing homozygous mutant clones were replicated to wells containing 50 µl/well of *Enterobacter aerogenes* in Dryl's medium with penicillin and streptomycin (Orias et al., 1983). The bacterial suspension is a 50-fold dilution of a stationary (overnight) culture into Dryl's medium. The bacteria serve as food to allow several cell doublings of the *Tetrahymena*, leaving the cells in a low osmotic strength buffer that is ideal for exocytotic screening. Replicated cells were incubated at 30°C for 2 days. Capsule formation was provoked by adding an equal volume of 0.02% Alcian Blue in 0.5 mM CaCl₂, followed by addition after 2-3 minutes of 20 µl 2% proteose peptone. Screening on an inverted microscope was at 100× magnification. For wells in which capsule formation was absent or markedly reduced, individual cells were cloned. Because up to four mating types are found in the progeny of a single cross, it was possible to obtain multiple mating types.

Table 1. Strains

| Strain | Mic | Mac |
|-----------|-------------------------------|-------------------------------------|
| CU428.1 | <i>mpr1-1/mpr1-1</i> | (mp-s, exo ⁺ , VII) |
| CU438 | <i>pmr1-1/pmr1-1</i> | (pm-s, exo ⁺ , IV) |
| B2086 | <i>MPR/MPR</i> | (mp-s, exo ⁺ , II) |
| SB210 | <i>gal1-1/gal1-1</i> | (dg-s, exo ⁺ , VI) |
| IA267 | <i>chx1-1/chx1-1</i> | (cy-s, exo ⁺ , III) |
| A*III | | (exo ⁺ , III) |
| A*V | | (exo ⁺ , V) |
| SB281 | <i>gal1-1/gal1-1</i> | (dg-r, exo ⁻ , III) |
| UC1 | <i>pmr1-1/pmr1-1</i> | (pm-r, exo ⁻ , sterile) |
| UC12.3 | <i>PMR/pmr1-1; CHX/chx1-1</i> | (pm-?, cy-?, exo ⁻ , VI) |
| MN173(IV) | <i>mpr1-1/mpr1-1</i> | (mp-r, exo ⁻ , IV) |
| MN173(V) | <i>mpr1-1/mpr1-1</i> | (mp-r, exo ⁻ , V) |
| MN175(II) | <i>mpr1-1/mpr1-1</i> | (mp-r, exo ⁻ , II) |
| MN175(IV) | <i>mpr1-1/mpr1-1</i> | (mp-r, exo ⁻ , IV) |

Abbreviations: mp, 6-methyl purine; pm, paromomycin; dg, 2-deoxygalactose; cy, cycloheximide; Mic, micronuclear diploid genotype; Mac, macronuclear-determined phenotype.

Mutagenesis and mass screening

Growing CU428.1 cells were exposed to nitrosoguanidine, washed and starved in Tris buffer exactly as above. Mutagenized cells were mixed with a starved culture of A*V in a final volume of 10 ml at 200,000 cells/ml. After 6 hours, 0.75 ml of 20% glucose was added. At 7 hours, cells were diluted fivefold into growth medium. At 10 hours, cells were sedimented at approx. 800 g for 5 minutes, resuspended in 18 ml of medium, and divided into six samples. After overnight incubation, 6-methyl purine was added to 15 µg/ml. After 4 days at 30°C, cells were sedimented, washed in DMC, and resuspended in an equal volume of DMC for 24 hours. 0.3 ml was transferred from each sample into a clear 5 ml conical tube. 7.5 µl of 1% Alcian Blue was added with vigorous mixing, followed after 30 seconds with 4 ml 0.25% proteose peptone and 0.5 mM CaCl₂. Cells were immediately pelleted at approx. 800 g for 3 minutes. After 5 minutes, approx. 1 ml was withdrawn from the top of each tube, containing swimming cells. This was centrifuged at approx. 600 g for 5 minutes, and resuspended in 300 µl DMC. After 8 hours, Alcian Blue treatment and panning were repeated, and 100 µl samples of cells were withdrawn from the meniscus of each sample. 48 individual cells were manually isolated from each sample into drop plates containing medium. This protocol is outlined in Fig. 2A. Cells were replicated to microtiter plates for overnight growth, and successively to plates containing *Enterobacter aerogenes* in Dryl's medium for testing, as described above.

Generation of progeny

MN175×wild type (F1). Starved cultures of MN175 and B2086 were mixed as above. At 9 hours, culture medium was added to prevent further pairing. The following morning, 0.5 ml was diluted 400-fold into medium containing 15 µg/ml 6-methyl purine. After 2 days, this dilution was repeated. After 4 days at 30°C, there were no survivors in a similarly treated control culture (B2086 alone). Survivors from the mating mixture were therefore a mixture of non-mated MN175 and (MN175×B2086)F1 heterozygotes. After 2 more days of drug selection, cells were tested for Alcian Blue-stimulated capsule formation, as described below. Since MN175 shows no capsule formation, the presence of capsules would indicate that sexual progeny can regain an exo⁺ phenotype. Heterozygous cells may become homogeneous for any particular macronuclear allele due to phenotypic assortment, but this effect is minor in the 20 generations undergone in this experiment (Doerder et al., 1975).

Mating type tests

Mating type tests were performed according to Orias and Bruns (1976) with tester strains provided by Ed Orias (UC Santa Barbara).

Conjugation rescue

Induction of mating was identical to that described above except that the buffer was DMC (0.1 mM Na₂HPO₄, 0.1 mM NaH₂PO₄, 0.65 mM CaCl₂, 0.1 mM MgCl₂, 0.2 mM sodium citrate, pH 7.1) (Orias et al., 1983), which is optimal for testing of capsule formation. At set intervals after mixing, samples were withdrawn, fixed with 0.2% formalin, and single cells and pairs were counted using a hemacytometer. For some experiments, cycloheximide was added to a concentration of 15 µg/ml from a 100× stock solution in ethanol, at the time point when about 50% of cells were paired. Cycloheximide prevents additional pair formation but leaves intact most previously formed pairs (Satir et al., 1986). For labeling with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR), starved cells were labeled prior to mating at a concentration of 10 µg/ml for 10 minutes (Cole, 1991).

Cell stimulation

Alcian Blue 8GX

Cells were pelleted (approx. 810 g, 45 seconds) washed once and starved in DMC for 2-4 hours at room temperature, prior to stimulation. 1% Alcian Blue was added to 0.025% and mixed vigorously. The mixture was diluted after 30 seconds with a minimum of 9 volumes of 0.25% proteose peptone and 0.5 mM CaCl₂. After 2 minutes, cells were sedimented and either resuspended for observation or fixed for immunofluorescence (IF). Stimulation of mating cell cultures was identical except that these cultures required no preincubation in DMC.

Dibucaine

Growing cells were pelleted and resuspended in approx. 1/10th volume of 10 mM Tris pH 7.0, 1 mM CaCl₂. Dibucaine (25 mM in water) was added to a final concentration of 2.5 mM and mixed by rapid swirling for 5 seconds, and the mixture was diluted at least tenfold in the same buffer. To detect the large aggregates of exocytosed protein, the mix was underlayered with 0.25 M sucrose and centrifuged at approx. 810 g for 3 minutes. Cells enter the sucrose layer while flocculent secreted protein accumulates at the interface. For direct visualization, cells were diluted into fixative for IF within 5 seconds after dibucaine addition.

A23187

4 µl of a 1 mg/ml stock solution (in ethanol) of A23187 was added to 400 µl growing cells. After 30 seconds, cells were fixed for IF.

Subcellular fractionation

Cells were grown to 2-3×10⁵/ml, then rapidly chilled and sedimented at 150 g for 5 minutes. All subsequent operations were performed cold. Cells were resuspended and washed once in 10 mM HEPES pH 7.0, and the pellet volume measured. The pellet was washed and resuspended in two volumes of buffer A (0.3 M sucrose, 10 mM HEPES pH 7.0, 28.8 mM KCl, 2 mM MgCl₂, 2 mM EGTA) (Fluka, Ronkonkoma, NY) containing the following protease inhibitors: leupeptin (0.5 µg/ml), antipain (12.5 µg/ml), E-64 (10 µg/ml) and chymostatin (10 µg/ml). Cells were passed through a ball-bearing cell cracker (Hans Issel, Palo Alto, CA) with a nominal clearance of 0.0005 inches. The homogenate was cleared for 10 minutes at 500 g. 0.4 ml samples of cleared lysates were applied to 9 ml continuous Nycodenz (Gibco BRL, Gaithersburg, MD) gradients (top solution: buffer A with additional 50 mM sucrose; bottom solution: 45% Nycodenz in buffer A) and centrifuged for 90 minutes at 120,000 g. 0.5 ml fractions were collected, and samples withdrawn for protein or enzyme assays, or for western blotting.

Enzyme and protein assays

Protein concentrations were determined using bicinchoninic acid (Pierce, Rockford, IL). Acid phosphatase was assayed using *p*-nitrophenol phosphate as described (Hunseler et al., 1987). The rate

of substrate conversion, rather than the end point, was determined by spectrophotometric measurements taken every 30 seconds for 15 minutes, using a BioTek kinetic plate reader. The absorbance increase was linear during this period.

SDS-PAGE and western blots

Equal cell numbers from growing cultures were sedimented and washed once with 10 mM HEPES, pH 7.0, containing protease inhibitors as above. Cell pellets were dissolved in SDS-containing sample buffer with 10 mM dithiothreitol at 100°C, and fractionated by SDS-PAGE (Laemmli, 1970) along with molecular weight standards (Gibco BRL, Gaithersburg, MD). Antibody blotting was according to Towbin et al. (1979), and antibodies were visualized using ¹²⁵I-labeled Protein A (1 mCi/ml, 10⁶ cpm per blot (ICN, Costa Mesa, CA) and quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The rabbit antisera specific for two granule markers, Gr11p (p40) and p80, have been described (Turkewitz et al., 1991). Rabbit antiserum against SerH was the gift of F. Paul Doerder (Cleveland State University, OH).

Immunofluorescence

Cells were fixed for 10 minutes in ice-cold 0.2% Triton X-100 in 50% ethanol. Blocking was for 30 minutes 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 minute incubation in 20% hybridoma supernatant in TBS. The monoclonal antibody 4D11, specific for the DCV protein p80, has been previously described (Turkewitz and Kelly, 1992), and was the gift of Marlo Nelson and Joseph Frankel (University of Iowa). Cells were then washed for 3×5 minutes in BSA/TBS, followed by a 30 minute incubation in 0.1% rhodamine isothiocyanate-coupled rabbit anti-mouse IgG (Calbiochem, La Jolla, CA). They were then washed as before, resuspended in Slowfade (Molecular Probes, Eugene, OR), and images were collected using epifluorescence optics. For quantitation of somatic rescue, fields of paired cells were identified under visible optics and then photographed using fluorescent optics, and rescue was scored from photographic prints.

RESULTS

Mutant screens

We screened homozygous mutagenized cells for *exo*⁻ mutants with defects in stimulus-dependent exocytosis of DCVs. Such mutants can be recognized under low magnification by their failure to form pericellular protein capsules following stimulation with Alcian Blue. Alcian Blue stimulates secretion and also appears to coagulate the released granule proteins in the form of blue-stained capsules (Tiedtke, 1976). Both docked granules and the capsule formed by released protein can be visualized by indirect IF using a mAb directed against the p80 protein in the DCV core (Fig. 1A,B). In initial screens, pairing cells were individually placed into and grown in 96-well plates, and then replicated into a buffer optimized for capsule formation. Of approximately 9000 clones screened, 11 mutants were identified by these methods.

A practical limit to screening large cell numbers was the need to hand-pick cell pairs before screening. Lacking a genuine selection, we sought to enrich for the desired mutants. Since wild-type cells become entrapped in secreted protein following stimulation, we exploited the difference between the mobility of *exo*⁻ (free-swimming) cells and wild type. Mass cultures of mutagenized homozygotes were divided into pools which were processed in parallel to obtain independent

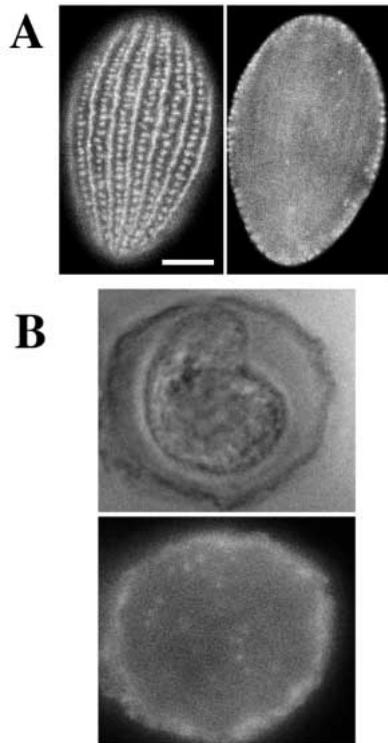


Fig. 1. Localization of DCVs in wild-type cells by indirect IF. (A) Characteristic patterns of DCVs in wild-type cells. Left, the plane of focus is at the cell surface, and DCVs appear in an ordered punctate pattern reflecting their docking at regularly spaced sites along cortical meridians. Right, the focus is shifted to the cell center. Punctate IF is limited to the cell borders, reflecting the fact that all DCVs are normally docked. (B) Cell following stimulation with Alcian Blue. Top, (visible optics), a cell can be seen trapped within a spherical capsule. Bottom, the same encapsulated cell is visualized by epifluorescence. Antibody-labeling of the capsule indicates that it consists of secreted DCV protein, while the degranulated cell is devoid of signal. Bar, 10 μ M.

isolates. The mobile fraction following stimulation was expected to be enriched in exo^- mutants, but also to contain any wild-type cells that had escaped their capsules. Such

Fig. 3. (A) Central section of MN173. Punctate IF throughout the cytoplasm reflects the presence of non-docked DCVs. The unlabeled large central spot is the macronucleus. (B) Central section of MN173 \times wild type conjugating pair, taken 1.5 hours after mixing. The IF pattern in both cells is identical, indicating that the cytoplasmic MN173 DCVs have redistributed to peripheral docking sites. (C) Central section of a pair of conjugating MN173 cells, taken 3 hours after mixing. Multiple mating types of a strain bearing a mutation of interest can be isolated as described in Materials and methods. Conjugation between the two MN173 cells does not result in redistribution of the cytoplasmic DCVs. Bar, 10 μ M.

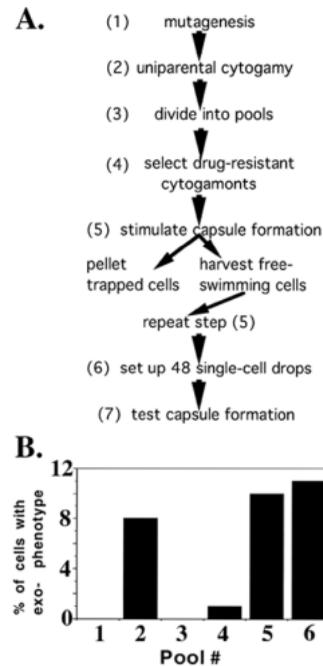
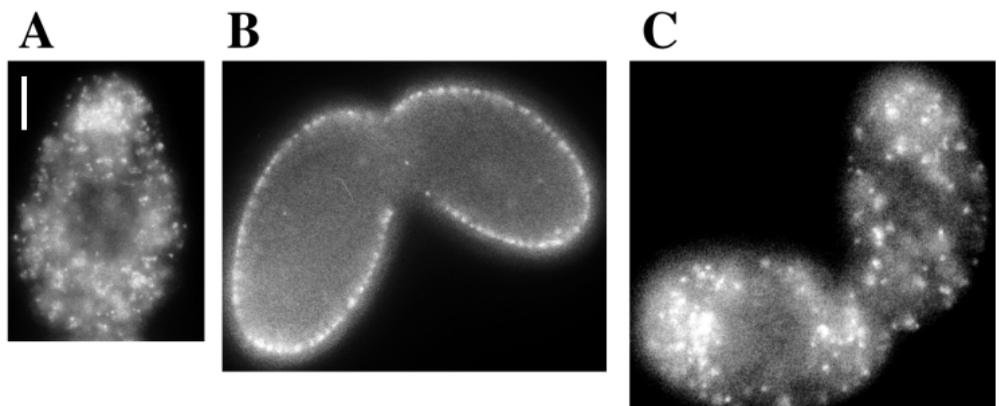


Fig. 2. (A) Outline of panning method used to enrich for exo^- mutants. (B) Exo^- mutant yield (%) following panning. 48 single cell clones from each of six pools were tested for capsule formation using Alcian Blue. Four pools each contained one or more exo^- mutant.

degranulated cells can regenerate docked secretory granules in several hours (Haddad and Turkewitz, 1997) and the panning step was therefore repeated after 8 hours. Swimming cells were isolated into drops, grown and tested as individual clones. An outline of this screen and its results are shown in Fig. 2A,B. Exo^- mutants constituted up to 10% of each pool. Most significantly, isolating 48 cells from each pool was sufficient to obtain at least one exo^- clone from $\geq 50\%$ of the pools. The efficiency of screening was therefore increased at least 17-fold, and it was not necessary to hand-pick pairs. Seven mutants were obtained by these methods. Since the screen is based on detecting protein release, the mutations may affect any step upstream of, or including, secretion. A number of the mutants

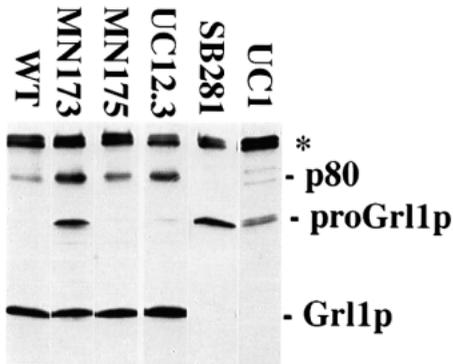


Fig. 4. Western blotting of whole cell lysates. SDS-containing lysates were prepared from the six cell lines indicated, and 250,000 cell equivalents were loaded for SDS-PAGE on a 10% polyacrylamide gel. After transfer to nitrocellulose, samples were blotted with antisera against p80 and against Gr11p (p40). The band marked by * is a non-DCV protein that is cross-reactive with the anti-p80 serum, and serves as a control for loading.

isolated appear to affect morphogenesis, resulting in accumulation of DCVs with aberrant and irregular shapes (not shown). These may be due to defects in DCV contents, but it will be difficult to assign such defects to particular steps until methods are developed for cloning of the affected genes (see Discussion). In this paper, we have therefore focused on a subset of mutants that could be evaluated using biochemical and morphological criteria.

An *exo*⁻ mutant with a defect in DCV transport

MN173 showed a complete absence of encapsulation following stimulation; a very small number of already evacuated (i.e. flimsy) capsules were seen. Cells were also tested for their sensitivity to dibucaine, a secretagogue that functions by a mechanism different from that of Alcian Blue (Orias et al., 1983). Dibucaine was also ineffective as a secretagogue, as seen by the absence of flocculent secretion seen in wild-type cells (not shown). The basis for the exocytosis defect is the defective localization of DCVs in these cells. MN173 DCVs are not docked at the plasma membrane where they can undergo rapid fusion, but instead accumulate in the cytoplasm (Fig. 3A). The DCVs themselves were morphologically wild type (Chilcoat et al., 1996). A biochemical measure of DCV maturation in wild-type cells is the proteolytic processing of core proproteins, which can be assessed by western blotting of cell lysates to detect both pro- and processed forms. Unexpectedly, MN173 showed accumulation of the proprotein form of the DCV protein Gr11p (Fig. 4), whereas in wild-type cells the precursor is rapidly and efficiently converted to the mature form. Accumulation of unprocessed proprotein is seen in cells defective in DCV assembly (Turkewitz et al., 1991), but this appears unlikely to represent a primary defect in MN173 (see Discussion). No other structures in the cells showed aberrant positioning, and the normal doubling rate suggests that organelle segregation during cell division is unimpaired.

DCV mislocalization in MN173 may be explained by a defect in transport to the cell periphery. We asked whether addition of heterologous cytosol could rescue the phenotype.

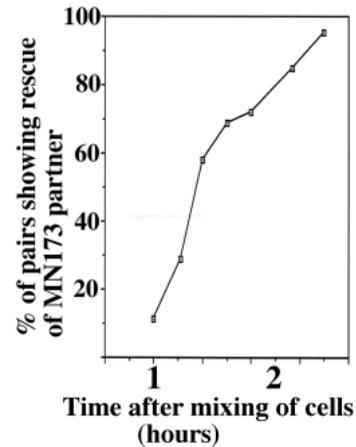


Fig. 5. Time course of conjugation rescue of the MN173 after pairing. Wild type and MN173 were mixed at $t=0$, and pair formation and rescue were measured as described in Materials and methods. Pair formation in this experiment began at approx. 1 hour and was largely complete by 2 hours.

A junction that forms between partners during mating (Maupas, 1889) permits exchange of meiotic pro-nuclei 5 hours after the onset of mating (Martindale et al., 1982); at earlier times, it permits transfer of cytosolic factors but not large organelles (Roberts and Orias, 1973). If a mutant defect can be rapidly and globally complemented by such somatic rescue, it implies involvement of a diffusible cytoplasmic factor rather than a membrane-associated protein (Beisson et al., 1980).

Synchronous pair formation was induced between MN173 and wild-type cells. Following conjugation, DCVs in MN173 were rapidly transported to the plasma membrane to produce a docked pattern indistinguishable from the wild type (Fig. 3B). Rescue depended upon the transfer of heterologous cytosol; no rescue occurred when two different mating types of MN173 were conjugated (Fig. 3C). The kinetics of rescue for a representative experiment are shown in Fig. 5. In this

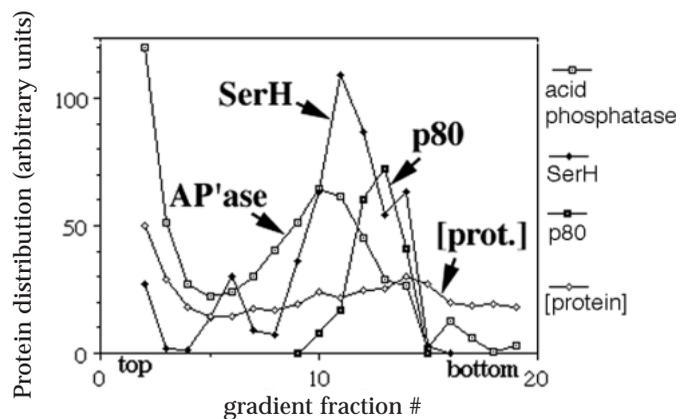
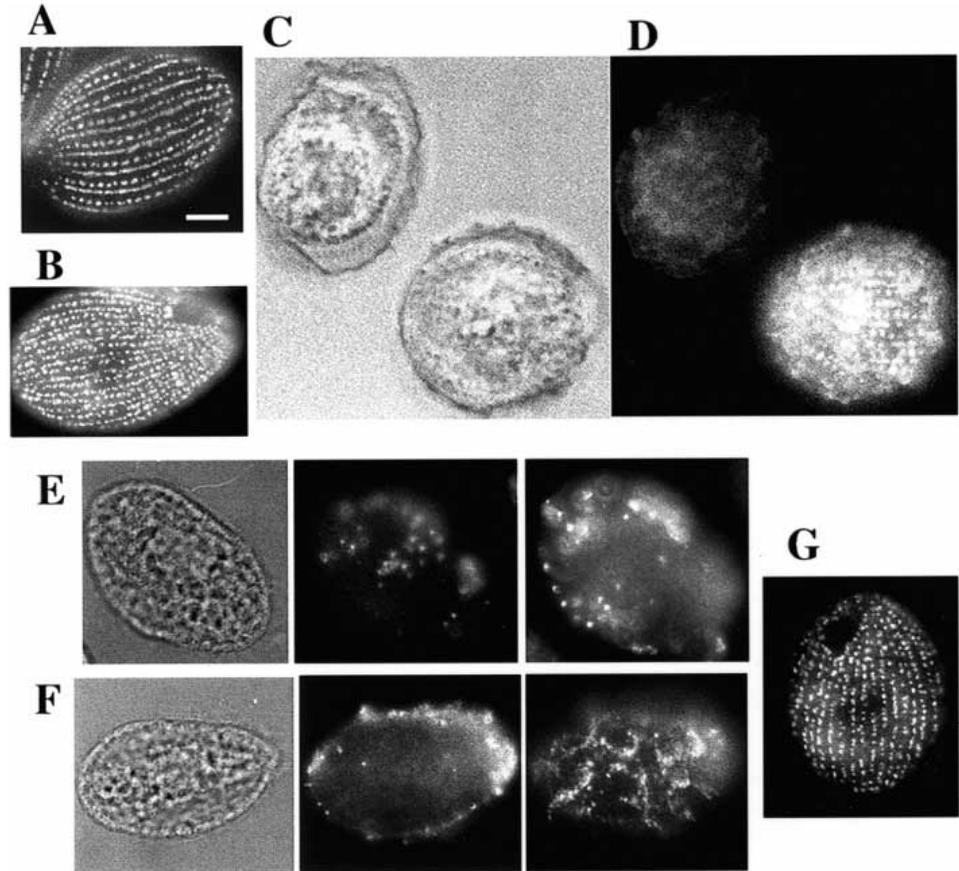


Fig. 6. Distribution of markers, separated by equilibrium density centrifugation of a post-nuclear supernatant from MN173. Distributions are shown for total protein, acid phosphatase activity, SerH (a phosphatidyl inositol-linked membrane protein) and p80 (a component of the DCV core). DCVs are most concentrated in fraction 13; the SerH peak is in fraction 11, and acid phosphatase activity is most concentrated in fraction 10.

Fig. 7. Localization of DCVs in UC12.3 and MN175 by indirect IF. The distribution of DCVs in UC12.3 (A) and MN175 (B) closely resembles that of wild type. However, following stimulation with Alcian Blue, a difference in DCV localization between wild type and UC12.3 can be seen, as shown in (C) (visible) and (D) (IF). A pair of cells is shown: the upper is wild type and the lower is UC12.3. These cells were positively identified because only the wild type had been pre-labeled with CFSE (not shown). After stimulation with Alcian Blue, both cells have formed visible capsules (C); this is observed for virtually all wild-type cells, and for approx. 5% of UC12.3. Visualization of the same pair by IF reveals a significant difference. In the upper, wild-type cell, the absence of punctate labeling reflects complete degranulation. In the lower, UC12.3 cell, the punctate pattern reflects persistence of a significant number of docked DCVs, in addition to the capsule. (E) Visible and IF images (two focal planes) of UC12.3 following 30 seconds of exposure to dibucaine, indicating extensive degranulation. (F) Visible and (two focal planes) of UC12.3 following 5 seconds of exposure to A23187, indicating extensive degranulation. (G) IF image of MN175 following dibucaine stimulation indicates no degranulation has occurred. Bar, 10 μ M.



experiment, pairing was first seen at 1 hour and reached 90% by 2 hours. The lag observed between pair formation and the rescue of DCV transport represents the time required for the rescuing factor(s) to be transferred and to fulfil their function. One can conclude from the data that this period cannot exceed 1 hour, and may be as short as several minutes.

MN173 offers a tool for *Tetrahymena* cell biology simply because cells can be homogenized without provoking exocytosis of a variable number of DCVs. In wild type, this results in release of the notoriously 'sticky' granule contents. MN173 therefore permits reliable subcellular fractionation of compartmental markers. Acid phosphatase and a phosphatidylinositol-linked cell surface protein, SerH, are two proteins whose trafficking has previously been suggested to involve DCVs (Bolivar and Guiard-Maffia, 1989; Tiedtke and Görtz, 1983). However, neither of these was concentrated in the same fraction as an authentic DCV marker, p80, when MN173 cell fractions were separated by equilibrium density centrifugation (Fig. 6). Similarly, neither SerH nor acid phosphatase was found to co-fractionate with p80 in wild-type homogenates (not shown). This suggests that DCVs are unlikely to be the major transport vesicles for these proteins.

Late stage exo^- mutants

Several exo^- mutants contain docked DCVs indistinguishable from those in wild-type cells. Two examples are UC12.3 and MN175 (Fig. 7A,B). The defects in UC12.3 and MN175 could be ordered based on several correlated criteria. Both UC12.3 and MN175 are defective in Alcian Blue-stimulated capsule

formation. Encapsulation of UC12.3 is less than 5% that of wild-type cells; MN175 is less than 0.1%. Interestingly, while encapsulated wild-type cells appear completely degranulated (Fig. 1B), all encapsulated UC12.3 cells still contain numerous docked DCVs (Fig. 7C). MN175 show no detectable degranulation (not shown), and therefore this mutation imposes a more stringent block.

Though insensitive to Alcian Blue, UC12.3 was strikingly sensitive to dibucaine, showing dramatic degranulation within seconds of exposure (Fig. 7D). This suggests that the defect in UC12.3 lies between the Alcian Blue response and the dibucaine response. Since dibucaine is likely to raise directly the level of intracellular Ca^{2+} (Matt and Plattner, 1983), UC12.3 may be defective in generating a signal leading to Ca^{2+} mobilization. Consistent with this, UC12.3 also degranulated in response to A23187, a Ca^{2+} ionophore (Fig. 7E).

In contrast, MN175 does not respond to dibucaine (Fig. 7F) or A23187 (not shown). The defective factor in MN175 is therefore proposed to function downstream of both Ca^{2+} and the UC12.3 factor.

UC12.3 and MN175 were further distinguished in their capacity to undergo somatic complementation. We crossed MN175 and UC12.3 with wild type or other mutants, and judged capsule formation and degranulation. These experiments required that we tag one partner before mixing, since the DCV patterns of the partners cannot be used to distinguish them. This was done with carboxyfluorescein diacetate succinimidyl ester (CFSE), resulting in labeling of many cellular structures at the emission wavelength of fluorescein. The 2^o antibody used to

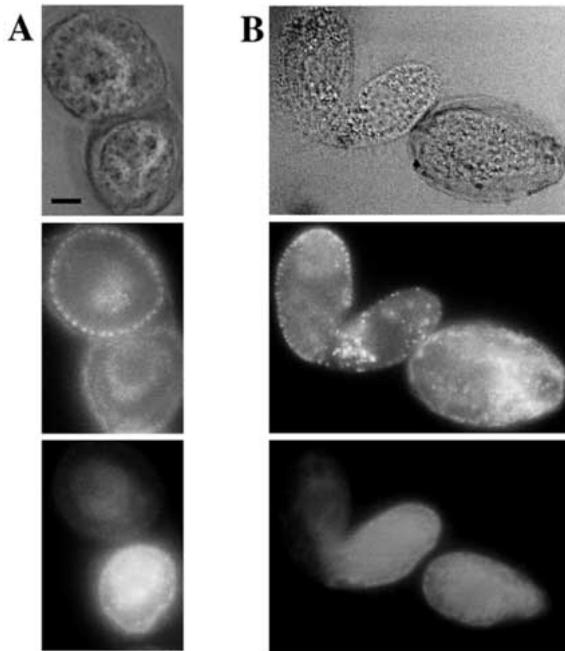


Fig. 8. UC12.3×wild type and MN175×wild type conjugating pairs, following stimulation with Alcian Blue. (A) UC12.3×wild type pair in which the wild-type cell is labeled with CFSE; fixed following Alcian Blue stimulation. Top (visible): both cells show encapsulation. Bottom: CFSE fluorescence identifies the lower cell as wild type. Central: IF indicates that the wild-type cell has undergone degranulation, while the UC12.3 cell still retains docked DCVs. (B) MN175×wild type pair (+ unpaired wild-type cell), in which the wild-type cells are labeled with CFSE; fixed following Alcian Blue stimulation. Top (visible): only the unpaired cell is encapsulated. In this case, the conjugating wild-type cell has already escaped from its capsule, which often occurs rapidly because the capsule in such pairs is open at the junction with the mutant cell. Bottom: CFSE fluorescence identifies both the individual cell and the right-hand member of the pair as wild type. Central: IF indicates that both wild-type cells have undergone degranulation, while the MN175 cell retains a set of docked DCVs. Bar, 10 μ M.

localize DCVs was rhodamine-coupled. Significant transfer of fluorescein-tagged structures occurs between conjugants, but the initially tagged cell could be unambiguously identified because of its more intense labeling and because some labeled structures are not transferred, most notably mitochondria and

cilia. CSFE labeling of control cells did not affect the progress of conjugation or capsule formation (not shown).

UC12.3 in conjugating pairs showed a markedly higher percentage of encapsulation, approx. 80% within 3 hours of mixing versus <5% in unpaired cells. This rescue did not produce complete degranulation: numerous docked DCVs are still visible in the encapsulated mutant cell, though the wild-type partner is completely emptied (Fig. 8A). The results suggest that a low level of exocytosis occurs over the entire cell surface. In contrast, MN175 cells showed no detectable degranulation or capsule formation after comparable periods of conjugation (Fig. 8B). Similarly, only UC12.3 showed rescue in MN175×UC12.3 crosses (not shown).

Although this result suggests that the MN175 defective factor is non-diffusible, the absence of somatic rescue could indicate a dominant mutation. Three observations argued against this. First, limited capsule formation was seen in conjugating MN175 cells after nuclear exchange had occurred at approx. 10 hours (not shown). This depended upon protein synthesis, since it was completely blocked by cycloheximide addition. Secondly, no inhibition of exocytosis was observed in the wild-type member of paired cells. Lastly, we crossed MN175 with wild type to produce a mixed culture of MN175 and (MN175×wild type) F1 progeny. A large fraction of this culture showed robust encapsulation upon stimulation (not shown), indicating that MN175 heterozygotes are exo^+ .

Exo⁻ mutants with defects in granule synthesis

A previously described mutant in *Tetrahymena*, SB281, contains no detectable DCVs (Fig. 9A) (Maihle and Satir, 1985) and shows aberrant processing of DCV proteins. In wild-type cells, proproteins are cleaved in the maturing granule to produce smaller polypeptides; this processing is absent in SB281 (Turkewitz et al., 1991). The SB281 defect can be at least partially rescued during conjugation, suggesting that it resides in a cytosolic factor required at a very early stage of granule formation (Sauer and Kelly, 1995).

A newly isolated mutant, UC1, is phenotypically similar to SB281. DCVs appear to be absent (Fig. 9B) and no DCV intermediates were detected by EM (not shown). Proprotein processing is also defective, as seen by the accumulation of proGr1p and absence of mature Gr1p (Fig. 4). A second granule marker, p80, appears to be absent. However, p80 epitopes may depend on processing (Turkewitz and Kelly, 1992). Although the phenotypic consequences are similar, the molecular defect in UC1 appears different from that in SB281.

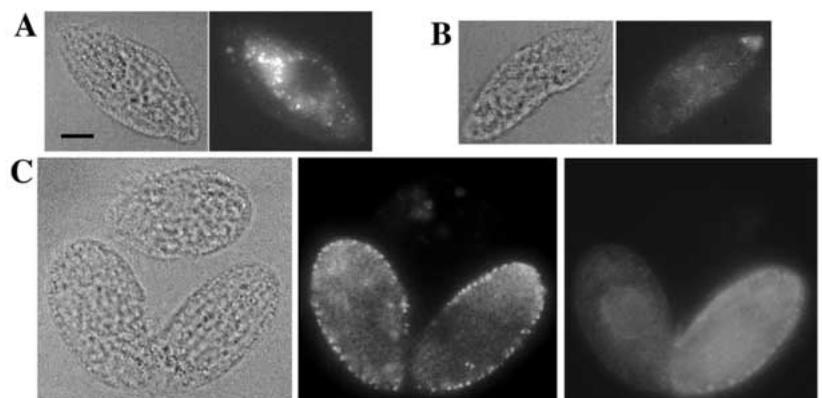


Fig. 9. DCV visualization in SB281, UC1 and SB281×UC1 pairs. (A) Visible and IF images of SB281, showing some punctate labeling limited to the cytoplasm. (B) Visible and IF images of UC1, showing very faint cytoplasmic labeling. (C) SB281×UC1 pair (+ unpaired SB281 cell), in which the UC1 cells are labeled with CFSE. Left: visible. Right: CFSE fluorescence identifies the right-hand cell as UC1, the left hand and free cells as SB281. Central panel: both SB281 and UC1 in the conjugating pair show phenotypic rescue, as shown in the appearance of docked DCVs. The unpaired SB281 cell shows faint cytoplasmic label. Bar, 10 μ M.

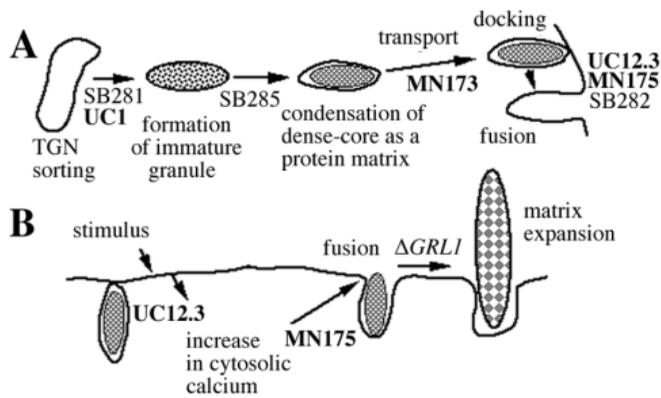


Fig. 10. Mutational dissection of the pathway of dense-core vesicle exocytosis in *Tetrahymena thermophila*. The cartoon illustrates the steps at which defects have been characterized in a set of mutant strains, including those described in this paper. (A) illustrates stages from initial DCV formation at the TGN, to fusion of docked DCVs. (B) focuses on late-acting mutations, and illustrates three successive steps following vesicle docking. References for the mutants not described in this paper are found in Turkewitz and Kelly (1992; SB282, SB285) and Chilcoat et al. (1996; $\Delta GRL1$).

Each mutant showed somatic complementation by the other (Fig. 9C). The rescue of UC1 by SB281 was as efficient as that seen with wild-type partners (not shown). While such rescue may also arise by intra-allelic complementation, it appears likely that UC1 now reveals a second cytosolic, diffusible factor required in early DCV biogenesis. These conclusions are summarized in Fig. 10.

DISCUSSION

With these results, the number of reported mutants affecting regulated secretion in *Tetrahymena* increases from 10 to 28, reinforcing the argument that *Tetrahymena* is well-suited for mutational analysis of this pathway. Two factors contributed to screening efficiency. The first was rapid generation of homozygous cells that express recessive traits (Cole and Bruns, 1992). The second was differential centrifugation to enrich for exo^- relative to exo^+ cells, since only the latter are encapsulated following stimulation under particular conditions. In our experience, 2000–3000 clones per day can be screened for capsule formation by an individual. Testing of 2000 cells (40 pools of 50) should yield at least 20 independent mutants, and multiple independent mutants may also be isolated from a single pool. The time required from initial mutagenesis through 2° screening is approximately 10 days. This should make it possible to do saturated screens in this pathway. More immediately, these techniques can be combined with novel methods for generating exo^- mutants to allow for direct cloning of the affected genes. One interesting possibility lies in induction of mutants by stable expression of antisense sequences (Sweeney et al., 1996). A small number of proteins that may be specific for granule exocytosis in mammalian cells have been identified using biochemical assays (Hay et al., 1995; Walent et al., 1992). The development of genetic tools for studying granule-specific factors in ciliates adds a new dimension to analysis of this pathway.

Since secretion is the last step in a pathway, the screen is sensitive to defects at many prior steps, as reflected in the variety of mutants that have been detected. Diffusible factors are involved at three distinct stages. First, at least two cytoplasmic factors appear to be involved in the earliest detectable phase of granule formation, and may act at the *trans*-Golgi network where DCV formation occurs (Garreau de Loubresse, 1993; Peck et al., 1993). These may be cytosolic coat proteins involved in the budding of immature secretory granules, such as clathrin (Orci et al., 1987). It should be noted that we have not yet demonstrated that each of the mutant defects is due to a single mutation. However, this appears likely in view of the complementation-rescue results; in addition, it would be consistent with previously examined cases (Gutiérrez and Orias, 1992). The normal morphology of these mutants and the absence of growth defects argue that the factors function specifically in regulated, and not constitutive, secretion. Both SB281 and UC1 maintain pools of DCV protein precursors, as indicated by accumulation of proGrl1p in an as-yet uncharacterized compartment. These precursors may be available for granule biogenesis as soon as the defective factor in each cell is supplied during conjugation.

A second cytosolic factor is involved in DCV transport to the plasma membrane, as demonstrated by the MN173 phenotype. DCV transport in these cells is an active process involving specific factors, because there is no detectable mispositioning of other organelles. Based on current knowledge of organellar traffic, this suggests the activity of cytosolic motors moving along cytoskeletal elements to the cell periphery (Rogers et al., 1997). The defect in MN173 could be in a motor, or in an interacting protein on the DCV membrane or cytoskeleton. Since transport recovered rapidly following the initiation of pairing with wild-type cells, a cytosolic factor is the best candidate. Imperfect synchrony of pair formation limits measurement of the precise rescue time, but we can conservatively conclude that complete rescue occurs within 1 hour, during which time approximately 8% of the bulk cytosol is estimated to transfer (McDonald, 1966). The rapidity of rescue in paired MN173 may suggest that the relevant factor is itself actively transported between cells. Alternatively, a small number of molecules may act enzymatically during DCV transport; for example, a motor may disengage from the granule after docking is complete. An interesting question is whether transport can be targeted for particular regions of the cell periphery. There is no indication that exocytosis normally occurs over the entire cell surface; instead, local stimulation leads to local degranulation (Erxleben et al., 1997). Given the large size of ciliate cells, efficient replacement may involve targeting the transport of newly synthesized DCVs to those same regions. One way this could be accomplished is through a transiently polarized cytoskeleton, akin to that seen in polarized cells (Mays et al., 1994).

MN173 also shows incomplete processing of a DCV proprotein, suggesting accumulation of DCVs that have not undergone complete maturation. DCV biogenesis in wild-type cells relies upon coordinated expression of a set of genes that are subject to both positive and negative regulation (Haddad and Turkewitz, 1997). The incomplete processing in MN173 seems unlikely to represent the primary defect, but may instead arise from disruption of regulatory signals whose generation depends upon docked DCVs.

UC12.3 and MN175 represent a mutant class not previously characterized in *Tetrahymena*. Both are defective in response to stimulation by Alcian Blue, though UC12.3 shows a low frequency of capsule formation. However, whereas encapsulated wild-type cells appear to be completely degranulated, degranulation of UC12.3 cells is partial. Since encapsulation does not indicate complete exocytosis, it may be necessary to consider alternative screening strategies to detect mutations that only partially inhibit exocytosis. A recently identified exocytosis-responsive promoter (Haddad and Turkewitz, 1997), linked to a structural gene conferring a selectable phenotype, could form the basis for such a screen.

The UC12.3 defect is bypassed with either dibucaine or A23187, suggesting that the mutation blocks the mobilization of intracellular Ca^{2+} in response to stimulation. Candidates for defective factors include signal transduction machinery as well as factors involved directly in Ca^{2+} release from the vesicular stores that closely underlie the plasma membrane (Erxleben and Plattner, 1994; Stelly et al., 1995). In fact, eight of the mutants recently obtained have a phenotype similar to UC12.3. The defect in UC12.3 can be partially complemented during conjugation: the frequency of capsule formation increases substantially, but complete degranulation does not occur.

MN175 has a tight exo^- phenotype as judged by capsule formation, and degranulation is not induced by either A23187 or dibucaine. The defect therefore appears to be downstream of Ca^{2+} mobilization, in a step very close to exocytosis. The mutation is recessive and the defect is not subject to somatic complementation. The factor is likely to act specifically in DCV exocytosis, based on the normal growth and morphology of the mutants.

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REFERENCES

- Beisson, J., Cohen, J., Lefort-Tran, M., Pouphe, M. and Rossignol, M. (1980). Control of membrane fusion in exocytosis: physiological studies on a *Paramecium* mutant blocked in the final step of the trichocyst extrusion process. *J. Cell Biol.* **85**, 213-227.
- Bolivar, I. and Guiard-Maffia, J. (1989). Cellular localization of the SerH surface antigen in *Tetrahymena thermophila*. *J. Cell Sci.* **94**, 343-354.
- Bonnemain, H., Gulik-Krzywicki, T., Grandchamp, C. and Cohen, J. (1992). Interactions between genes involved in exocytotic membrane fusion in *Paramecium*. *Genet.* **130**, 461-470.
- Bruns, P. J. (1986). Genetic organization of *Tetrahymena*. In *The Molecular Biology of Ciliated Protozoa* (ed. J. Gall), pp. 27-44. Academic Press, New York.
- Cassidy-Hanley, D., Bowen, J., Lee, J. H., Cole, E., VerPlank, L. A., Gaertig, J., Gorovsky, M. A. and Bruns, P. J. (1997). Germline and somatic transformation of mating *Tetrahymena thermophila* by particle bombardment. *Genetics* **146**, 135-147.
- Chilcoat, N. D., Melia, S. M., Haddad, A. and Turkewitz, A. P. (1996). Gr1p, 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. *J. Cell Biol.* **135**, 1775-1787.
- Cole, E. S. (1991). Conjugal blocks in *Tetrahymena* pattern mutants and their cytoplasmic rescue. *Dev. Biol.* **148**, 403-419.
- Cole, E. S. and Bruns, P. J. (1992). Uniparental cytogamy: a novel, efficient method for bringing mutations of *Tetrahymena* into homozygous expression with precocious sexual maturity. *Genetics* **132**, 1017-1031.
- Doerder, F. P. and Berkowitz, M. S. (1987). Nucleocytoplasmic interactions during macronuclear differentiation in ciliate protists: genetic basis for cytoplasmic control of *serH* expression during macronuclear differentiation in *Tetrahymena thermophila*. *Genetics* **117**, 13-23.
- Doerder, F. P., Lief, J. H. and Doerder, L. E. (1975). Appendix: A corrected table for macronuclear assortment in *Tetrahymena pyriformis*, syngen 1. *Genetics* **80**, 263-265.
- Erxleben, C., Klauke, N., Flötenmeyer, M., Blanchard, M.-P., Braun, C. and Plattner, H. (1997). Microdomain Ca^{2+} activation during exocytosis in *Paramecium* cells. Superposition of local subplasmalemmal calcium store activation by local Ca^{2+} influx. *J. Cell Biol.* **136**, 597-607.
- Erxleben, C. and Plattner, H. (1994). Ca^{2+} release from subplasmalemmal stores as a primary event during exocytosis in *Paramecium* cells. *J. Cell Biol.* **127**, 935-945.
- Gaertig, J. and Gorovsky, M. A. (1995). DNA mediated transformation in *Tetrahymena*. In *Cilia and Eukaryotic Flagella* (ed. W. Dentler), pp. 559-569. Academic Press, New York.
- Garreau de Loubresse, N. (1993). In *Early Steps of the Secretory Pathway in Paramecium*, vol. 2 (ed. H. Plattner), pp. 27-60. JAI Press, Greenwich, CT.
- Gutiérrez, J. C. and Orias, E. (1992). Genetic characterization of *Tetrahymena thermophila* mutants unable to secrete capsules. *Dev. Genet.* **13**, 160-166.
- Haddad, A. and Turkewitz, A. P. (1997). Analysis of exocytosis mutants indicates close coupling between regulated secretion and transcription activation in *Tetrahymena*. *Proc. Nat. Acad. Sci. USA* **94**, 10675-10680.
- Halban, P. A. and Irminger, J. C. (1994). Sorting and processing of secretory proteins. *Biochem. J.* **299**, 1-18.
- Hall, D. H. and Hedgecock, E. M. (1991). Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in *C. elegans*. *Cell* **65**, 837-847.
- Hay, J. C., Fiset, P. L., Jenkins, G. H., Fukami, K., Takanawa, T., Anderson, R. A. and Martin, T. F. J. (1995). ATP-dependent inositolide phosphorylation required for Ca^{2+} -activated secretion. *Nature* **374**, 173-177.
- Hunseler, P., Scheidgen-Kleyboldt, G. and Tiedtke, A. (1987). Isolation and characterization of a mutant of *Tetrahymena thermophila* blocked in secretion of lysosomal enzymes. *J. Cell Sci.* **88**, 47-55.
- Knoll, G., Braun, C. and Plattner, H. (1991). Quenched flow analysis of exocytosis in *Paramecium* cells: time course, changes in membrane structure, and calcium requirements revealed after rapid mixing and rapid freezing of intact cells. *J. Cell Biol.* **113**, 1295-1304.
- Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680-682.
- Maihle, N. J. and Satir, B. H. (1985). Protein secretion in *Tetrahymena thermophila*: characterization of the secretory mutant strain SB281. *J. Cell Sci.* **78**, 49-65.
- Martindale, D. W., Allis, C. D. and Bruns, P. J. (1982). Conjugation in *Tetrahymena thermophila*. *Exp. Cell Res.* **140**, 227-236.
- Matt, H. and Plattner, H. (1983). Decoupling of exocytotic membrane fusion from protein discharge in *Paramecium* cells. *Cell Biol. Int. Rep.* **7**, 1025-1031.
- Maupas, E. (1889). La rajeunissement karyogamique chez les cilies. *Arch. Zool. Exp. Gen.* **7**, 149-517.
- Mays, R. W., Beck, K. A. and Nelson, W. J. (1994). Organization and function of the cytoskeleton in polarized epithelial cells: a component of the protein sorting machinery. *Curr. Opin. Cell Biol.* **6**, 16-24.
- McDonald, B. B. (1966). The exchange of RNA and protein during conjugation in *Tetrahymena*. *J. Protozool.* **13**, 277-285.
- Orci, L., Ravazzola, M., Amherdt, M., Perrelet, A., Powell, S. K., Quinn, D. L. and Moore, H.-P. H. (1987). The trans-most cisternae of the Golgi complex: a compartment for sorting of secretory and plasma membrane proteins. *Cell* **51**, 1039-1051.
- Orias, E. and Bruns, P. J. (1976). Induction and isolation of mutants in *Tetrahymena*. *Meth. Cell Biol.* **13**, 247-282.
- Orias, E., Flacks, M. and Satir, B. H. (1983). Isolation and ultrastructural characterization of secretory mutants of *Tetrahymena thermophila*. *J. Cell Sci.* **64**, 49-67.
- Orias, E. and Hamilton, E. P. (1979). Cytogamy: an inducible, alternate pathway of conjugation in *Tetrahymena thermophila*. *Genetics* **91**, 657-671.
- Peck, R. K., Swiderski, B. and Tourmel, A.-M. (1993). Involvement of the trans-Golgi network, coated vesicles, vesicle fusion, and secretory product condensation in the biogenesis of *Pseudomicrothorax* trichocysts. In

- Membrane Traffic in Protozoa.* (ed. H. Plattner), pp. 1-26. JAI Press, Greenwich, CT.
- Pollack, S.** (1974). Mutations affecting the trichocysts in *Paramecium aurelia*. I. Morphology and description of the mutants. *J. Protozool.* **21**, 352-362.
- Roberts, C. T. and Orias, E.** (1973). Cytoplasmic inheritance of chloramphenicol resistance in *Tetrahymena*. *Genetics* **73**, 259-272.
- Rogers, S. L., Tint, I. S., Fanapour, P. C. and Gelfand, V. I.** (1997). Regulated bidirectional motility of melanophore pigment granules along microtubules in vitro. *Proc. Nat. Acad. Sci. USA* **94**, 3720-3725.
- Satir, B. H., Reichman, M. and Orias, E.** (1986). Conjugation rescue of an exocytosis-competent membrane microdomain in *Tetrahymena thermophila* mutants. *Proc. Nat. Acad. Sci. USA* **83**, 8221-8225.
- Sauer, M. K. and Kelly, R. B.** (1995). Conjugation rescue of exocytosis mutants in *Tetrahymena thermophila* indicates the presence of functional intermediates in the regulated secretory pathway. *J. Euk. Microbiol.* **42**, 173-183.
- Skouri, F. and Cohen, J.** (1997). Genetic approach to regulated exocytosis using functional complementation in *Paramecium*: identification of the ND7 gene required for membrane fusion. *Mol. Biol. Cell* **8**, 1063-1071.
- Stelly, N., Halpern, S., Nicolas, G., Fragu, P. and Adoutte, A.** (1995). Direct visualization of a vast cortical calcium compartment in *Paramecium* by secondary ion mass spectrometry (SIMS) microscopy: possible involvement in exocytosis. *J. Cell Sci.* **108**, 1895-1909.
- Sweeney, R., Fan, Q. and Yao, M.-C.** (1996). Antisense ribosomes: rRNA as a vehicle for antisense RNAs. *Proc. Nat. Acad. Sci. USA* **93**, 8518-8523.
- Tiedtke, A.** (1976). Capsule shedding in *Tetrahymena*. *Naturwissenschaften* **63**, 93-94.
- Tiedtke, A. and Görtz, H.-D.** (1983). Acid phosphatase associated with discharging secretory vesicles (mucocysts) of *Tetrahymena thermophila*. *Eur. J. Cell Biol.* **30**, 254-257.
- Towbin, H., Staehlin, T. and Gordon, J.** (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.
- Turkewitz, A. P. and Kelly, R. B.** (1992). Immunocytochemical analysis of secretion mutants of *Tetrahymena* using a mucocyst-specific monoclonal antibody. *Dev. Genet.* **13**, 151-9.
- Turkewitz, A. P., Madeddu, L. and Kelly, R. B.** (1991). Maturation of dense core granules in wild type and mutant *Tetrahymena thermophila*. *EMBO J.* **10**, 1979-87.
- van der Bliek, A. M. and Meyerowitz, E. M.** (1991). Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. *Nature* **351**, 411-414.
- Walent, J. H., Porter, B. W. and Martin, T. F. J.** (1992). A novel 145 kD brain cytosolic protein reconstitutes Ca²⁺-regulated secretion in permeable neuroendocrine cells. *Cell* **70**, 765-775.