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Conjugation rescue of an exocytosis-competent membrane microdomain in *Tetrahymena thermophila* mutants

(secretory mutants/membrane assembly/vesicle/microdomains)

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ABSTRACT **Conjugation-rescue experiments with two *Tetrahymena thermophila* mutants (exo⁻) incapable of exocytosis (SB255, SB258) have been used to dissect regulatory steps in assembly of a functional membrane microdomain, the fusion rosette. "Rescue" refers to the recovery of a secretory activity. Exo⁻ mutants fail to secrete mucus normally (form capsules) when stimulated by the secretagogue alcian blue and are blocked before the assembly of a functional fusion rosette in the cell membrane. Two criteria are used to assay recovery of the wild-type (exo⁺) phenotype: (*i*) the conjugant's ability to form capsules when stimulated and (*ii*) the presence of assembled rosettes, which disperse upon stimulation. Conjugation of exo⁺ × SB258 results in restoration of secretion in 60% of the mutant conjugants and reappearance of assembled rosettes. Secretory capacity is restored in the SB258 cell within one-half hour of firm pair formation. This restoration is not due to new gene expression or continued protein synthesis, since it occurs when SB258 is crossed to a "star" strain (A*), which has defective micronuclei and therefore cannot contribute wild-type genes, and restoration occurs in the presence of cycloheximide during conjugation. Conjugation of exo⁺ × SB255 reveals a real but inefficient restoration of exocytic capacity in the exo⁻ conjugant and a significant decrease of exocytic capacity in the exo⁺ conjugant. SB255 × SB258 crosses also show a low but significant rescue of exocytic competence, indicating that different components of the exocytic mechanism are affected in the two mutants. This cross leads to restoration of rosette assembly and function in one of the partners, presumably SB258. These results provide data about some of the steps necessary for rosette assembly and suggest that transferable factors that promote and/or inhibit exocytosis are present in these cells.**

Specific microdomains within the cell membrane, formed, controlled, and regulated by mechanisms about which very little currently is known, are apparently critical for membrane function. An example of a structurally well-characterized microdomain is the fusion rosette, whose freeze-fracture signature is an intramembrane particle (IMP) array. The rosette functions in the ciliates *Tetrahymena* and *Paramecium* during stimulus transduction in exocytosis (1–4). In *Tetrahymena*, prior to the actual membrane fusion event that signals exocytosis, a secretory vesicle (the mucocyst) docks at a specific site below the cell membrane. The fusion rosette then assembles in the cell membrane above the docked mucocyst. When a secretory stimulus is presented to the cell, membrane fusion and exocytosis occur at the site of the rosette. Although presumably a multiplicity of cytoplasmic factors and transmembrane signals are involved in the steps leading to exocytosis, the rosette stands in a key position along the pathway: successful membrane fusion and exocytosis have never been observed in the absence of the rosette (5). However, neither the regulation of assembly nor the precise function of assembled rosettes in exocytosis is currently understood. We have isolated 10 stable exocytosis-deficient mutants (exo⁻), potentially blocked at any of several different positions in the transduction pathway in *Tetrahymena* (5). All mutant cells were derived from SB210. Two of these mutants, SB255 and SB258, are used in this study. Cells of mutant strain SB255 lack assembled rosettes and have a drastically reduced number of secretory vesicles (mucocysts), few of which are docked. Cells of mutant strain SB258 also lack assembled rosettes; however, clusters of rosette-sized particles and the normal number of docked mucocysts are present. In this study, we use these mutants, their ultrastructure and physiology, to begin dissection of the regulatory steps involved in assembly of the functional fusion rosette.

METHODS

Exo⁻ mutants were isolated on the basis of their failure to secrete mucus normally when stimulated by the secretogogue alcian blue (5). Conjugation or pair formation by sexually mature strains of *Tetrahymena thermophila* is a process that can be induced under appropriate starvation conditions such as transfer into Tris buffer (6) or modified Dryl's solution (7). In these experiments, early stationary-phase cultures (48 hr at 30°C) of exo⁺ cells (SB210 and A*) and mutant exo⁻ cells (SB258 and SB255) were transferred to a modified Dryl's solution and starved for 16 hr prior to mixing of the appropriately selected two conjugants. Since the strain A* cells have defective micronuclei and fail to give rise to meiotic products (8), they are used as control partners to test whether expression of new micronuclear genes is necessary for rescue to wild-type phenotype.

Mating mixtures were tested every 30 min for both total pairs formed (a measure of the course of conjugation) and the fraction of these pairs found within capsules after alcian blue treatment. Capsule formation is a measure of the secretory capacity of the conjugants. Under specific ionic conditions, the secretagogue alcian blue induces synchronous release of all competent secretory vesicles and, in addition, stains the secreted product blue. An easily visible blue capsule surrounding the live cells serves as a criterion of secretion. Alcian blue treatment and quantitation of encapsulated cells and pairs was done as described (see ref. 5: quantitative test of capsule formation). Capsule formation by a mutant (exo⁻) conjugant is used as an indicator of restoration to exo⁺ phenotype. Alcian blue stimulation in exo⁺ × exo⁻ crosses could result in three different responses (Fig. 1): (*i*) If repair to the exo⁺ phenotype occurs in the exo⁻ conjugant, the pair should be surrounded by a double capsule (Fig. 1*A*), and/or there should be naked pairs that have escaped from their capsules leaving double empty capsules in the medium.

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Abbreviations: IMP, intramembrane particle; R-IMP, rosette IMP.

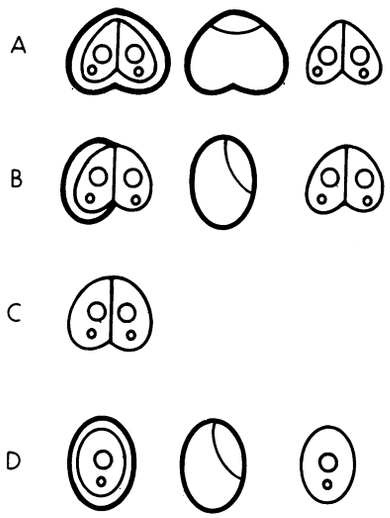

FIG. 1. Possible responses expected in conjugation rescue experiments in which exo$^+$ × exo$^-$ mating mixtures were stimulated to release mucocyst content with alcian blue. (*A*) The exo$^-$ mutant partner has been rescued and regained exo$^+$ phenotype. (*Left*) The conjugating pair is surrounded by a double capsule. (*Right*) A double empty capsule from which the conjugating pair has escaped (rare). (*B*) Absence of rescue. (*Left*) One conjugant, presumably the exo$^+$ cell, is surrounded by a capsule (rare). (*Right*) Single empty capsules are expected, in part since the conjugating pair can easily escape through the large opening of the capsule. (*C*) Inhibition of exo$^+$ function by the exo$^-$ cell. Neither cell forms capsules. (*D*) Response of unmated single cells in exo$^+$ × exo$^-$ crosses. (*Left*) Single cells with capsules. (*Center* and *Right*) Single empty capsules and single cells without capsules.

Double empty capsules are very rare. (*ii*) If repair of the exo$^-$ cell does not take place, only one of the conjugants (exo$^+$) would form capsules (Fig. 1*B*). In practice, a capsule around only one member of the pair is rarely observed, in part because the exo$^+$ conjugant can easily escape through the large opening in the capsule. Thus, one would observe either naked pairs or empty single capsules in this cross (Fig. 1*D*). (*iii*) If the exo$^+$ conjugant is inhibited by exo$^-$ factor(s) neither of the conjugants will form capsules (Fig. 1*C*). In exo$^-$ × exo$^-$ crosses, encapsulation of one or both conjugants would indicate repair. As an example, the percentage of cells found in the various categories with time is shown in Table 1.

A* cells have a defective micronucleus; in conjugation they fail to produce meiotic products and thus fail to contribute any nuclear genes to their sexual progeny (9). Crosses of exo$^-$ to A* cells were done to test whether restoration of the exo$^+$ phenotype to the exo$^-$ conjugant is due to gene transfer from the exo$^+$ conjugant.

Samples for freeze fracture analyses were taken ≈6 hr after initiation of pair formation and conjugants were fixed as described (5). Unequivocal identification of individual conjugant partners could often be obtained from replicas of cells in which the fracture had run along the cell membrane of one conjugant, then changed and cross-fractured the other conjugant. The extent of recovery in these is judged in high magnification freeze-fracture electron micrographs of the membranes of the two conjugants by the presence of any of the following signatures of exocytosis-related structures: rosette-sized IMPs, clusters of rosette-sized IMPs, assembled rosettes, exocytic openings, etc.

RESULTS

Conjugation Kinetics and Restoration of Secretory Capacity. The kinetics of conjugation and the changes in secretory capacity of conjugating pairs, as indicated by pairs within double capsules, are shown in Fig. 2. A control cross between two exo$^+$ strains, SB210 × A*, is shown in Fig. 2 (*Upper*). Four hours after mixing, ≈95% of the cells had formed pairs and ≈65% were found within double capsules when tested for secretion. When SB258 (exo$^-$) and A* (exo$^+$) were crossed, double capsules were observed (Fig. 2 *Lower*). The appearance of double capsules requires a massive release of mucocysts by both conjugants and implies that the exo$^-$ conjugant has phenotypically changed to the exo$^+$ competent phenotype (5). The appearance of double capsules lagged only about an hour behind pair formation, suggesting that the

Table 1. Distribution of cells found in various categories (pictured at left) as a function of time after mixing SB210 and SB258 cells

	SB210 × SB258						
	2 hr	2.5 hr	3 hr	3.5 hr	4 hr	5 hr	6 hr
	—, 2, —	6, 8, 12	17, 14, 32	20, 23, 21	33, 30, 30	30, 28, 30	29, 30, 27
	36, 40, 48	41, 40, 51	36, 44, 38	38, 33, 48	45, 47, 58	40, 44, 51	32, 35, 32
	36, 42, 48	47, 48, 63	53, 58, 70	58, 56, 69	78, 77, 88	70, 72, 77	61, 65, 72
	—, 5, —	13, 17, 19	32, 24, 46	34, 41, 30	42, 38, 34	43, 39, 29	47, 47, 46
	15, 19, 19	14, 18, 14	13, 9, 12	14, 15, 17	19, 7, 4	10, 7, 7	10, 14, 22
	43, 34, 32	36, 28, 21	30, 25, 18	25, 24, 13	3, 13, 8	20, 18, 12	26, 21, 19
	6, 5, 1	3, 6, 4	4, 8, 1	3, 5, 1	—, 3, —	—, 1, —	3, —, —

Each group of three represents results from three independent experiments. In each experiment, we counted 100–200 cells and calculated the percentage of cells in each depicted category.

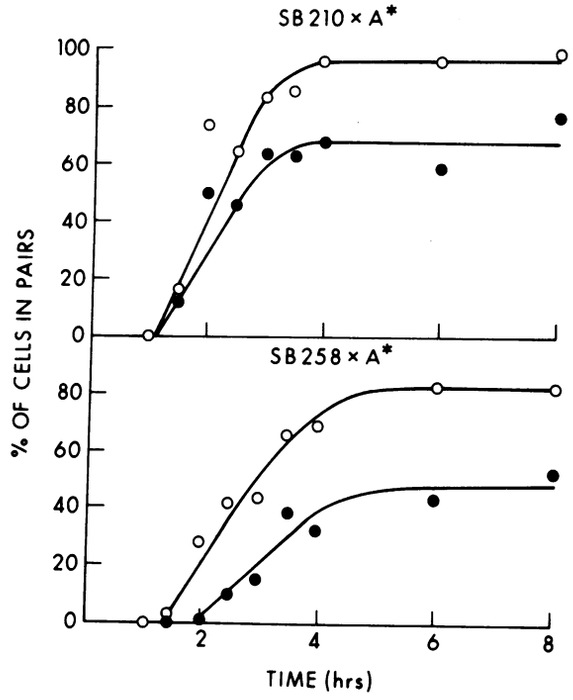

FIG. 2. Kinetics of conjugation and changes in secretory capacity of conjugating pairs, as indicated by pairs within double capsules. (*Upper*) Cross between control cells exo$^+$ (SB210) and exo$^+$ (A*) cells. (*Lower*) Cross between exo$^-$ cells (SB258) and exo$^+$ cells (A*); the kinetics of pairing differ from the control cross in proceeding at a slightly slower rate and fewer cells (≈80%) pair. Note the lag between pair formation and restoration of exocytic capacity to the exo$^-$ cells. ○, % of cells in the mixture that are involved in pairs (encapsulated or not); ●, % of cells encapsulated in the mixture; 100% equals all the cells that are mixed.

restoration of the exo$^-$ competent phenotype occurs relatively rapidly after pair formation. Since the A* mate cannot contribute any genes (9), the appearance of exocytic competence of the SB258 mate must depend on gene-product transfer. The lag period is probably a measure of the time necessary to transfer factor(s) from exo$^+$ to exo$^-$ conjugants to complete the restoration. After ≈5 hr, pairs within double capsules constituted 45% of the population when tested with alcian blue. This demonstrates that full repair of the exo$^-$ phenotype is taking place in >50% of all the conjugating pairs. The percentage of exo$^-$ conjugants rescued was higher (67%; data not shown) if SB258 was crossed to exo$^+$ SB210 cells instead of to A* cells. In any case, the percentages were not very different from those observed in the full exo$^+$ cross (SB210 × A*; Fig. 2 *Upper*).

Similar experiments, in which exo$^-$ SB255 was crossed to exo$^+$ cells, resulted in a low but significant percentage of rescue (3% double capsules). Crosses between SB258 and SB255 proved negative when tested for capsule formation. However, it is also important to take into account that massive mucocyst release is required to give recognizable capsules.

Effects of Cycloheximide. Cycloheximide was added to conjugating cells in experiments designed to determine whether protein synthesis was a requirement for recovery from inhibition of exocytosis at the start of conjugation or for rescue. At 15 μg/ml, cycloheximide completely inhibits incorporation of radiolabeled leucine into trichloroacetate-precipitable material in *Tetrahymena*. Cycloheximide blocks conjugation immediately upon addition of the drug (10). In the SB210 × A* cross, when cycloheximide was added at time zero, no pairs were formed at any time, although ≈80% of single cells kept in cycloheximide became encapsulated when tested with alcian blue 2 hr after mixing (data not shown). This is the frequency of capsule formation predicted for a 1:1 mixture wt/A* cells, indicating that cycloheximide has no effect on stimulus–exocytosis events in individual cells in this system. In the cross SB258 × A*, cycloheximide addition at time zero also completely inhibited pair formation. About 37% of the mixture of single cells is recorded in capsules; again, the percentage predicted for a 1:1 mixture of these cells (data not shown).

Although cycloheximide inhibited new pair formation (Fig. 3), it had little or no effect on the secretory capacity of pairs already formed. Fig. 4 (*Upper*) shows that in the control cross the secretory capacity of such pairs was unchanged in the presence of the drug. When cycloheximide was added 2.5 hr after mating SB258 × A* (Fig. 4 *Lower*), rescue of the secretory capacity of the mutant conjugant occurred with high efficiency. Within 30 min after drug addition (i.e., by 3 hr), 70% of the remaining pairs formed capsules upon stimulation. In contrast, in untreated matings, the percentage of pairs in capsules remained lower than 70% but continued to increase while pair formation continued, until at least 4 hr after mixing. The youngest committed pair formed 2.5 hr after mixing has regained secretory capacity within 0.5 hr. This illustrates clearly that cycloheximide does not affect the secretory events within the time of exposure and that continued protein synthesis after firm pair formation is not required for rescue.

Membrane Signatures of Conjugation Rescue. The assembly of the fusion rosette was investigated in freeze-fracture replicas during conjugation rescue. The membranes of wild-type and A* cells contain rosettes at sites of secretory organelle docking. When these cells are stimulated, the rosette particles disperse and exocytic openings appear, as described (2, 3). The individual IMPs of a rosette (R-IMP) represent a unique size class of particles clearly distinguishable from other IMPs present in the membrane (11). Unmated

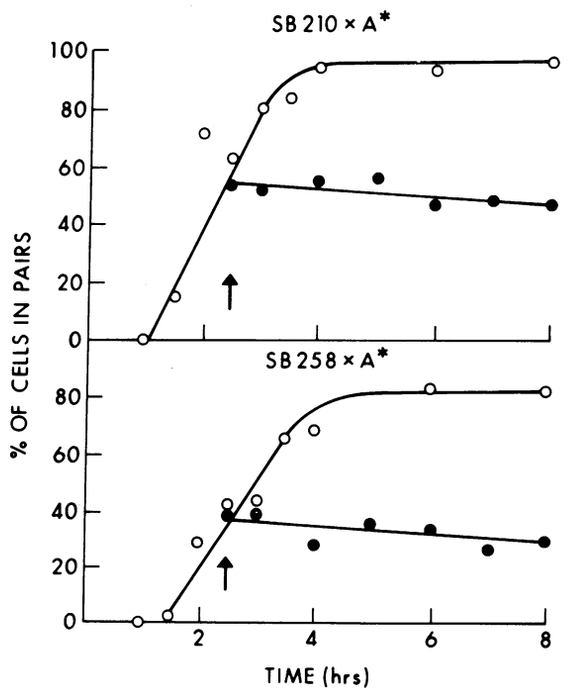

FIG. 3. Effect of cycloheximide addition (15 μg/ml) on pair formation. The drug was added 2.5 hr after the initial mixing of the two conjugants (arrow). Shown here are SB210 × A* (*Upper*) and SB258 × A* (*Lower*). In all cases, pair formation stopped immediately upon drug addition. ○, Untreated control; ●, cycloheximide-treated cells.

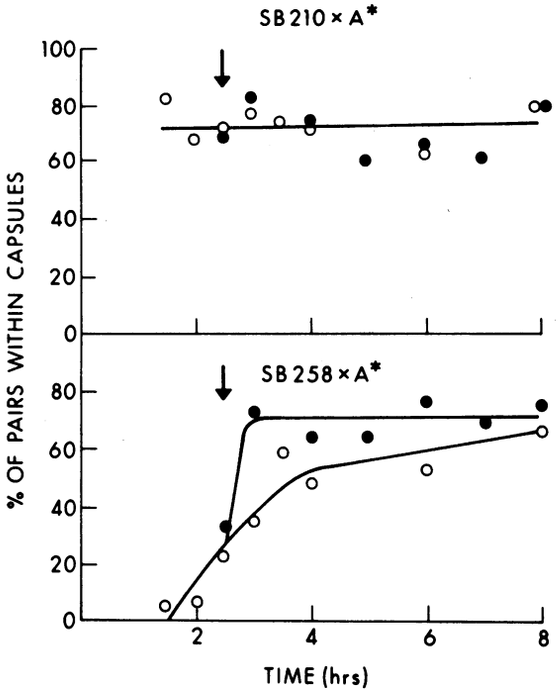

FIG. 4. Effect of cycloheximide on the rescue of exocytic capacity. Capsule formation of already formed pairs is not affected by addition of cycloheximide in both control cross (SB210 × A*) and in the exo$^-$ (SB258) × exo$^+$ (A*) cross. Symbols are the same as in Fig. 3; same experiment as in Fig. 3.

SB258 cells had no fully assembled rosettes, but clusters of 5 or more R-IMPs were consistently observed above docked secretory organelles. In contrast, SB255 cells contained only a few secretory organelles and occasional small clusters of R-IMPs (5). This difference may point to the necessity of having a docked secretory organelle present beneath the membrane at this stage of the assembly in order for individual R-IMPs to assemble into clusters.

Fig. 5*A* shows an example of a freeze-fracture replica of a pair of conjugants from the cross SB210 × SB258 6 hr after initiation of pair formation. Two areas where the membranes of the conjugating pair SB210 × SB258 have locally fused are seen (arrows). These areas form the pores that provide cytoplasmic continuity between the two conjugants. Exocytic openings (arrowheads) are seen in each conjugant, thus indicating that repair of the specific membrane microdomain had accompanied physiological restoration of the mutant. At higher magnification, assembled rosettes were also seen (not shown).

Comparable freeze-fracture replicas of conjugants from the cross SB210 × SB255 were analyzed. Consistent with the inefficient restoration, the mutant conjugant in each pair usually contained very few docked secretory organelles and there was correspondingly little evidence of restoration of features of the exocytic microdomain. Neither wild-type nor SB258 crosses increased the low level of rosettes or restored detectable exocytic profiles to the SB255 conjugant except in the few cases in which rescue was observed (Fig. 5*C*). In the exo$^+$ conjugant (SB210 × SB255, the number of exocytic openings was reduced, and intact fusion rosettes were correspondingly increased compared to control crosses.

Analyses of membranes from the cross between the two mutants SB255 × SB258 showed numerous rosettes (Fig. 5*B*). Presumably, components transferred from the SB255 cell had restored exocytic capacity to mutant SB258. These rosettes were found always at the correct position along 2° meridians and in between 1° meridians, where they normally

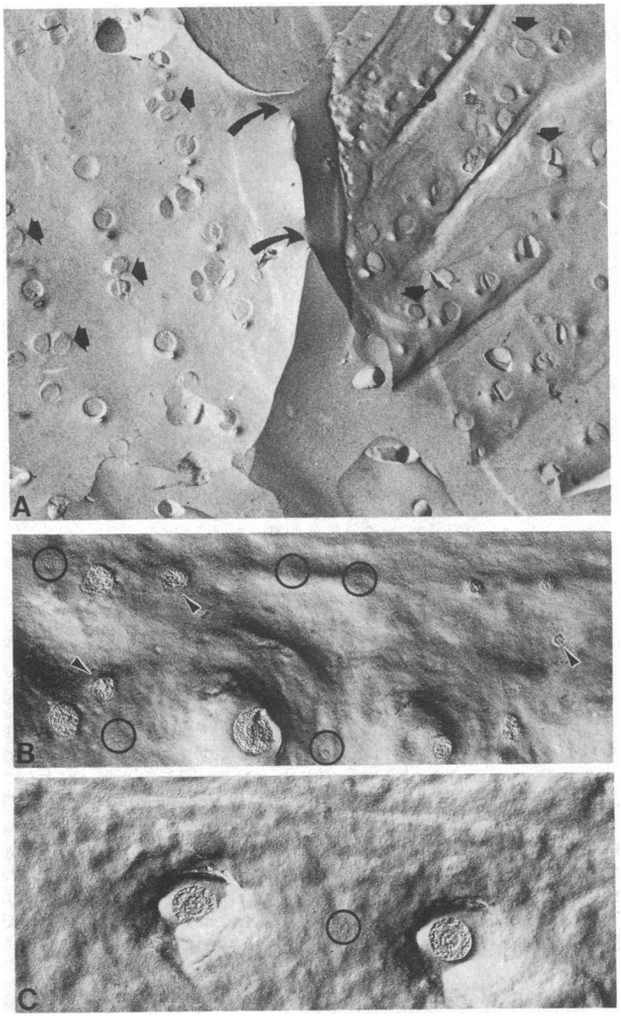

FIG. 5. (*A*) Protoplasmic fracture faces (PF) of the plasma membranes of a conjugating pair, SB210 × SB258, 6 hr after initiation of pair formation. Curved arrows point to local areas where the membranes of the two conjugants have fused. Exocytic openings (arrowheads) are now present in both cells, indicating that the mutant has been rescued by components from the exo$^+$ conjugant. (×7700.) (*B*) PF from a cross between exo$^-$ mutants SB255 and SB258. This membrane fracture shows the presence of both assembled rosettes (circles) and exocytic openings (arrowheads), presumably in the SB258 conjugant. Therefore SB258 has been complemented by components supplied by SB255, enabling it to assemble functional exocytic membrane microdomains. (×18,000.) (*C*) Corresponding PF of the exo$^-$ conjugant SB255. In this case, assembled rosettes or exocytic openings are rarely observed. This particular replica shows formation of one rosette between two cross-fractured cilia—i.e., along a 1° meridian. None is observed along the 2° meridian located above the cross-fractured cilia. (×18,000.)

are observed in exo$^+$ cells (1, 2, 11). Although this conjugant is incapable of forming a capsule, when stimulated, exocytic profiles (arrowheads) were also observed. Therefore, SB255 is capable of partial complementation of SB258 so that functional microdomains are formed. This suggests that different components of the exocytic mechanism are affected in the two mutants.

DISCUSSION

Restoration of exocytic competence in exo$^-$ mutants in *Tetrahymena* is clearly possible during conjugation with wild-type (exo$^+$) cells. The process was analyzed physiologically by capsule formation around the conjugating pair and structurally by assembly of a typical fusion rosette within the

mutant membrane, followed by its dispersion around exocytic openings.

Two lines of evidence exclude the possibility that the rescue observed requires wild-type gene transfer: (*i*) exocytic competence of SB258 conjugants develops within 30 min after firm pair formation, while the exchange of gamete pronuclei does not occur until ≈4 hr beyond that time (12). (*ii*) A* conjugants cannot form gamete pronuclei and thus cannot contribute any genetic information to their mate (9). Yet, they restore exocytosis competence to SB258 cells. Thus, exocytic competence must be restored by the transfer of exo$^+$ gene products. Since both the cytoplasms and the cell membranes of the two conjugants are in continuity, our experiments do not distinguish whether this transfer is due to cytoplasmic exchange or diffusion along the cell membranes.

The low efficiency of rescue of SB255 may be a consequence of the fact that exocytosis in this mutant is blocked at an early step, so that most of these mutant cells have very few docked mucocysts when firm pairs are formed with exo$^+$ cells. Thus, full restoration may require a more complex set of events for this mutant.

The apparent inhibition of exocytic capacity of exo$^+$ conjugants by the SB255 conjugant, as shown by freeze fracture, is noteworthy. It is possible that SB255 produces an inhibitor of exocytosis. Alternatively, the exo$^+$ component(s) required to restore exocytosis competence to SB255 may function stoichiometrically and may be present in near-limiting amounts. Pairing between a wild-type cell and SB255 cell may simply lead to a redistribution of the active (wild-type) molecules between the two cells, with a consequent dilution and lowered capacity of the exo$^+$ conjugant to form capsules. Either of these phenomena could also help explain the inefficient rescue of SB255 by exo$^+$ cells, and that of SB258 by SB255 (compared to SB258 rescue by exo$^+$ cells).

Restoration of exo$^+$ competence in SB258 does not require an extended period of protein synthesis. In the presence of cycloheximide, full exocytic competence is achieved within 30 min of firm pair formation (Fig. 4). This time is comparable to the 1-hr lag between loose pair formation and restoration of exocytic competence in untreated cells (Fig. 2). Thus, restoration in SB258 may well depend entirely on gene products already transcribed and translated prior to firm pair formation. The faster attainment of the rescue plateau in cycloheximide-treated pairs (compared to nontreated pairs; Fig. 4) is attributed to the block in pair formation and, consequently, the more-perfect mating synchrony caused by cycloheximide. Since pairs continue to form in the untreated population, there is a (constantly decreasing) fraction of the pairs that have recently formed, and in which rescue has not yet taken place. Such newly formed pairs are absent from the cycloheximide-treated population.

As illustrated in Fig. 6, the assembly process of the fusion rosette is clarified by these experiments. The exo$^-$ mutants studied contain clusters of R-IMPs above docked organelles, but no clusters where there is no organelle. Clustering of R-IMPs is probably analogous to the clustering of receptors into coated pits (13). R-IMPs may move randomly within the membrane until caught by cytoplasmic proteins, which perhaps are also bound to secretory vesicle membrane receptors. The R-IMPs and occupied mucocyst membrane receptors are thus brought into specific apposition. The membrane receptors are generally far from the docking site in SB255, so that R-IMPs do not usually cluster in this mutant. The next step in the assembly is to transform the clusters of R-IMPs into rosettes. This step is blocked in SB258, but it can be repaired by factors contributed by the wild-type partners or by SB255.

A similar phenomenon (conjugation rescue) has been shown in *Paramecium* for restoration of membrane excitability, ciliary motility, and trichocyst release using light microscopic observations (14–16). With the experiments described in this report, we have shown that rescue of a membrane microdomain responsible for stimulus transduction during exocytosis in *Tetrahymena* can occur at conjugation, and we have identified several important sequential steps that involve endogenous factors that can be transferred from cell to cell, which may promote or inhibit this process. As other exo$^-$ mutants are characterized, detailed mapping of the exocytic pathway and biochemical identification of the various factors involved should prove feasible.

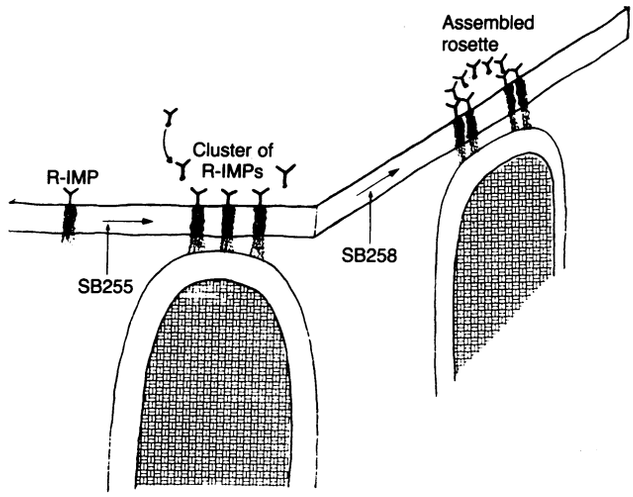

FIG. 6. Steps in the assembly process of the exocytic membrane microdomain (fusion rosette), as clarified by the mutant studies in *Tetrahymena*. The first step in forming a competent exocytic fusion membrane domain is clustering of R-IMPs above docked secretory organelles. The exo$^-$ mutants studied contain clusters of R-IMPs above docked organelles (SB258) but no clusters where there is no organelle (SB255). The next step is to transform the clusters of R-IMPs into rosettes. This step is blocked in SB258, but it can be repaired by factors contributed by the wild-type cells SB210 or A*, or by SB255.

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