CONTROL OF MEMBRANE FUSION IN EXOCYTOSIS

Physiological Studies on a Paramecium Mutant Blocked in the Final Step of the Trichocyst Extrusion Process

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ABSTRACT

Previous studies on exocytosis in Paramecium using mutants affecting trichocyst extrusion permitted us to analyze the assembly and function of three intramembrane particle arrays ("ring" and "rosette" in the plasma membrane, "annulus" in the trichocyst membrane) involved in the interaction between these two membranes.

Using a conditional mutation, nd9, which blocks rosette assembly and prevents exocytosis at the nonpermissive temperature, we have analyzed the effect of temperature on the secretory capacity of nd9 cells. By combining several techniques (physiological studies, microinjections, inhibition of fatty acid synthesis, and freeze-fracture analysis) we demonstrate (a) that the product of the mutated allele nd9 is not thermolabile but that its activity is dependent upon temperature-induced changes in the membrane lipid composition and (b) that the product of the nd9 locus is a diffusible cytoplasmic component whose interaction with both plasma membrane and trichocyst membrane is required for rosette assembly and exocytosis.

The data provide physiological evidence for the existence of a molecular complex(es) linking the two membranes and involved in the control of membrane fusion; we discuss the possible nature and function of these links.

For three main reasons, the trichocysts of Paramecium constitute a model system for analysis of the mechanisms of exocytosis and membrane fusion. Firstly, a number of mutations are available that block the trichocyst cycle at various stages (trichocyst development, attachment to the plasma membrane, or exocytosis). Secondly, exocytosis can be triggered at will and observed under the light microscope at low magnification. Thirdly, before exocytosis (i.e., the fusion of trichocyst membrane with plasma membrane), the region of contact between the two membranes is marked by several ultrastructural differentiations whose presence and/or organization can be affected by mutation and whose function can therefore be studied.

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These ultrastructural differentiations, which have been described by various authors (2, 5, 16, 26, 31), are the following: (a) On the plasma membrane, at the sites where trichocysts are normally attached and rest until their excretion is triggered, two intramembrane particle arrays are revealed by freeze-fracture: a double “ring,” 300 nm in diameter, of ~80 particles, and, in the center of the ring, a “rosette” of 8–10 larger particles. (b) On the tip of the attached trichocyst, an “annulus” of several tightly packed rows of particles is present. (c) Between trichocyst tip and plasma membrane, some electron-dense material referred to as “connecting material” apparently linking the region of the rosette and the apex of the trichocyst membrane, is visible on favorable thin sections.

Previous analysis of a first set of three mutants, t1, tam6, and nd9 (6), provided some information about the assembly and/or function of the ring and rosette on the plasma membrane. (a) Before any interaction with the trichocyst, a precursor of the ring (in the form of a “parenthesis”) is present in the plasma membrane at each presumptive site of trichocyst attachment. (b) Upon attachment of the trichocyst to the plasma membrane, the parenthesis is transformed into a ring and the central rosette is assembled. (c) The rosette is necessary for membrane fusion and exocytosis.

By electron microscopy, cytochemistry, and x-ray microanalysis, Plattner et al. (27), demonstrated the presence of a Ca++-activated ATPase activity at the site of trichocyst attachment to the plasma membrane. The use of a range of mutants enabled the Ca++-ATPase activity to be more precisely located in the rosette.1

In the experiments reported here, we analyze the site and model of action of a thermosensitive mutation, nd9. This mutation has already been shown to block rosette assembly, appearance of the connecting material, and Ca++-ATPase activity at the nonpermissive temperature (5, 23; footnote 1). Our results show that the product of the mutated gene nd9 is abnormal at both permissive and nonpermissive temperatures and that its conditional activity is dependent upon the lipid composition of the membranes. We confirm that the mutation affects a cytoplasmic component whose wild-type form can restore, by microinjection, exocytotic capacity in nd9 cells grown at the nonpermissive temperature as first observed by Außerheide (3), and we show that this cytoplasmic factor is readily diffusible. We demonstrate that this cytoplasmic factor (product of the locus nd9), which is necessary for rosette assembly and membrane fusion, interacts with both trichocyst membrane and plasma membrane. These results suggest that membrane fusion would depend upon the activity of a molecular complex linking the two membranes. We discuss the possible function and nature of this molecular complex and its relationships with rosette assembly.

MATERIALS AND METHODS

Strains

The wild-type strain from which all mutants were derived was stock d4-2 of P. tetraurelia, a derivative of stock 51 carrying the allele k in the stock 51 genetic background. The following nuclear mutations were used: nd9, previously described (5), is a thermosensitive mutation characterized by normal attached trichocysts unable to be excreted at the nonpermissive temperature (27°C), and excreted at the permissive temperature (18°C). tam 38, previously described (30), is characterized by abnormal trichocysts unable to attach to the plasma membrane; as shown by microinjection experiments, the mutation affects the trichocysts but neither the cytoplasm nor the plasma membrane.2 tam 6, previously described (6), is characterized by normal trichocysts unable to attach to the plasma membrane. This mutation is somewhat leaky; a minority of trichocysts can attach and be excreted; the mutational defect lies in the plasma membrane, whereas the trichocysts are functionally normal. The three mutations correspond to three independent loci (M. Rossignol and J. Beisson, unpublished observations).

Aside from the three strains respectively homozygous for the nd9, tam 6, and tam 38 mutations, two double mutant strains were constructed: tam 38–nd9, in which the tam 38 phenotype (unattached abnormal trichocysts) is epistatic to the nd9, and tam 6–nd9, in which the tam 6 mutation (normal unattached trichocysts) is epistatic to the nd9 mutation.

Culture Conditions

Cells were grown according to the usual procedure (33) in a Scotch grass infusion or in Cerophyline infusion bacterized the day before use with Klebsiella pneumoniae. The standard temperature of growth was 27°C, unless otherwise specified. Cultures referred to as grown at 27° or 18°C were maintained at the designed temperature for ≥10 fissions before the beginning of the experiment.

1 Plattner, H., K. Reichel, H. Matt, J. Beisson, M. Pouflchile, and M. Lefort-Tran. Genetic dissection of the final exocytotic steps in Paramecium cells: cytochemical localization of Ca++-ATPase activity over preformed exocytosis sites. J. Cell Sci. Manuscript submitted for publication.

2 Lefort-Tran, M., M. Pouflchile, M. Rossignol, K. Außerheide, and J. Beisson. Genetic analysis of membrane differentiation in Paramecium. Mutations affecting the organization and function of exocytosis sites. Manuscript in preparation.
**Test of Discharge Ability**

The capacity of cells to excrete trichocysts was assayed by the routine picric acid test (28). One drop of a saturated solution of picric acid was added to one drop of cell suspension on a slide, then examined under the microscope (dark field) at a low magnification (× 100). The number of excrated trichocysts was counted or estimated, as illustrated in Fig. 2.

**Microinjection Technique**

The technique used was that described by Knowles (18) and Koizumi (20) and first applied to microinjection of trichocysts by Aufderheide (3).

As previously demonstrated (3), this method permits one to determine whether a particular mutation causes an autonomous defect of the trichocyst, an autonomous defect of the nontrichocyst compartment (cortex and/or cytoplasm), or a nonautonomous defect, repairable in the mutant upon injection of wild-type cytoplasm. All the studied mutants so far analyzed fall into these three categories, and the mutation nd9 belongs to the third one: 27°C nd9 cells, unable to excrete their trichocysts, regain discharge ability when injected with wild-type cytoplasm (3).

The recipient cell is injected with a small volume (~5,000 µm³) of “cytoplasm” sucked out of the donor cell immediately before injection. This sample of injected cytoplasm may contain a few trichocysts; when the sample is taken from a tam 6 cell loaded with unattached trichocysts, it generally contains dozens of trichocysts. In contrast, when the sample is taken from a cell carrying the tam 39 mutation (abortive trichocysts), it can be considered as devoid of trichocysts. According to Aufderheide’s method (3), microinjected cells were tested individually for their discharge ability by the picric acid test 2 h after injection.

**Electron Microscopy and Freeze-Fracture Techniques**

**nd9 Cells**

Cells cultivated at 15°C (control cells and cerulenin-treated cells) were gently harvested by centrifugation. The pellets, cooled for 90 s at 0°C, were incubated for 90 min at 0°C in 10 mM phosphate buffer, pH 7.1, containing 0.5% glutaraldehyde, then washed, transferred to increasing concentrations of glycerol in the same buffer until a 30% final concentration was reached, and kept overnight at 4°C.

Samples were frozen in liquid Freon 22 cooled by liquid N₂, and stored in liquid N₂. Freeze-fracture was performed by standard techniques using a Balzers apparatus (BA 360 M; Balzers Corp., Nashua, N. H.) at ~100°C. The replicas were examined in a Hitachi HU 12 A electron microscope at 75 kV.

**Cerulenin Treatment**

Cerulenin, purchased from Makor Chemical Co., Jerusalem, Israel, was prepared and kept at 4°C as a stock solution (10 mg/ml) in 30 mM phosphate buffer, pH 7.2, and added to the cell suspension to a final concentration of 25 µg/ml. At this concentration, the effect of the drug on growth and survival is the following: (a) When the drug was applied to growing populations maintained at constant temperature (27° or 15°C), multiplication was blocked without any notable death over a 24-h period; (b) when applied to populations transferred from 27° to 15°C, addition of the drug was lethal for growing populations but not for cells in stationary growth phase, at least over a 48-h period. Consequently, stationary phase cells were used to analyze specific effects of the drug on secretory processes.

**RESULTS**

The mutant nd9 displays a thermosensitive capacity for exocytosis: when grown at 27°C or above, nd9 cells are unable to excrete their trichocysts, although they appear structurally normal and are “attached” to the plasma membrane; when grown at 18°C or below, nd9 cells discharge their trichocysts normally upon appropriate stimulation. These alternative phenotypes, (exo)/(exo+), are paralleled by the absence/presence of rosettes in the plasma membrane (5) and by the absence/presence of Ca++-ATPase activity and connecting material (see footnotes 1 and 3). The connecting material, shown in Fig. 1, is visible only on favorable thin sections, i.e., in sections exactly perpendicular to the plasma membrane (pm), between the subpellicular alveoli (a). Between the plasma membrane and the trichocyst tip, an electron-dense connecting material (arrow) is visible on favorable section planes. × 110,000.

1 Matt, H., H. Plattner, K. Reichel, M. Lefort-Tran, and J. Beisson. Genetic dissection of the final exocytosis steps in *Paramecium* cells: trigger experiments. *J. Cell Sci.* Manuscript submitted for publication.
icular to the cell surface along the trichocyst main axis, in their narrow zone of contact. In contrast, the presence of rosettes, visible on P-fracture faces of the plasma membrane, at each trichocyst docking site, may be taken as a most reliable cytological index of exocytosis capacity in nd9 cells. Because in wild-type cells rosette formation is induced upon trichocyst attachment to the cell surface, the absence of rosettes in nd9 cells at nonpermissive temperatures might result a priori either from a defect in the plasma membrane, from a defect in the trichocyst, or from a defect in some nonmembrane interacting factor. To determine the site of action of the mutation nd9 and to dissect the factors involved in rosette assembly and exocytosis capacity, we have analyzed the (exo⁺) => (exo⁻) phenotypic changes in nd9 cells in four ways. (a) We have studied the variation of the phenotype of nd9 cells as a function of temperature. (b) We have investigated by "complementation" experiments whether the product of the gene nd9 is normal at permissive temperatures. (c) We have studied the effects of cerulenin, an inhibitor of fatty acid biosynthesis (23), on the assembly of the rosettes in nd9 cells transferred from the nonpermissive to the permissive temperature. (d) We have analyzed by microinjection experiments the site of action of the mutation.

Variation of the Phenotype of nd9 Cells as a Function of Temperature

The phenotype of nd9 cells, i.e., their capacity to excrete trichocysts, is determined by the picric acid test (see Materials and Methods). As shown in Fig. 2, a range of responses can be observed, from absence of any expelled trichocysts to the formation of a dense fringe around the cell, composed of hundreds of excreted trichocysts (wild-type or sub-wild-type response). No attempt was made to count precisely the excreted trichocysts, and the phenotypes were classified in five categories (0, 1, 2, 3, and 4) as defined in the legend of Fig. 2.

The effect of temperature on the nd9 phenotype was first studied in populations maintained under stable temperature conditions. Fig. 3 depicts the distribution of phenotypic categories in exponential or early stationary growth phase after 2-4 d of continuous growth at a given temperature, between 18°C and 27°C. 18°C-20°C defines the upper boundary of permissive temperatures at which all cells belong to categories 3 and 4; 26°C-27°C defines the lower boundary of nonpermissive temperatures at which all cells belong to category 0. Between 27°C and 18°C, nd9 cells are capable of excreting more and more trichocysts as temperature decreases; however, this evolution is not linear, and a sharp transition between leaky (exo⁻) phenotypes and sub-wild-type phenotypes is observed at 22°C. In "aging" cells, i.e., beyond 2-5 d in stationary phase, the mutant phenotype becomes leakier: for instance, at 27°C, the phenotype will shift to type 1 class, or at 22°C, the phenotype will shift to types 3 and 4.

When the cells are transferred from permissive to nonpermissive temperature or vice versa, a com-
Phenotypic evolution of nd9 cells as a function of temperature. Each histogram represents the distribution of phenotypes, as defined in Fig. 2, within a population of nd9 cells, in exponential or early stationary phase of growth, observed after stabilization at each temperature.

Complete change of phenotype \((\text{exo}^-) \leftrightarrow (\text{exo}^+)\) is regularly observed; the kinetics of this transformation have been studied. Temperature shifts, \(27^\circ \text{C} \) to \(18^\circ \text{C}\) or \(13^\circ \text{C}\), and \(18^\circ \text{C} \) to \(27^\circ \text{C}\), were applied to either exponentially growing cells or stationary phase cells. Table I gives the results of one set of such experiments, which are consistent with the results obtained in three other series of experiments. Three main facts emerge from these data. (a) In exponentially growing cells (Table I A), the change from the \((\text{exo}^-)\) towards the \((\text{exo}^+)\) phenotype proceeds more rapidly, in terms of number of cell generations, than the reverse change. Conversely, in stationary phase cells (Table I B), the \((\text{exo}^-)\) phenotype is lost in most cells after 96 h at the permissive temperature, whereas the \((\text{exo}^+)\) phenotype is fully retained after 125 h at the nonpermissive temperature. (b) The change from \((\text{exo}^-)\) to \((\text{exo}^+)\) phenotype proceeds faster in shifts from \(27^\circ \text{C} \) to \(13^\circ \text{C}\) than it does in shifts from \(27^\circ \text{C}\) to \(18^\circ \text{C}\). In particular, most of cells become \((\text{exo}^+)\) in less than one cell generation after transfer to \(13^\circ \text{C}\). (c) Until completion of the phenotypical change, the population remains heterogeneous: the recovery (or loss) of secretory capacity is not in all-or-none phenomenon at the cell level, but most probably affects each trichocyst attachment site individually.

**Analysis of the Restoration of the Secretory Capacity of nd9 Cells at Permissive Temperature**

The thermosensitive expression of the nd9 mutation can be a result of either the conditional activity of a thermolabile polypeptide coded by the gene nd9 or an indirect effect of temperature on the activity of this gene product. In the following experiments, we analyzed whether the product of the gene nd9 was functionally active at permissive temperatures and whether the lipid composition of the membrane affected the expression of the nd9 mutation.

**Permissive Temperature Does Not Restore a Normal nd9 Gene Product**

If the product of the gene nd9 had a normal activity in nd9 cells grown at the permissive temperature, it should be able to restore secretory capacity in \(27^\circ \text{C} \) nd9 cells, as does wild-type or nd9+ cytoplasm injected into \(27^\circ \text{C} \) nd9 cells (3) (see Material and Methods). To ascertain the properties of the cytoplasm in \(18^\circ \text{C} \) nd9 cells, we
carried out two types of experiments: "natural" injection of 18°C nd9 cytoplasmic factors, and microinjection of cytoplasm taken from 18°C nd9 cells devoid of functional trichocysts.

(a) Natural injection. It is known that during conjugation, in which the two mates remain tightly attached for 5–6 h, molecules are exchanged between the partners (7). Except for the migratory gametic nuclei, no organelles such as mitochondria or trichocysts are exchanged under normal conditions. When pairs of conjugants formed in a cross nd9 × wt (grown and maintained at 27°C) were examined in the course of conjugation, it was observed that all nd9 conjugants expressed an (exo') phenotype by the 2nd h of pairing, i.e., before nuclear exchange had taken place. A similar result was observed for nd9 cells crossed with nd9 cells of complementary mating type grown at 27°C. These observations demonstrate that the (exo') phenotype of 27°C nd9 cells can be efficiently and quickly complemented by diffusible products from various cellular types carrying an normal nd9' gene.

In contrast, when nd9 cells grown at 18°C were crossed with nd9 cells of complementary mating type grown at 27°C, the 27°C nd9 partner never acquired an (exo') phenotype, even at the end of

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### Table 1

**Kinetics of Evolution of the Phenotype of nd9 Cells in Exponential or Stationary Phase of Growth in Temperature Shift Experiments**

| Temperature shift | Time | Cells/ml | Phenotypes | Cells/ml | Phenotypes |
|-------------------|------|----------|------------|----------|------------|
|                   |      |          | 0 1 2 3 4  |          | 0 1 2 3 4  |
| Exponential       |      |          |            |          |            |
|                   |      |          |            |          |            |
| 27°C–18°C         | 0    | 88       | 100        | 4,050    | 100        |
|                   | 8    | 129      | 57 32 11   | 3,985    | 67 24 9    |
|                   | 15   | 155      | 27 33 23 17| 3,880    | 73 23 2 2  |
|                   | 24   | 235      | 8 10 12 28 |          |            |
|                   | 48   | 1,100    | 2 1 1 9 87 |          |            |
|                   | 54   |          |            | 4,160    | 2 1 3 11 83|
|                   | 96   |          |            |          |            |
| 27°C–13°C         | 0    | 255      | 100        | 6,000    | 100        |
|                   | 4    | 285      | 98 2       |          |            |
|                   | 20   | 300      | 6 14 9 44 31| 5,960    | 1 28 60 11 |
|                   | 24   | 275      | 1 14 9 28 49|          |            |
|                   | 27   | 372      | 3 2 1 3 91 |          |            |
|                   | 45   |          |            | 6,020    | 2 1 9 51 37|
|                   | 48   |          |            | 6,000    | 1 1 98     |
|                   | 72   |          |            |          |            |
| 18°C–27°C         | 0    | 96       | 15 85      | 3,940    | 9 91       |
|                   | 12   | 310      | 16 17 14 34 19| 4,090    | 12 88      |
|                   | 23   | 700      | 22 32 20 23 3 |          |            |
|                   | 25   | 1,450    | 49 34 9 8  |          |            |
|                   | 29   | 4,420    | 52 32 13 3 |          |            |
|                   | 47   |          |            | 4,050    | 12 88      |
|                   | 53   |          |            |          |            |
|                   | 61   |          |            | 4,420    | 100        |
|                   | 73   |          |            | 4,040    | 15 85      |
|                   | 125  |          |            | 4,050    | 12 88      |

These experiments were carried out on nd9 populations growing or having reached their plateau in 1 liter of culture fluid. Time 0 corresponds to the transfer of the population from one temperature to the other. At various times after transfer, cell counts were made on 2 × 1 ml samples of the population and the phenotypes of ~100 cells were determined by the picric acid test (see Materials and Methods). The phenotypic classes 0–4 correspond to those defined in Fig. 2 and its legend. The figures represent the percentage of cells in each phenotypic class.
a 6-h pairing period at 27°C or a 10-h pairing period at 18°C. In control crosses, nd9 27° × wt grown at 18°C, the (exo+) phenotype of nd9 cells was restored as efficiently as in crosses nd9 27° × wt 27°C. These results show that, despite their (exo+) phenotype, nd9 cells grown at 18°C do not contain a normal or active nd9 gene product equivalent to the product of an nd9* gene.

(b) Microinjection of 18°C nd9 cytoplasm devoid of trichocysts. To carry out this experiment, the double mutant nd9–tam 38 was used as a source of 18°C nd9 cytoplasm. Such cells contain only a few abortive trichocysts because of the mutation tam 38, so that the probability of transferring trichocysts along with the sample of cytoplasm is low; furthermore, it is known that tam 38 trichocysts cannot "mature" (i.e., become attached and capable of being excreted) in tam 38* cytoplasm. A control experiment (Table II, series 1) first showed that the cytoplasm taken from tam 38 donors grown at 18°C was as efficient as wild-type cytoplasm in restoring secretory capacity of 27°C nd9 cells. Then, 27°C nd9 cells were injected with cytoplasm from 18°C tam 38–nd9 donors; as shown in Table II, series 2, none of them was capable of excreting even a single trichocyst. These results demonstrate that the nd9 product present in 18°C nd9–tam 38 cells is inactive and cannot restore exocytosis capacity in a 27°C nd9 cell.

Taken together, these two sets of experiments (natural and experimental injections) show that permissive temperatures, although they restore exocytotic capacity in nd9 cells, do not restore the activity of the product of the mutant gene nd9. Furthermore, these results provide a satisfactory explanation for the observation by Aufderheide (3), confirmed here in Table II, series 3, that injection of cytoplasm from an 18°C nd9 cell into a 27°C nd9 recipient cell results in no or abortive responses. Series 4–12 define the requirements for secretory capacity in nd9 cells. Series 4–8 are control experiments: series 4 and 5 indicate that tam 6 trichocysts are functional and can be excreted by nd9–tam 38 cells; series 6 demonstrates that the double-mutant tam 6–nd9 trichocysts are functional and can be excreted by a tam 38 cell; series 7 and 8 show that 27°C nd9 cells can excrete 18° but not 27°C nd9–tam 6 trichocysts. These control experiments show that the tam 6 mutation does not affect the expression of the nd9 mutation in the trichocysts, and that the tam 38 mutation does not modify the properties of plasma membrane in nd9–tam 38 recipient cells. Series 9–12 demonstrate that neither 27°C trichocysts nor 27°C plasma membranes carry an autonomous deficiency (see text).

### Table II

**Determination of the Site of Action of the Mutation nd9 by Microinjection Experiments**

| Series | Donor | Recipient | Picric acid response | No. of positive responses | No. of tested cells |
|--------|-------|-----------|----------------------|--------------------------|---------------------|
| 1      | tam 38, 18°C | nd9, 27°C | + to +++              | 17                       | 22                  |
| 2      | nd9–tam 38, 18° | nd9, 27° | –                     | 0                        | 23                  |
| 3      | nd9, 18° | nd9, 27° | +                     | 1                        | 13                  |
| 4      | tam 6, 18° | nd9–tam 38, 18° | +                   | 3                        | 4                   |
| 5      | tam 6, 27° | nd9–tam 38, 27° | +                   | 3                        | 8                   |
| 6      | nd9–tam 6, 27° | tam 38, 18° | +                     | 3                        | 4                   |
| 7      | nd9–tam 6, 27° | nd9, 27° | –                     | 0                        | 5                   |
| 8      | nd9–tam 6, 18° | nd9, 27° | +                     | 6                        | 6                   |
| 9      | nd9–tam 6, 27° | nd9–tam 38, 27° | –                   | 0                        | 11                  |
| 10     | nd9–tam 6, 18° | nd9–tam 38, 18° | + to ++              | 8                        | 8                   |
| 11     | nd9–tam 6, 18° | nd9–tam 38, 27° | +                     | 11                       | 11                  |
| 12     | nd9–tam 6, 27° | nd9–tam 38, 18° | +                     | 8                        | 9                   |

A sample of cytoplasm taken from the donor cell is injected into the recipient cell. 2 h after injection, the surviving recipients are triggered with picric acid and trichocyst extrusion is observed. Picric acid responses range from – (no trichocyst expelled) to + or ++ (at least one trichocyst expelled or more, up to ~20) and +++ (many trichocysts expelled, from ~50 to several hundred). When donor cells carry the tam 38 mutation, no trichocysts or nonfunctional trichocysts are injected. When the donor carries the tam 6 mutation, many trichocysts are injected into the recipient cell. In recipient cells carrying the tam 38 mutation, all trichocyst attachment sites are free and the picric acid response indicates whether the injected trichocysts can be attached and excreted. Series 1–3 show that nd9* cytoplasm taken from a tam 38 donor efficiently repairs the exocytotic capacity of 27°C nd9 recipient cells. In contrast, no repair is observed in 18°C nd9–tam 38 cytoplasm devoid of trichocysts. The limited positive response obtained in series 3 corresponds to excretion by the recipient cell of the few injected 18°C nd9 trichocysts. Series 4–12 define the requirements for secretory capacity in nd9 cells. Series 4–8 are control experiments: series 4 and 5 indicate that tam 6 trichocysts are functional and can be excreted by nd9–tam 38 cells; series 6 demonstrates that the double-mutant tam 6–nd9 trichocysts are functional and can be excreted by a tam 38 cell; series 7 and 8 show that 27°C nd9 cells can excrete 18° but not 27°C nd9–tam 6 trichocysts. These control experiments show that the tam 6 mutation does not affect the expression of the nd9 mutation in the trichocysts, and that the tam 38 mutation does not modify the properties of plasma membrane in nd9–tam 38 recipient cells. Series 9–12 demonstrate that neither 27°C trichocysts nor 27°C plasma membranes carry an autonomous deficiency (see text).
results in excretion by the recipient 27°C nd9 cells of a few trichocysts; it is reasonable to assume that in this situation, the few excreted trichocysts did not reflect a limited repair by the 18°C nd9 cytoplasm but corresponded to the excretion of the few 18°C nd9 trichocysts present in the injected sample of cytoplasm.

**Cerulenin Prevents Restoration of the Exocytotic Capacity of nd9 Cells at Permissive Temperatures**

The experiments reported above show that permissive temperatures do not restore the activity of the product of the gene nd9. Therefore, the thermosensitive expression of the nd9 mutation must be a result of some indirect effect of temperature. It is known that cells maintain their membrane properties over a range of temperatures by adjusting their content of unsaturated fatty acids, which increases as temperature decreases (13). In particular, this phenomenon has been demonstrated in another protozoan, *Tetrahymena* (12). It seemed therefore interesting to investigate whether the thermosensitive expression of the nd9 mutation depends upon such temperature-induced changes in the membranes. To test this hypothesis, we used cerulenin, an antibiotic extracted from *Cephalosporium caerulens*, which has been shown to inhibit fatty acid synthesis in a variety of organisms (24), including mammalian cells (15).

The drug (25 µg/ml) was added to stationary-phase cells at the time of temperature shift (see Materials and Methods). We first studied the effect of the drug on the secretary capacity of (exo+) cells: wild-type cells grown at 27°C and nd9 cells grown at 18°C continued to express fully their (exo+) phenotype 48 h after transfer to 15°C in the presence of cerulenin. Then, we analyzed the effect of cerulenin on nd9 cells transferred from 27°C to 15°C in the following way: A population of nd9 cells that had reached its plateau at 27°C and expressed a homogeneous (exo-) phenotype 48 h after transfer to 15°C in the presence of cerulenin. Then, we analyzed the effect of cerulenin on nd9 cells transferred from 27°C to 15°C in the following way: A population of nd9 cells that had reached its plateau at 27°C and expressed a homogeneous (exo-) phenotype was divided into two aliquots, one supplemented with cerulenin, the other serving as control, and both were transferred to 15°C. The evolution of the exocytotic capacity was followed and, after 39 h, both populations were fixed for freeze-fracture.

The results are given in Table III and Figs. 4-8. Table III shows that in this particular experiment (as in preliminary ones), the appearance of exocytotic capacity was almost totally blocked by cerulenin in nd9 cells transferred from 27°C to 15°C. Figs. 4-5 show fracture faces from control cells, and Fig. 6, fracture faces from cerulenin-treated nd9 cells. These figures show that rosettes are absent from the treated cells but present in a number of trichocyst attachment sites in the control cells. Fig. 8 summarizes the observations and gives the distribution of the number of rosette particles in 44 occupied sites of treated cells, and in the same number of sites in control cells. Fig. 8 also shows that the number of rosette particles in cerulenin-treated cells is remarkably similar to that observed in untreated nd9 cells maintained at 27°C. It can be noted that in the control cells not all sites have a rosette. This is in agreement with the data of Table III, which indicate that after 39 h, when these cells were fixed, the (exo-) phenotype was not yet fully expressed. When the control cells were fixed, they were in the course of phenotypic transformation, and various stages of rosette assembly are therefore observed.

In a parallel experiment, the effect of an inhibitor of protein synthesis was studied. At the concentration of 100 µg/ml, puromycin completely blocks cell division. It was observed that addition of 100 µg/ml of puromycin to a 27°C stationary-phase nd9 population transferred to 15°C did not affect its phenotypic evolution towards an (exo+) phenotype.

These observations indicate that, in the mutant nd9, (a) the recovery of the (exo+) phenotype at permissive temperature is paralleled by the progressive assembly of the rosettes and (b) rosette assembly and exocytotic capacity are blocked by cerulenin, whose presumed action is to block adaptation of the lipid composition of membranes to low temperatures.

**Localization of the Site of Action of the nd9 Mutation**

We next wanted to determine to which cellular

### Table III

| Time after transfer (h) | Control | Cerulenin-treated |
|------------------------|---------|-------------------|
|                        | Cells/ml | Phenotypes | Cells/ml | Phenotypes |
| 0                      | 7,550    | 100          | 7,550    | 100        |
| 22                     | 7,000    | 8            | 6,950    | 94          |
| 39                     | 6,660    | 2            | 6,800    | 46          |

Cell counts and determination of phenotypes as in Table I.
FIGURE 4 and 5  Cerulenin treatment experiment: control cells. (a) Cryofracture of the plasma membrane P face (PMP) of the untreated mutant, 39 h after transfer from the nonpermissive (27°C) to the permissive (15°C) temperature. Trichocyst sites (arrows) are normally aligned along the ciliary rows; some unoccupied sites are collapsed in "parentheses." Rosettes are present and nearly complete. ci, Cross-fractured cilium. × 27,000. Fig. 4b and c, enlargement of two sites of Fig. 4a (asterisk with arrow). × 78,600. Fig. 5 shows, as a comparison, typical trichocyst site on P face of a wild-type cell, with its fully assembled rosette. × 76,500.

compartment the product of the gene nd9 belongs. That the mutational defect in 27°C nd9 cells is repaired by injection of wild-type cytoplasm does not allow us to ascertain its localization; the nd9 gene product might be either a readily exchangeable component of either trichocyst or plasma membrane or a truly cytoplasmic, nonmembrane molecule.
In Aufderheide's microinjection method (3), a mutational defect can be localized in the trichocyst compartment if the trichocysts that are unable to be excreted in the mutant cannot be excreted even if introduced into a competent recipient cell. Because of the reparability of the nd9 defect by nd9+ cytoplasm, the actual functional properties of nd9 trichocysts can only be ascertained in the absence of nd9+ products. We therefore carried out four series of microinjections in which the properties of nd9 trichocysts and plasma membranes could be separately tested in the absence of nd9+ products. We used tam-38-nd9 cells as recipients and tam 6-nd9 cells as donors. tam 6-nd9 cells are a con-
venient source of trichocysts because their cytoplasm is loaded with unattached trichocysts. In tam 38-nd9 cells, the trichocysts are abortive and unattached because of the mutation tam 38; in these cells, all membrane sites are free so that any excreted trichocyst is unambiguously an injected one. We first carried out control experiments (Table II, series 4–8) to ascertain that the mutations tam 38 and tam 6 did not introduce any bias (see legend of Table II). Then, we injected tam 6-nd9 trichocysts from 18° or 27°C cells into 27° or 18°C nd9-tam 38 cells. The results are given in Table II, series 9–12. The only combination that yielded a negative result was the injection of 27°C tam 6-nd9 trichocysts into a 27°C tam 38-nd9 recipient cell. In contrast, the trichocyst–plasma membrane interaction was functional in the other three combinations: 27° → 18°C, 18° → 27°C or 18° → 18°C. Because we know that the nd9 gene product is by itself inactive at both 18° and 27°C, these results demonstrate that in an nd9 cell, the exocytotic function is restored, provided either the trichocyst or the plasma membrane, or both, were formed at permissive temperature.

DISCUSSION
For most secretory systems, the analysis of exocytosis is based upon cytological, immunocytochemical, or biochemical techniques that permit one to identify and localize structural components or particular molecular species but generally do not permit the assessment of their in vivo function. The foremost interest of the Paramecium trichocyst system lies in the availability of mutations affecting various steps between development of the secretory vesicle and its final extrusion upon stimulation. Each locus identified by a mutation defines a particular function that might not be detected by other methods. The study of the conditional mutant nd9 reveals the role of a cytoplasmic diffusible product that controls membrane fusion by interacting with both the plasma membrane and the trichocyst membrane.

The mutant nd9 has normal trichocysts (regularly inserted at their docking site beneath the plasma membrane) that cannot be excreted at nonpermissive temperature (≥27°C) but that are normally excretable at permissive temperatures (≤18°C). At nonpermissive temperatures, the (exo−) phenotype is correlated with the absence of rosettes in the plasma membrane and with the absence of both Ca++-ATPase activity at the site of trichocyst attachment to the plasma membrane and of connecting material, a fibrous electron-dense structure in the narrow space (~15–30 nm) between the trichocyst tip and the plasma membrane (see footnotes 1 and 3). In this study, we have analyzed the function of the nd9 gene product and its site of action. We will first discuss the significance of the data and propose an interpretation of our results; we will then discuss the possible nature of the nd9 factor, its relationship with rosette assembly, and its possible function in the control of membrane fusion.

Mode of Action of the nd9 Gene Products:
Facts and Interpretation
The following four points concerning the mode of action of the nd9 product have been established:

(a) The product of the gene nd9 is a diffusible cytoplasmic component. Aufderheide (3) first observed that injection of wild-type cytoplasm into a 27°C nd9 cell restored its exocytotic capacity. This repair phenomenon provides a biological assay that might permit us to identify the molecular species involved (14). The microinjection experiments reported here fully confirm Aufderheide’s results. Furthermore, a similar repair phenomenon was demonstrated during conjugation of 27°C nd9 cells paired with one of various cell types carrying an nd9+ gene. The rapid diffusion of the nd9+ product was indicated by the restoration of the (exo+) phenotype in the 27°C nd9 conjugant by the 2nd h of conjugation, which is about the time required for electrical coupling, according to Y. Naitoh (personal communication).

(b) Permissive temperatures do not restore a functional nd9 product. Whether by conjugation with an 18°C nd9 partner or by microinjection of cytoplasm taken from an 18°C nd9-tam 38 donor (devoid of functional trichocysts), the exocytotic capacity of 27°C nd9 cells is not restored. The limited repair observed by Aufderheide (3) and confirmed here (Table II, series 3) after microinjection of 18°C nd9 cytoplasm into a 27°C nd9 recipient cell is therefore a result of the few 18°C trichocysts present in the injected cytoplasm sample and not to the transfer of some functional 18°C nd9 product. It can therefore be concluded that the normal (exo+) phenotype of nd9 cells grown at permissive temperatures is a result of an indirect effect of temperature on the nd9 product, and it is reasonable to assume that the activity of

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FIGURE 8 Distribution of the number of particles per rosette in P faces of cerulenin-treated and control nd9 cells. The dotted line histogram represents the number of particles per rosette in P faces of nd9 cells maintained at 27°C as established in a previous experiment (5). For the latter histogram, the values of the ordinate were adjusted to the same number (44) of rosettes as in the other two histograms.

The nd9 product is restored by some temperature-sensitive molecular interaction.

This conclusion is in agreement with the phenotypic evolution of nd9 cells in temperature shift experiments. If temperature acted directly on the conformation and activity of the nd9 product, one would expect to observe a rather synchronous evolution within the population; however, Table I shows that this is not the case. Furthermore, the long stability of the (exo+) phenotype in stationary cells transferred from 18° to 27°C (Table I B) is best explained by assuming that the nd9 product is part of a molecular complex that cannot be formed, but can continue to function, at nonpermissive temperatures, once formed under permissive conditions.

(c) The (exo+) phenotype of nd9 cells at permissive temperatures depends upon temperature-induced changes in the lipid composition of the membranes. This statement is based upon the observed blockage by cerulenin of secretory capacity and rosette assembly in nd9 cells transferred from 27° to 15°C. We did not actually study the effect of cerulenin on fatty acid synthesis, and therefore we can only assume that the drug acts on Paramecium as it does on other organisms (15, 24). However, we have studied the effects of cerulenin on growth and survival of Paramecia (see Material and Methods) and the observed effects are precisely what would be expected from a blockage of fatty acid synthesis. On the basis of this reasonable assumption, the blockage by the drug of exocytotic capacity (Table III) and rosette assembly (Figs. 6 and 8) seems quite significant. Because, as previously discussed, the (exo+) phenotype of nd9 cells grown at permissive temperature is not a result of the restoration of the activity of the nd9 factor per se, it may be concluded that the (exo+) phenotype results from some interaction between the nd9 factor and membranes. Efficient interactions require low-temperature membranes, richer in unsaturated fatty acids, and are impossible with more “rigid,” high-temperature membranes. That the (exo-) phenotype becomes leaky in aging nd9 cells fits this interpretation well, because it is known that the percentage of unsaturated fatty acids increases in late stationary-phase Paramecium cells (P. Mazliak and J. Beisson, unpublished observations; 17).

(d) The (exo+) phenotype is restored either by 18°C-formed trichocysts or by an 18°C-formed plasma membrane. This is demonstrated by microinjection experiments (Table II, series 9–12) in which 18° or 27°C nd9 trichocysts are transferred into 18° or 27°C recipient nd9 cells. Assuming that the activity of the nd9 product depends on its interaction with membranes, as inferred above, this means that either membrane is sufficient and therefore that both membranes interact with the nd9 factor. This rules out the possibility that the nd9 product might be a component of the rosette particles, which are integral proteins of the plasma membrane.

These results and conclusions led us to the following interpretation, illustrated in Fig. 9, of the mode of action of the nd9 gene product. This
The product exists as a free diffusible molecule that, in wild-type and nd9 cells grown at permissive temperatures (18°C), interacts with this gene product, whether normal (●) or mutated (○), with plasma membrane (PM) and trichocyst membrane (TM) in the polymerization of the nd9 gene product (alone or with associated proteins) into a "filament" connecting the two membranes. In nd9 cells grown at nonpermissive temperatures, this interaction is prevented. The right part of the scheme illustrates the results of the microinjection experiments (Table II, series 11-12), which demonstrate that in "mixed" nd9 cells (18°C PM with 27°C TM, or vice versa), polymerization (i.e., activation of the nd9 gene product) takes place.

Biochemical characterization of the nd9 product should be the next step. Meanwhile, the function of this product can be discussed.

**Possible Function of the Structures Containing the nd9 Product**

The existence of some structure linking plasma membrane and trichocyst, which is here deduced from physiological data, may be correlated with the cytologically identified connecting material (Fig. 1) that is absent in 27°C nd9 cells (see footnotes 1 and 3). Whether this connecting ma-
terial contains the \textit{nd9} product remains of course
to be demonstrated. Nevertheless, it is particularly
interesting to point out that in one of the best
studied exocytosis systems, the chromaffin cells of
bovine adrenal medulla, cytological and biochem-
ical data (4, 8, 9) have shown the existence of
structures or molecular complexes linking mem-
branes about to fuse. In the freeze-fracture study
of Aunis et al. (4), the observed fibrous structure
is interpreted as "a stage of recognition and an-
choring during which granule-plasma membrane
connections are formed just before membrane fu-
sion." In their biochemical study of "synexin,"
which permits in vitro fusion of chromaffin gran-
ules, Creutz et al. (8, 9) propose that because of
their Ca\textsuperscript{2+}-dependent self-aggregating properties,
the cross-linked polymers of synexin would also
bridge the plasma membrane and secretory gran-
ules and by bringing the two membranes into more
intimate contact would permit or induce fusion.
The idea that a link connecting the membranes is
required to promote fusion under specific condi-
tions (such as an increased internal Ca\textsuperscript{2+} concen-
tration) deserves consideration. It is indeed strik-
ing that, in models of membrane fusion (1, 10),
nothing accounts for the fact that, all of a sudden,
the two membranes are in close contact as required
for fusion of the lipid matrix. It is therefore tempt-
ing to speculate that, in all membrane fusion pro-
cesses, molecular complexes of the synexin type are
present, long before (as in the \textit{Paramecium} tricho-
cyst system) or just before (in most other secretory
systems) membrane fusion. The complexes may
react to the stimulus (Ca\textsuperscript{2+}?) by further aggrega-
tion or cross-linking so as to either establish a
more intimate membrane/membrane contact, in-
duce molecular rearrangements in the membranes,
or both, facilitating fusion.

Such connecting material might be the still un-
known site of action of Ca\textsuperscript{2+}, whose role in mem-
brane fusion seems general (29). Trichocyst extru-
sion is also Ca\textsuperscript{2+}-dependent (22) and may be
triggered by an increase in internal Ca\textsuperscript{2+} concen-
tration. Indeed, although the stimuli that trigger
exocytosis are varied, all of them, as pointed out
by Plattner et al.,\textsuperscript{5} first induce reversal of ciliary
beating, reflecting membrane depolarization and
Ca\textsuperscript{2+} influx (23).

\textbf{Relationships between the nd9 Gene Product
and Rosette Assembly and Function}

Previous data (5) had shown that normally ro-
settes are assembled wherever trichocysts become
attached at their submembrane docking site. In all
the mutants characterized by attached trichocysts
that are unable to be excreted, no rosette is assem-
bled (5; see footnote 3). These facts indicate that
rosettes are required for membrane fusion. A
Ca\textsuperscript{2+}-ATPase activity was found in the region of
trichocyst attachment to the plasma membrane
(27), and was more precisely located in the rosette
particles.\textsuperscript{4} Because the mutant \textit{nd9} at nonpermis-
sive temperatures lacks both rosettes and func-
tional products of the locus \textit{nd9}, the question arises
as to their relationship and respective roles in
membrane fusion.

As was demonstrated by freeze-fracture studies
of cerulenin-treated \textit{nd9} cells, rosette assembly is
prevented by the drug. If our interpretation of the
modification of the \textit{nd9} product (in terms of its
interaction with membranes) is correct, the ceru-
lenin effect indicates that rosette assembly depends
on previous formation of the fibers linking tricho-
cyst and plasma membrane. Preliminary observa-
tions show that rosette assembly seems to proceed
through progressive aggregation of smaller intra-
membrane particles. Rosette assembly and the
appearance of the Ca\textsuperscript{2+}-ATPase activity might
therefore result from some modification in the
local state of the plasma membrane, induced by
the formation of the linking fibers. Involvement of
actin filaments bound to the particles of the rosette
and responsible for the stability of the aggregate
is also possible, as it seems to be for surface
antigens in lymphocytes (11, 19).

If, as discussed above, the \textit{nd9} fibers were the
target of Ca\textsuperscript{2+} ions, promoting membrane fusion
as soon as their concentration rises, the question
becomes what the function of the rosette might be.
This particle array at the potential site of mem-
brane fusion seems to be a special feature of
\textit{Paramecium} and \textit{Tetrahymena}, because the prev-
alent view is that fusion occurs at particle-free
zones of membranes (1, 10, 21, 25). This peculi-
arity, however, may be related to the fact that
trichocysts or mucocysts are "ready-to-fire", stably
attached secretory vesicles. The rosette therefore
might not be a "fusion-rosette" as proposed by
Satir et al. (32), but an "antifusion rosette": its
function (or one of its functions) would be to
restrain membrane fusion until triggered by the
proper stimulus. The Ca\textsuperscript{2+}-ATPase activity might
correspond to a Ca\textsuperscript{2+} pump maintaining local low
Ca\textsuperscript{2+} concentrations and thus helping to prevent
membrane fusion. In 27°C \textit{nd9} cells, despite the
absence of rosettes, exocytosis would not take
place because of the absence of the Ca\textsuperscript{2+} target necessary to bring about membrane fusion.

In conclusion, membrane fusion in trichocyst excretion seems to be controlled by two molecular complexes, one in the plasma membrane (the rosette) that is needed because of the stable proximity of the secretory vesicle, and one between the plasma membrane and the trichocyst that might be common to all exocytotic systems and would be required to promote membrane fusion. The main interest of our results is to provide genetic and physiological arguments in support of the biochemical (8, 9) and cytological (4) evidence for a fibrous material connecting membranes about to fuse.

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