

# ***N*-Ethylmaleimide-Sensitive Factor Is Required to Organize Functional Exocytotic Microdomains in Paramecium**

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## ABSTRACT

In exocytosis, secretory granules contact plasma membrane at sites where microdomains can be observed, which are sometimes marked by intramembranous particle arrays. Such arrays are particularly obvious when membrane fusion is frozen at a subterminal stage, *e.g.*, in neuromuscular junctions and ciliate exocytotic sites. In *Paramecium*, a genetic approach has shown that the “rosettes” of intramembranous particles are essential for stimulated exocytosis of secretory granules, the trichocysts. The identification of two genes encoding the *N*-ethylmaleimide-sensitive factor (NSF), a chaperone ATPase involved in organelle docking, prompted us to analyze its potential role in trichocyst exocytosis using a gene-silencing strategy. Here we show that NSF deprivation strongly interferes with rosette assembly but does not disturb the functioning of exocytotic sites already formed. We conclude that rosette organization involves ubiquitous partners of the fusion machinery and discuss where NSF could intervene in this mechanism.

**E**XPORT of molecules from eukaryotic cells follows a secretory pathway from the endoplasmic reticulum to the plasma membrane, which is carried out by vesicle budding from donor compartments and fusion with target compartments. The process of membrane fusion had long been a black box until complementary approaches, biochemistry in neurendocrine tissues and genetics in yeast, led to the discovery of essential protein partners. Numerous proteins and protein complexes have been discovered in these studies. A central one, *N*-ethylmaleimide-sensitive factor (NSF), has been found as a ubiquitous ATPase involved in membrane fusion events throughout intracellular traffic, including the terminal step of exocytosis at the plasma membrane. Together with soluble NSF attachment proteins (SNAPs), NSF acts as a chaperone in the disassembly of a tight complex of SNAP receptors (SNAREs). SNAREs are *trans*-membrane proteins, which are associated in *trans* configuration between the two membranes to be fused (SÖLLNER *et al.* 1993a,b; HANSON *et al.* 1997; WEBER *et al.* 2000). Yet the precise docking/fusion/recycling step at which NSF acts is still under debate (UNGERMANN *et al.* 1998; BOCK and SCHELLER 1999; JAHN and SÜDHOF 1999). Part of the discrepancies comes from the different levels of the traffic at which membrane fusion is

studied. In yeast, the most extensively studied cell type, molecular mechanisms are given for homotypic fusion of vacuolar membranes (WICKNER and HAAS 2000) and for heterotypic membrane fusion during exocytosis (GROTE *et al.* 2000). Due to differences in recycling after fusion in these pathways, the step at which NSF dissociates SNAREs can be different.

To address this question, the study of *Paramecium* has two main advantages. First, it is one of the rare cell models easily amenable to genetics, an approach with which new genes can be discovered without any *a priori* assumptions about their primary structure, location, or function. Second, in *Paramecium*, trichocyst exocytotic membrane fusion can be isolated from previous steps of the traffic pathway (reviewed by VAYSSIÉ *et al.* 2000). After biogenesis, trichocysts migrate to the plasma membrane and are anchored to predetermined sites. When examined by freeze fracture and transmission electron microscopy, the organization of a microdomain can be observed at each exocytosis site in the plasma membrane and between the two membranes to be fused. A ring of particles delineates the exocytosis site, and a rosette of particles, as well as a connecting material between trichocyst and cell membrane, marks the focal point where membrane fusion will be initiated (BEISSON *et al.* 1976; OLBRICHT *et al.* 1984; POUPHILE *et al.* 1986). These particle arrays could be the landmark of possible lipidic microdomains, as recently described (LANG *et al.* 2001). The system remains frozen in this prefusion state, until external stimulation occurs (Figure 1). Strong evidence that these microdomains are essential for membrane fusion has been provided by the isolation and study

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AJ347751 and AJ347752 for *PtNSF1* and *PtNSF2*, respectively.

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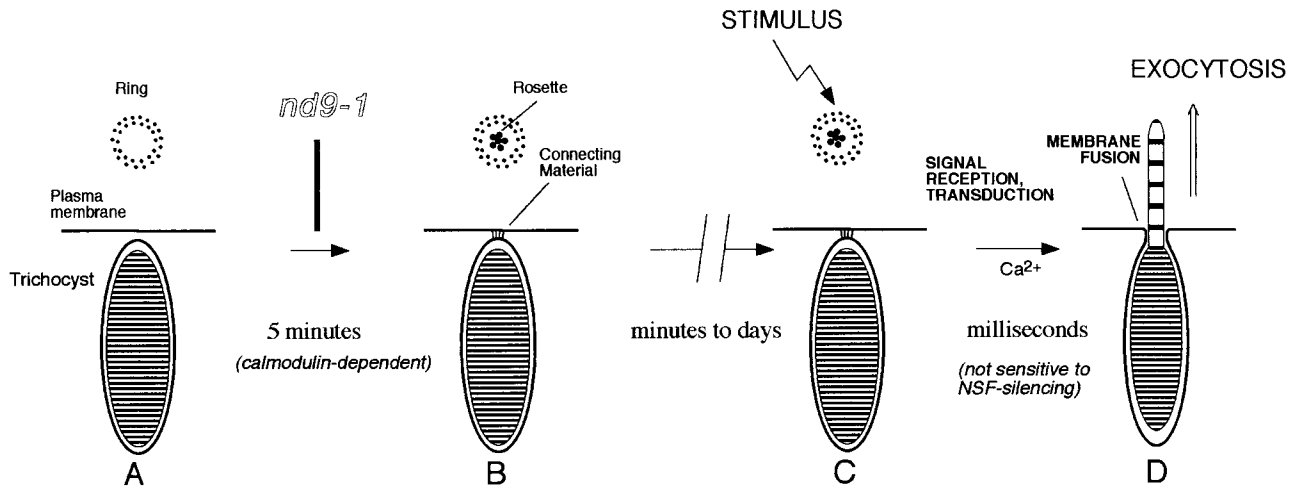


FIGURE 1.—Schematic representation of the trichocyst exocytotic pathway at the plasma membrane in *Paramecium*. (A–D) Four stages are represented on the scheme. Exocytotic sites with (A) positioned or (B) attached trichocysts or (C and D) undergoing exocytosis are shown. On the top are schematized the corresponding arrays of intramembranous particles visible in freeze-fracture electron microscopy. After trichocyst insertion at its site, small intramembranous particles are recruited to form rosette particles at the center of preexisting rings and the connecting material assembles between trichocyst and plasma membrane, in a calmodulin-dependent way (KERBOEUF *et al.* 1993). This requires only ~5 min after the arrival of a trichocyst, but for the ensemble of the docking sites in a cell, hours are required since trichocysts are docked nonsynchronously (PLATTNER *et al.* 1993). The fact that this assembly occurs hours after a shift to permissive temperature in the mutant *nd9-1* is not the result of weaker efficiency of the machinery in the mutant but of the fact that the mutant Nd9-1p molecules await a physiological change in membrane lipid composition, which takes hours to occur (BEISSON *et al.* 1980; FROISSARD *et al.* 2001). Exocytosis sites then remain unchanged, even for days (BEISSON *et al.* 1976), until a stimulus (C) triggers exocytosis, which occurs within milliseconds (D), in a way not sensitive to NSF silencing (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results).

of thermosensitive “nondischarge” (*nd*) mutants, which lack these arrays correlatively with their deficiency in exocytosis. The recent characterization of two *NSF* genes in *Paramecium* (DESSEN *et al.* 2001; R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results), *PtNSF1* and *PtNSF2*, provided new tools to link ultrastructural features pertinent to trichocyst exocytosis to current concepts of exocytosis in other systems.

Using a gene-silencing approach, we showed that NSF is essential for membrane traffic, in particular for the endoplasmic reticulum to Golgi transition, for endocytosis, and phagocytosis (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results). Apparently, no effect on trichocyst exocytosis could be detected in wild-type cells in which *PtNSF* genes have been silenced. In this work, we developed a strategy to bypass the early defects of *PtNSF* gene silencing and focus on the last steps of microdomain organization, using the thermosensitive mutant *nd9-1*, which is able to assemble rosettes when shifted to permissive temperature, independently of cell growth or even protein synthesis (BEISSON *et al.* 1980; FROISSARD *et al.* 2001). We showed that NSF action is essential for rosette assembly, suggesting that SNARE disassembly could be a prerequisite for this process.

## MATERIALS AND METHODS

**Strains and culture conditions:** The wild-type strain was *Paramecium tetraurelia* stock d4-2, derived from stock 51 (SONNEBORN 1974). The thermosensitive *nd9-1* mutant (permissive temperature <20°, nonpermissive one >25°), previously described (BEISSON *et al.* 1980; FROISSARD *et al.* 2001), belongs to the *nd* category of mutants in which trichocysts are attached to the cortex, but in which membrane fusion is impaired. Cells were grown at 27° or 18°, as indicated, in grass infusion (wheat grass powder; Pines International, Lawrence, KS), bacterized with *Klebsiella pneumoniae* the day before use, and supplemented with 0.4 µg/ml β-sitosterol (SONNEBORN, 1970).

**Monitoring exocytosis:** To visualize individual cells with their own discharged trichocysts, a saturated solution of picric acid is used as a fixing secretagogue. Discharged trichocysts remain clustered around the cell surface and can be visualized easily under dark-field light microscopy with a 10× objective.

**Homology-dependent gene silencing:** The silencing of a particular gene can be triggered experimentally in *Paramecium* by microinjection of the corresponding coding DNA, without flanking sequences, into the macronucleus of wild-type cells (RUIZ *et al.* 1998; GALVANI and SPERLING 2001). *PtNSF* gene silencing was performed in either wild-type or *nd9-1* mutant cells by microinjection into the macronucleus of concentrated PCR DNA corresponding to the open reading frame of the *PtNSF1* gene. Since silencing effect is dose-dependent and we do not accurately control the amount of DNA introduced through microinjection, we took advantage of the clear blockage in food vacuole formation to recognize and isolate silenced cells; see below.

**PCR on genomic DNA for microinjection:** The open reading

frame of *PtNSF1* was amplified from total wild-type DNA (prepared from log-phase cultures according to DUHARCOURT *et al.* 1995), using polymerase chain reaction made with the Expand Long Template PCR system kit (Roche Diagnostics, Mannheim, Germany). The reaction (50  $\mu$ l), adjusted to a concentration of nucleotides corresponding to the Paramecium A + T rich genome composition (740 nM dATP and dTTP; 260 nM dCTP and dGTP), contained 150 ng of genomic DNA, 50 pmol of each primer, and 3 units of polymerase mix. The primers used were as follows: primer 1, 5'-TCTAACTAT TACCAGTTGCTC-3'; primer 2, 5'-GGTGATTCATAATCACT GTAG-3'.

Amplification was performed with 1 cycle of denaturation (92°, 2 min); 10 cycles of denaturation (92°, 10 sec), annealing (55°, 30 sec), and extension (68°, 3 min 30 sec); and then 20 cycles of denaturation (92°, 10 sec), annealing (55°, 30 sec), and extension (68°, 3 min 30 sec + 15 sec/cycle), with a final extension (68°, 7 min).

PCR amplification of the  $\gamma$ -tubulin open reading frame was performed as described by RUIZ *et al.* (1999).

The products obtained were purified by the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), filtered on Millex-GV (0.22  $\mu$ m; Millipore, Bedford, CA), precipitated, and resuspended in water at a final concentration between 10 and 30  $\mu$ g/ $\mu$ l.

**DNA microinjection:** Microinjection of filtered concentrated PCR DNA was made under an inverted Nikon phase-contrast microscope, using a Narishige micromanipulation device and Eppendorf air-pressure microinjector. For silencing experiments in the wild type, cells were treated with a solution of aminoethyl dextran (PLATTNER *et al.* 1984) at 0.01% to stimulate trichocyst exocytosis before microinjection, to avoid further discharge that could disturb the microinjection procedure. Cells recover their full exocytotic capacity within 7 hr, a much shorter time than the minimal 18–24 hr required for silencing to appear. After microinjection, cells were cloned at 27° (or 18°, as indicated) and followed for growth rate, global phenotype, phagocytosis ability, and exocytosis. For silencing experiments on the *nd9-1* mutant, cells grown at 27° (deficient for exocytosis) were directly microinjected without prior treatment. Cells were cloned and cultured various times at 27° before shift to 18° (see RESULTS).

**Monitoring phagocytotic activity:** Living cells were observed 10 min after addition of India ink in the culture medium. Food vacuoles appear as black dots in the cytoplasm under the dissecting microscope. Control cells make approximately one food vacuole per minute and therefore appear full of black dots. In the case of phagocytosis inhibition, cells remain totally white.

**Freeze-fracture electron microscopy:** As NSF-silenced cells rapidly lose their viability, few cells are available for freeze fracturing. We therefore developed a procedure in which small pools of cells could be processed by transfer of cells in different solutions and onto copper plates for rapid freezing. To prevent unwanted stimulation during the fixation step, the cell medium was adjusted to 20 mM MgCl<sub>2</sub> with a 1 M stock solution for 3 min before transfer into 1% glutaraldehyde, 10 mM Na-phosphate buffer pH 7.0 for 1 hr at room temperature and two washes in the same buffer without glutaraldehyde. Fixation was used in this experiment since it allows rosette particles to partition into the plasmatic fracture face, like the other particles (double ring) of the exocytotic site (LEFORT-TRAN *et al.* 1978). Cells were placed onto hydrophilic 10- $\mu$ m-thick round copper plates and then rapidly covered by another plate. The rest of the treatment, including freeze fracture and electron microscopy, was as previously described by BONNEMAIN *et al.* (1992).

## RESULTS

**NSF deprivation does not interfere with membrane fusion when microdomains are already assembled:** Two *PtNSF* genes sharing 87% identity at the DNA level and 94% identity at the protein level have been identified: *PtNSF1* and *PtNSF2* (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results). In Paramecium, silencing experiments, conducted by microinjection into the macronucleus of concentrated PCR DNA corresponding to the open reading frame of the *PtNSF1* gene (see MATERIALS AND METHODS), are supposed to affect the expression of both *PtNSF1* and *PtNSF2* genes, since cosilencing is assumed for genes sharing  $\geq 85\%$  identity in nucleotide sequence [extrapolated from RNAi experiments in the nematode (PARRISH *et al.* 2000; also discussed by RUIZ *et al.* 1998)]. To assess the role of NSF in trichocyst membrane fusion, silencing experiments were performed on wild-type cells. Twenty-four hours after microinjection, silenced cells maintained at 27° have a recognizable phenotype, as shown by R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN (unpublished results). They have undergone only two fissions (instead of four in the uninjected control), appear morphologically altered, are unable to form food vacuoles, as assayed with India ink, and display altered overall membrane traffic in electron microscopy. Longer incubations provoke increase of the altered phenotype and eventually cell death within 48 hr postmicroinjection (this time is doubled if cultures are performed at 18°). Therefore, *PtNSF* genes appear to be essential in Paramecium.

When monitored with picric acid 24 hr at 27° after microinjection and even later, cells displayed normal exocytosis (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results). Small pools of cells, selected for efficient silencing by inability to form food vacuoles, were processed for rapid freezing and freeze fracture. Observation of the replica in electron microscopy showed that all silenced cells displayed normal rosettes, as illustrated in Figure 2, b–e. The silencing of NSF was therefore unable to interfere with the exocytotic process in cells where the machinery is already in place (Figure 1, C and D) before silencing is installed. In addition, this indicates that NSF silencing is unable to destabilize rosettes that are already assembled. This is in convergence with the fact that the trichocyst exocytosis sites are very stable, once built, and can remain functional, even under conditions in which they would not assemble, *e.g.*, nonpermissive temperature in thermosensitive *nd* mutants (BEISSON *et al.* 1980; KERBOEUF *et al.* 1993).

***PtNSF* gene silencing blocks exocytosis recovery in the *nd9-1* mutant:** The lack of effect of *PtNSF* silencing on preformed exocytosis sites lead to the question of its effect on the assembly of this site (Figure 1, A and



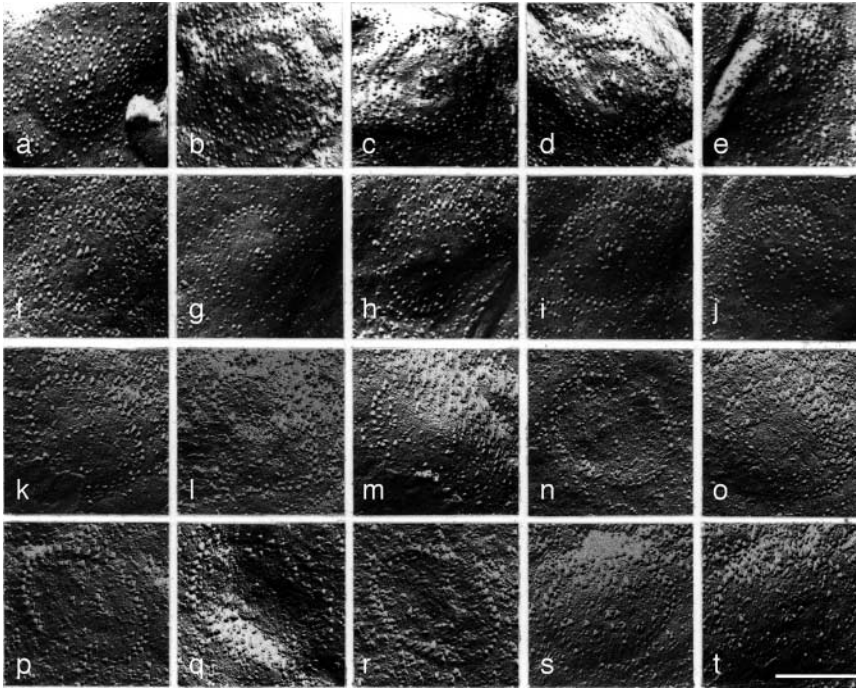


FIGURE 2.—NSF silencing effect on rosette assembly in freeze-fracture replicas. (a) Typical wild-type exocytosis site with a rosette of 13-nm intramembranous particles located at the center of a double ring of 8-nm particles (PLATTNER and ZINGSHEIM 1983; BEISSON *et al.* 1976). (b–e). No difference is seen in exocytotic sites of wild-type cells after NSF silencing. (f–j) Exocytotic sites of *nd9-1* cells 24 hr after temperature shift from 27° to 18°. Rosettes are variably assembled according to the site observed, translating the fact that exocytosis recovery is not fully completed. (k–t) Exocytotic sites of NSF-silenced *nd9-1* cells during exocytosis recovery. No rosette is assembled at all. Only one or two particles can be seen in some sites. Bar, 200 nm.

B). We first tried to follow the recovery of normal exocytosis in PtNSF-silenced wild-type cells, when sites are rebuilt after complete discharge of trichocysts using the vital secretagogue aminoethyl-dextran. However, the block in membrane traffic and in trichocyst biogenesis induced by PtNSF silencing (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results) precludes this experiment. In addition, the very fragile NSF-silenced cells are killed by the aminoethyl-dextran treatment.

To study rosette assembly under NSF deprivation, we took advantage of the genetic possibilities of *Paramecium*, with its panel of exocytosis mutants (*nd*) blocked in membrane fusion (VAYSSIÉ *et al.* 2000) in which we found the ideal one to address this question. In the thermosensitive mutant *nd9-1*, we can disconnect rosette assembly from early steps of trichocyst biogenesis, migration, and attachment to the cortex. Indeed, when *nd9-1* cells are shifted from nonpermissive (27°) to permissive (18°) temperature, rosette particles can be assembled, even in the absence of cell growth (stationary phase) and of protein synthesis (presence of puromycin). This indicates that rosette components are present in the membrane of the mutant, even at 27°, but that the mutational defect prevents their assembly and possible interactions of these components with the cytosolic protein Nd9-1p (BEISSON *et al.* 1980; FROISSARD *et al.* 2001). PtNSF gene-silencing experiments were therefore undertaken in the *nd9-1* mutant, during rosette assembly and exocytosis recovery, to isolate this particular step from all the other membrane fusion events upstream of it.

The first goal was to synchronize two different kinetics, the establishment of PtNSF gene silencing and the recovery of exocytosis upon temperature shift. After several attempts (Figure 3), a protocol was obtained in which the appearance of cells unable to form food vacuoles in silenced cells coincided with one-half of the recovery of exocytosis capacity in control *nd9-1* cells shifted to permissive temperature. *Nd9-1* mutant cells, equilibrated for at least 3 days at the nonpermissive temperature of 27° to have a fully expressed phenotype, were microinjected in their macronucleus with the coding DNA corresponding to the PtNSF1 gene. Cells were cultured at this temperature for another 16 hr before being shifted to 18°. Twenty-four hours later, transformed cells with silenced phenotype (no food vacuole formation) were obtained. These cells were unable to discharge their trichocysts (Figure 3, experiment 3).

To be sure that this defect in exocytosis recovery was not due simply to general metabolism alteration in silenced cells committed to die, we made a control experiment using  $\gamma$ -tubulin-silenced cells. Indeed, such cells stop duplicating their ciliary basal body, yield smaller cells after division, and eventually die after two fissions (RUIZ *et al.* 1999). *nd9-1* cells preequilibrated at 27° were microinjected with  $\gamma$ -tubulin-silencing DNA, left for 24 hr at 27°, and transferred to 18° when silencing was already expressed, as assessed by the small size of the cells. Twenty-four hours later, both control and silenced cells underwent exocytotic recovery, indicating that this process was unaffected when cells were close to death for a reason other than PtNSF gene silencing.

**Effect of PtNSF gene silencing at the ultrastructural**

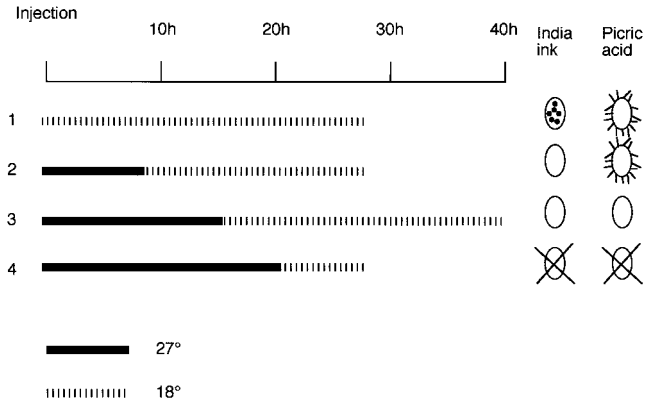


FIGURE 3.—Synchronization of *PtNSF* gene silencing with exocytosis recovery in *nd9-1* cells. *nd9-1* cells preequilibrated at 27° for 3 days were microinjected with *PtNSF* gene-silencing DNA and shifted at various times from 27° to 18° after microinjection. In all cases, uninjected control *nd9-1* cells (not shown) were able to ingest India ink in food vacuoles and in experiments 1–3, partial exocytosis of 50–300 trichocysts was recovered. The time of exposure at 18° in experiment 4 was too short to initiate recovery. *PtNSF* gene silencing was inefficient in experiment 1 because the full time at 18° slowed down its establishment. *PtNSF* gene silencing was efficient in experiment 2 (as revealed by the incapacity to form food vacuoles) but appeared too late compared to exocytosis recovery. In experiment 3, we obtained (i) a good synchrony between silencing establishment and exocytosis recovery in control cells and (ii) a clear-cut negative effect of NSF silencing on trichocyst exocytosis. In experiment 4, the cells died before the test, suggesting that silencing was already triggered after 21 hr at 27° and that this is rapidly lethal for cells submitted to a temperature shift, which induces thorough membrane traffic.

**level in the *nd9-1* mutant:** The data accessible using freeze fracture of *Paramecium* plasma membrane are twofold. They permit determination of the proportion of sites occupied by a trichocyst and, among these sites, checking of the appearance of the rosettes. The state of occupancy of exocytotic sites can be determined easily since parenthesis-shaped particle arrays denote empty sites, free of trichocysts, whereas ring-shaped arrays indicate the presence of a trichocyst anchored at the site (BEISSON *et al.* 1976). We counted the numbers of parentheses and rings in control and *PtNSF1*-silenced cells submitted to the temperature shift. Out of 119 observable sites in controls, 36 (30%) were parentheses and 83 (70%) were rings. In silenced cells, out of 136 sites, 81 (60%) were parentheses and 55 (40%) were rings. When silencing is established, no new trichocyst can be formed and attached to the cortex [because the traffic is blocked very early, at the step of vesicle budding from the endoplasmic reticulum (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results)]. Hence, the total number of rings remains constant upon residual cell growth and division, yielding a decreased proportion in each descent cell. The fact that the number of rings in silenced cells is

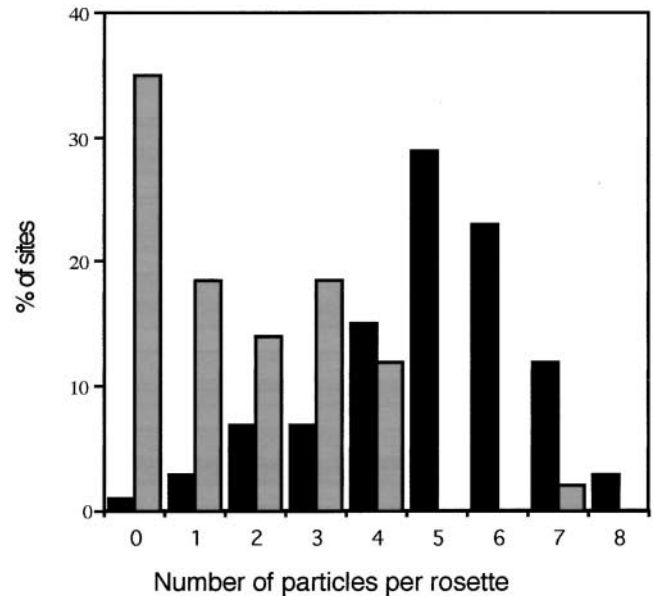


FIGURE 4.—Effect of *PtNSF* gene silencing on rosette assembly in *nd9-1* cells shifted to 18°. The histogram plots the percentage of rings against the number of rosette particles they contain. Solid bars show the control *nd9-1* strain (73 sites) and shaded bars show the *PtNSF*-silenced ones (49 sites).

roughly one-half that of control suggests that only one cell division has occurred after silencing establishment.

Second, exocytotic sites with attached trichocysts (rings) normally display a rosette of approximately eight particles in wild-type cells, indicating that the site is functional and can undergo membrane fusion. Mutant sites in *nd* cells, such as *nd9-1* at 27°, are devoid of rosette particles. From an intermediate situation in which only a few particles are assembled, for instance, during temperature shifts of thermosensitive *nd* mutants, it has been assumed that more than four particles per rosette are needed to allow exocytotic membrane fusion (LEFORT-TRAN *et al.*, 1981). It was crucial to know whether the failure of trichocyst discharge recovery in silenced *nd9-1* cells was due to a failure of rosette assembly or to a downstream block. Among the ring-shaped sites, 73 were analyzable for presence of rosette particles in control cells and 49 in *PtNSF*-silenced cells. The repartition of the number of rosette particles per site clearly shows that rosette formation is inhibited in *PtNSF*-silenced cells (Figure 4), as illustrated in Figure 2, f–j (control cells) and k–t (*PtNSF*-silenced cells). The fact that these cells do not discharge any trichocyst also confirms the observation that more than four rosette particles are necessary to allow membrane fusion.

## DISCUSSION

*Paramecium* is a unicellular organism with many differentiated functions in the same cytoplasm and displays a complex role of membrane traffic orchestrated through

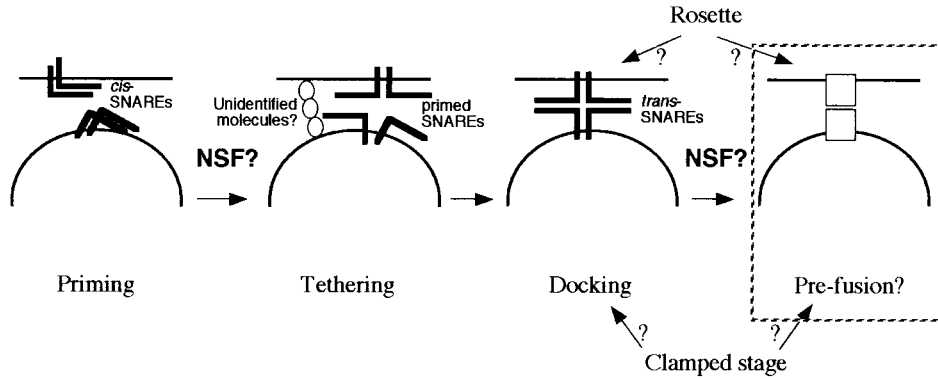


FIGURE 5.—Molecular events proposed for trichocyst docking and membrane fusion. The cartoon integrates features generally accepted or hypothesized to explain events in the scheme of rosette assembly in *Paramecium*, as shown in Figure 1, A and B. Concomitant or successive steps of SNARE priming (*cis*-SNARE complex disassembly) and trichocyst tethering to the plasma membrane precede docking (*trans*-SNARE interactions). As discussed in the text, protein complexes downstream from *trans*-SNARE assembly have been postulated in some systems (UNGERMANN *et al.* 1998; PETERS *et al.* 2001) and are represented here as a box. Therefore, NSF may have two possible sites of action: (i) to disrupt *cis*-SNAREs before tethering/docking and (ii) to disrupt *trans*-SNARE downstream interactions, if they do exist, to perform membrane fusion. The stage that is clamped (primed) in *Paramecium*, identifiable by the presence of a rosette, is either the *trans*-SNARE complex or any downstream complex. In either case, the rosette is a visualization of the presence of such a complex.

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the NSF-associated machinery (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results). The question remained to know whether an NSF-associated machinery was also involved in the terminal step of regulated exocytosis, namely trichocyst discharge, and more precisely in the assembly of specialized arrays defining a microdomain visible in electron microscopy, the rosette. Previous work did not give any clues in this direction since the few *ND* genes already cloned by functional complementation all appeared to be novel for membrane fusion (*ND7*, SKOURI and COHEN 1997; *ND9*, FROISSARD *et al.* 2001). In addition, NSF silencing in wild-type cells did not give any effect on stimulated exocytosis of trichocysts already attached to the cortex (R. KISSMEHL, M. FROISSARD, H. PLATTNER, M. MOMAYEZI and J. COHEN, unpublished results; this work).

The existence of *Paramecium* conditional mutants such as *nd9-1*, in which rosette assembly can be specifically manipulated, permits unique insights into the role of essential proteins, such as NSF, in the very last steps of exocytotic membrane fusion. *nd9-1* cells silenced for NSF at the restrictive temperature were unable to recover exocytosis and to assemble rosettes when shifted to the permissive temperature. Two conclusions can be drawn from these results. First, like most other membrane fusion events, trichocyst exocytosis requires an NSF/SNAP/SNARE machinery. Second, at least one NSF-dependent stage lies upstream from rosette assembly whereas the steps downstream from rosette assembly up to membrane fusion seem less sensitive to NSF deprivation (see the synthetic scheme in Figure 5), although it is not excluded that NSF tightly bound to exocytotic sites remain active during silencing experiments.

The direct role of NSF is to disassemble complexes between v-SNAREs and t-SNAREs, although the precise step within the sequence of docking-priming-fusion-recycling at which the disassembly takes place is still

being debated (HANSON *et al.* 1997; UNGERMANN *et al.* 1998; BOCK and SCHELLER 1999; JAHN and SÜDHOF 1999). Two main classes of membrane fusion occur in the cell. One, called homotypic, involves identical partner membranes (as is the case with vacuoles in yeast); the other one, called heterotypic, involves different membranes (*e.g.*, endoplasmic reticulum with Golgi membrane, secretory vesicle with plasma membrane). In homotypic fusion, it is known that v- and t-SNARE complexes exist *in cis* on the membrane and have to be dissociated to allow interaction *in trans*. This is less evident in heterotypic fusion, for which such complexes need to be disassembled only after fusion, when they become *cis*-complexes on the mixed membrane.

Here, we found NSF action to be essential for rosette assembly, indicating that SNARE complex disassembly occurs prior to rosette particle interactions. Two possible targets are discussed for the action of NSF in trichocyst exocytosis: *cis*-SNARE complexes that may exist before membrane interaction or *trans*-SNARE complexes assembled during this interaction.

On the one hand, there is strong evidence for a priming role of NSF and  $\alpha$ -SNAP through *cis*-SNARE complex disassembly (HANSON *et al.* 1997; XU *et al.* 1999). This role is supported by the observations of WEBER *et al.* (2000) that only *cis*-SNARE complexes can be disassembled by NSF and  $\alpha$ -SNAP, in this case after membrane fusion. Such a role is possible in trichocyst exocytosis, if *cis*-SNARE complexes actually exist on trichocyst and/or plasma membranes, although it is heterotypic membrane fusion. In this case, rosette particles would reflect the presence of *trans*-SNARE complexes.

On the other hand, other studies plead in favor of a role of *trans*-SNARE complex disassembly before membrane fusion. First, mutant  $\alpha$ -SNAPs that prevent the stimulation of the ATPase activity of NSF, but not the binding to the SNARE complex, prevent disruption of this complex, and exocytotic membrane fusion, al-



though the wild-type molecule drives both events (BARNARD *et al.* 1997). Second, in homotypic fusion of vacuolar vesicles in yeast, *trans*-SNARE complexes have been shown to be disassembled by NSF and  $\alpha$ -SNAP (UNGERMANN *et al.* 1998), without interference with docking at the plasma membrane or downstream membrane fusion. In this case, rosette particles would be post-SNARE complexes.

Recently, it has been proposed that complexes between V0 subunits of proton pump ATPases in both membranes represent a calmodulin-sensitive post-SNARE fusion machinery (PETERS *et al.* 2001). In this context, the hypothesis of a role of NSF in *trans*-SNARE disassembly before ultimate proteolipid channel interactions seems very attractive for trichocyst exocytosis. Rosette particles visible in fractured membranes are more likely to correspond to membrane proteins with several transmembrane domains such as V0 than to SNARE proteins with single transmembrane helices. In addition, the assembly of the rosette is dependent on calmodulin (KERBOEUF *et al.* 1993).

The connections between membranes visible in electron microscopy might be not restricted to *Paramecium*, as they could correspond to a transient step in membrane fusion in other models. Indeed, such intermembrane links and intramembranous particles have sometimes been caught (AUNIS *et al.* 1979; HEUSER *et al.* 1979; CHANDLER and HEUSER 1980). This aspect, which makes it possible to focus on the mechanisms occurring in a space limited to a few cubic nanometers within the last milliseconds encompassing triggered membrane fusion, highlights the interest in *Paramecium*. Owing to the synergy between genetics and postgenomics, using gene silencing, *Paramecium* appears now to be the ideal model to unravel pre- and post-SNARE events in heterotypic membrane fusion.

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