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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,"[1] 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*, *tam8*, 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 Aufderheide (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 excretable 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.[2] 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 Cerophyl 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. Pouphile, and M. Lefort-Tran. Genetic dissection of the final exocytosis steps in *Paramecium* cells: cytochemical localization of Ca^{2+}-ATPase activity over preformed exocytosis sites. *J. Cell Sci.* Manuscript submitted for publication.

[2] Lefort-Tran, M., M. Pouphile, M. Rossignol, K. Aufderheide, 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 excreted 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, reparable 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 μm^3) 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 38* 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 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 N_2, and stored in liquid N_2. 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 10 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.

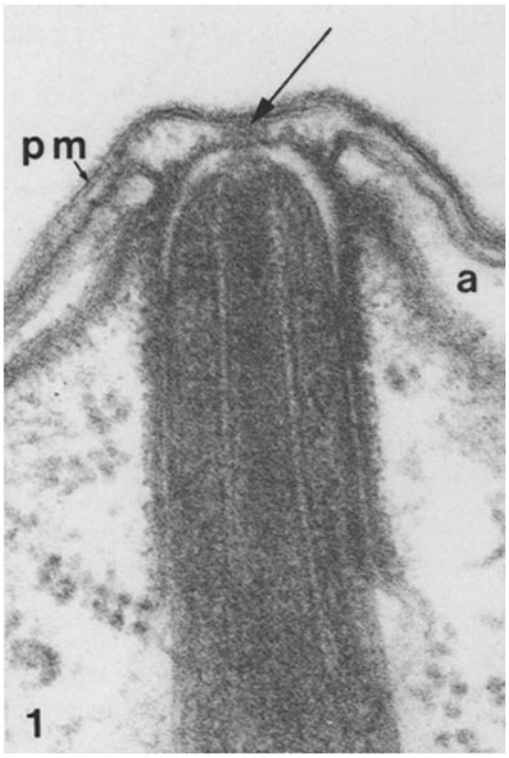

FIGURE 1 Longitudinal section through the apical part of a trichocyst attached to the plasma membrane (*pm*), between the subpellicular alveoli (*a*). Between the plasma membrane and the trichocyst tip, an electron-dense connecting material (arrow) is visible on favorable section planes. × 110,000.

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 perpen-

[3] 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.

dicular 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° and 27°C. 18°–20°C defines the upper boundary of permissive temperatures at which all cells belong to categories 3 and 4; 26°–27°C defines the lower boundary of nonpermissive temperatures at which all cells belong to category 0. Between 27° 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-

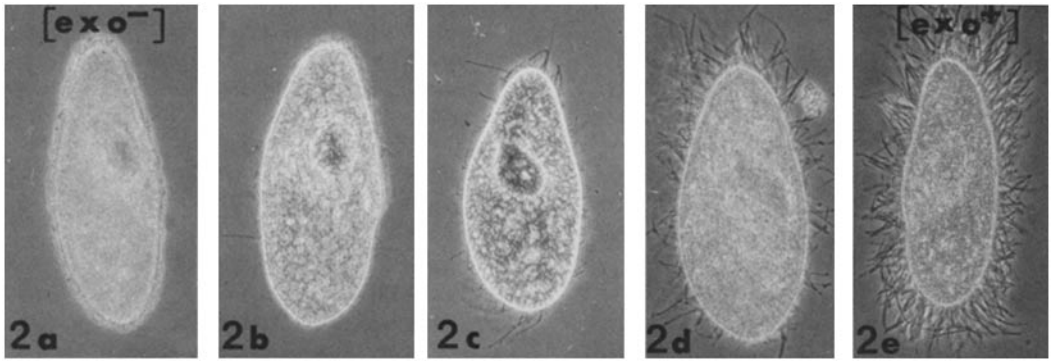

FIGURE 2 Range of variation of the *nd9* phenotype. The secretory capacity of *nd9* cells is assayed by the picric acid test (see Material and Methods). When the cells change conditions, the cellular phenotype shifts from a phenotype (exo^-) with no expelled trichocysts (2*a*) to a wild-type or sub-wild-type phenotype (exo^+) as shown in 2*e*. Fig. 2*b*–2*d* represent intermediate phenotypic classes corresponding respectively to: 1–5 excreted trichocysts per cell; >5; more than ~20 but still not forming a continuous fringe as in 2*e*. These five categories will be referred to as classes 0, 1, 2, 3, and 4 (exo^+).

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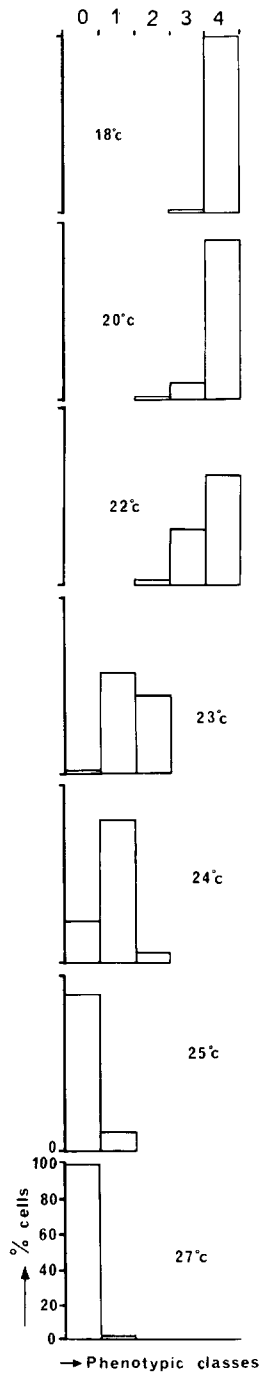

FIGURE 3 Phenotypic evolution of *nd9* cells as a function of temperature. Each histogram represents the distribution of phenotypes, as defined in Fig. 2, within a population of *nd9* cells, in exponential or early stationary phase of growth, observed after stabilization at each temperature.

plete change of phenotype (exo$^-$) ⇆ (exo$^+$) is regularly observed; the kinetics of this transformation have been studied. Temperature shifts, 27° to 18° or 13°, and 18° to 27°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 (exo$^-$) towards the (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 (exo$^-$) phenotype is lost in most cells after 96 h at the permissive temperature, whereas the (exo$^+$) phenotype is fully retained after 125 h at the nonpermissive temperature. (*b*) The change from (exo$^-$) to (exo$^+$) phenotype proceeds faster in shifts from 27° to 13°C than it does in shifts from 27° to 18°C. In particular, most of cells become (exo$^+$) in less than one cell generation after transfer to 13°C. (*c*) Until completion of the phenotypical change, the population remains heterogeneous: the recovery (or loss) of secretory capacity is not in all-or-nonphenomenon 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°C *nd9* cells, as does wild-type or *nd9*$^+$ cytoplasm injected into 27°C *nd9* cells (3) (see Material and Methods). To ascertain the properties of the cytoplasm in 18°C *nd9* cells, we

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TABLE I

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

		A Exponential						B Stationary					
			Phenotypes						Phenotypes				
Temperature shift	Time	Cells/ml	0	1	2	3	4	Cells/ml	0	1	2	3	4
	h		%						%				
27°–18°C	0	88	100					4,050	100				
	8	129	57	32	11								
	15	155	27	33	23	17							
	24	235	8	10	12	42	28	3,985	67	24	9		
	48							3,880	73	23	2	2	
	54	1,100	2	1	1	9	87						
	96							4,160	2	1	3	11	83
27°–13°C	0	255	100					6,000	100				
	4	285	98	2									
	20	300	6	14	9	44	31						
	24							5,960	1	28	60	11	
	27	275	1	14	9	28	49						
	45	372	3	2	1	3	91						
	48							6,020	2	1	9	51	37
	72							6,000			1	1	98
18°–27°C	0	96				15	85	3,940				9	91
	12	310	16	17	14	34	19						
	23	700	22	32	20	23	3						
	25							4,090				12	88
	29	1,450	49	34	9	8							
	47	4,420	52	32	13	3							
	53							4,050				12	88
	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.

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* 27°C cells paired to different mutants (e.g., *nd6*, *nd7*, *tam 8*, etc.). 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 a 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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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

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 ranges 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 therefore 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 secretory 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° 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° 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.

Table III

Kinetics of Evolution of the Phenotype of Stationary Phase nd9 Cells Transferred from 27° to 15°C in Absence or Presence of Cerulenin (25 μg/ml)

Time after transfer	Control						Cerulenin-treated					
	Cells/ml	Phenotypes					Cells/ml	Phenotypes				
		0	1	2	3	4		0	1	2	3	4
h		%						%				
0	7,550	100					7,550	100				
22	7,000	8	42	29	21		6,950	94	6			
39	6,660			2	29	69	6,800	46	54			

Cell counts and determination of phenotypes as in Table I.

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

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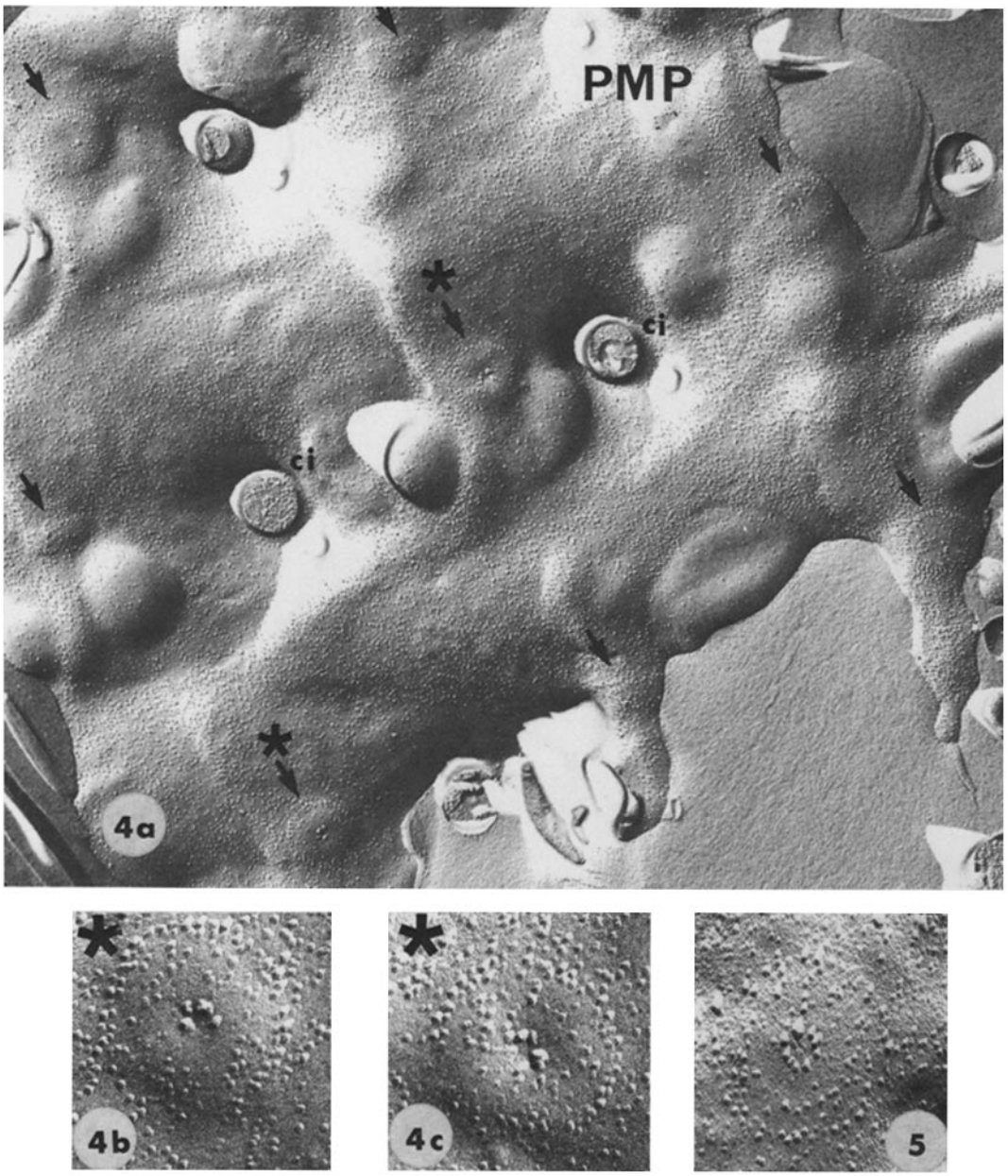

FIGURE 4 and 5 Cerulenin treatment experiment: control cells. (*a*) Cryofracture of the plasma membrane P face (*PMP*) of the untreated mutant, 39 h after transfer from the nonpermissive (27°C) to the permissive (15°C) temperature. Trichocyst sites (arrows) are normally aligned along the ciliary rows; some unoccupied sites are collapsed in "parentheses." Rosettes are present and nearly complete. *ci*, Cross-fractured cilium. × 27,000. Fig. 4 *b* and *c*, enlargement of two sites of Fig. 4 *a* (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.

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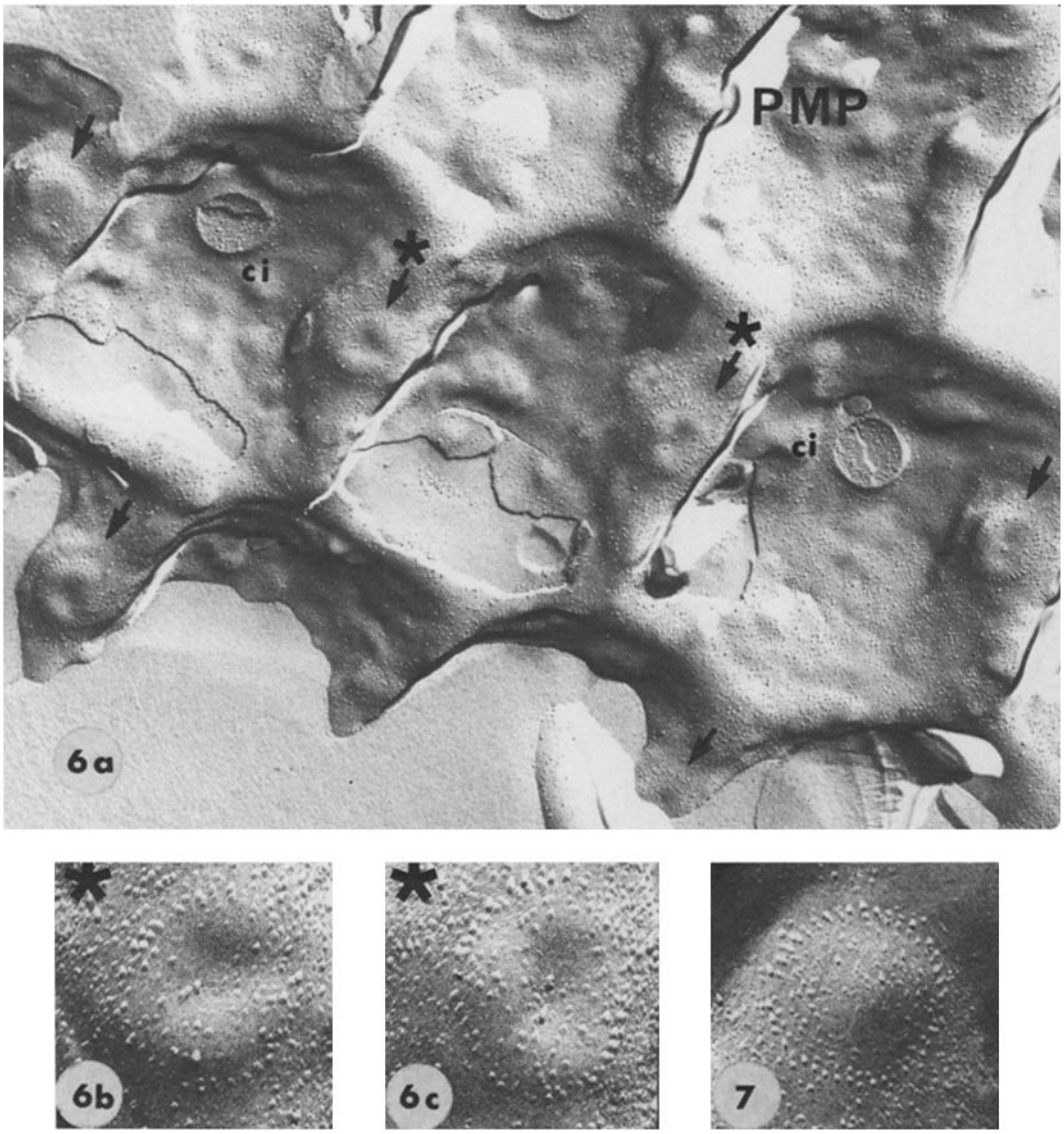

FIGURES 6 and 7 Cerulenin treatment experiment: treated cells, fixed 39 h after temperature shift in presence of cerulenin. (*a*) The P-fracture face (*PMP*) displays the regularly aligned trichocyst sites (arrows), with outer ring of particles, without rosette. *ci,* Cross-fractured cilium. × 27,700. Fig. 6 *b* and *c*, enlargements of two sites (asterisk with arrow) of Fig. 6 *a*. × 78,000. Fig. 7 shows, as a comparison, a typical site of the mutant *nd9* grown and maintained at 27°C. × 76,500.

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 introduct 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

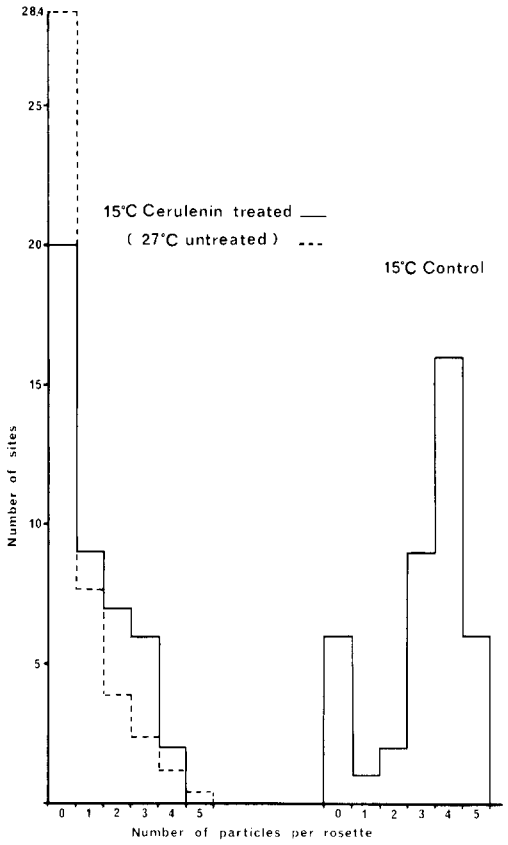

FIGURE 8 Distribution of the number of particles per rosette in P faces of cerulenin-treated and control *nd9* cells. The dotted line histogram represents the number of particles per rosette in P faces of *nd9* cells maintained at 27°C as established in a previous experiment (5). For the latter histogram, 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

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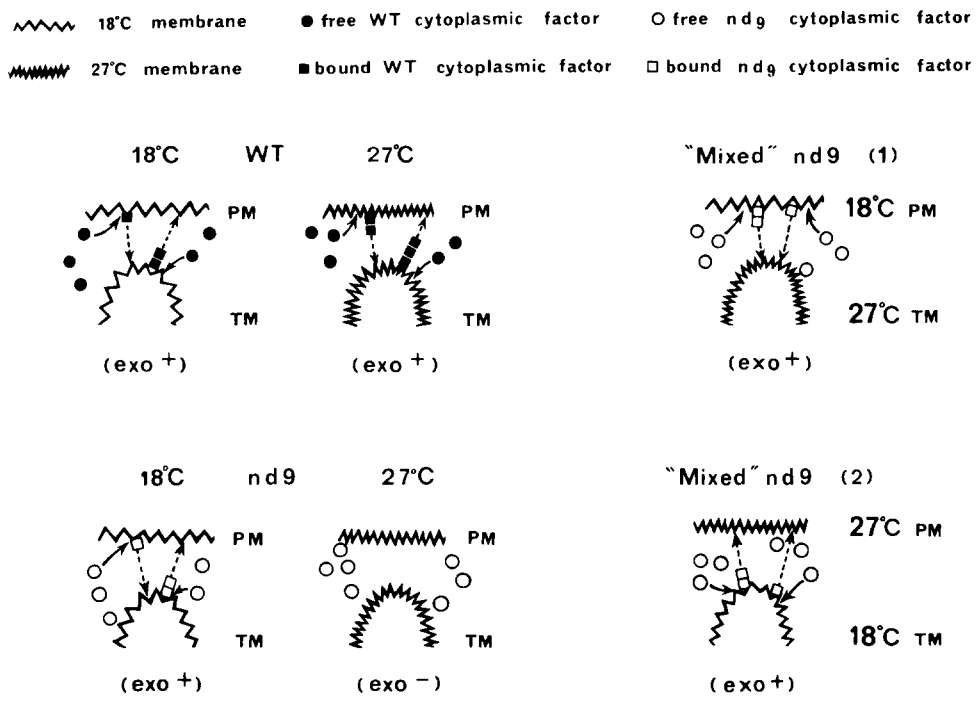

FIGURE 9 Interpretation of the function of the *nd9* gene product. In wild-type cells (*WT*) or in *nd9* cells at permissive temperatures (18°C), interaction of this gene product, whether normal (●) or mutated (○), with plasma membrane (*PM*) and trichocyst membrane (*TM*) results in the polymerization 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.

product exists as a free diffusible molecule that, in wild-type and *nd9* cells grown at permissive temperatures, polymerizes upon interaction with both trichocyst and plasma membranes. The polymerized "fibers" might include either *nd9* products only or other molecular species as well. Once anchored on either membrane, the fibers grow and reach the other membrane. In the mutant at nonpermissive temperatures, the lipid composition of the membranes would not permit anchorage and polymerization of the free mutated molecules. However, once polymerized at permissive temperatures, the fibers would remain functional at nonpermissive temperatures. In the right-hand part of Fig. 9, the situation in the "mixed" *nd9* cells constructed by microinjection is illustrated. This interpretation accounts for all the facts, and its assumed molecular basis is quite credible because it implies only well documented processes such as protein-lipid interactions (and their modulation according to the lipid composition of the membrane), anchorage of proteins in the membrane and polymerization into fibers, and conformational changes of a polypeptide chain upon polymerization or integration into a molecular complex. 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 *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 biochemical data (4, 8, 9) have shown the existence of structures or molecular complexes linking membranes 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 anchoring during which granule-plasma membrane connections are formed just before membrane fusion." In their biochemical study of "synexin," which permits in vitro fusion of chromaffin granules, Creutz et al. (8, 9) propose that because of their Ca^{++}-dependent self-aggregating properties, the cross-linked polymers of synexin would also bridge the plasma membrane and secretory granules 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 conditions (such as an increased internal Ca^{++} concentration) deserves consideration. It is indeed striking 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 tempting to speculate that, in all membrane fusion processes, molecular complexes of the synexin type are present, long before (as in the *Paramecium* trichocyst system) or just before (in most other secretory systems) membrane fusion. The complexes may react to the stimulus (Ca^{++}?) by further aggregation or cross-linking so as to either establish a more intimate membrane/membrane contact, induce molecular rearrangements in the membranes, or both, facilitating fusion.

Such connecting material might be the still unknown site of action of Ca^{++}, whose role in membrane fusion seems general (29). Trichocyst extrusion is also Ca^{++}-dependent (22) and may be triggered by an increase in internal Ca^{++} concentration. Indeed, although the stimuli that trigger exocytosis are varied, all of them, as pointed out by Plattner et al.,[3] first induce reversal of ciliary beating, reflecting membrane depolarization and Ca^{++} influx (23).

Relationships between the nd9 Gene Product and Rosette Assembly and Function

Previous data (5) had shown that normally rosettes 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 assembled (5; see footnote 3). These facts indicate that rosettes are required for membrane fusion. A Ca^{++}-ATPase activity was found in the region of trichocyst attachment to the plasma membrane (27), and was more precisely located in the rosette particles.[1] Because the mutant *nd9* at nonpermissive temperatures lacks both rosettes and functional products of the locus *nd9*, the question arises as to their relationship and respective roles in membrane fusion.

As was demonstrated by freeze-fracture studies of cerulenin-treated *nd9* cells, rosette assembly is prevented by the drug. If our interpretation of the modification of the *nd9* product (in terms of its interaction with membranes) is correct, the cerulenin effect indicates that rosette assembly depends on previous formation of the fibers linking trichocyst and plasma membrane. Preliminary observations show that rosette assembly seems to proceed through progressive aggregation of smaller intramembrane particles. Rosette assembly and the appearance of the Ca^{++}-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 *nd9* fibers were the target of Ca^{++} 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 membrane fusion seems to be a special feature of *Paramecium* and *Tetrahymena*, because the prevalent view is that fusion occurs at particle-free zones of membranes (1, 10, 21, 25). This peculiarity, 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^{++}-ATPase activity might correspond to a Ca^{++} pump maintaining local low Ca^{++} concentrations and thus helping to prevent membrane fusion. In 27°C *nd9* cells, despite the absence of rosettes, exocytosis would not take

place because of the absence of the Ca^{++} 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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