MEMBRANE PARTICLE ARRAYS ASSOCIATED WITH THE BASAL BODY AND WITH CONTRACTILE VACUOLE SECRETION IN CHLAMYDOMONAS

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ABSTRACT

Freeze-fracture replicas reveal that five distinct types of intramembranous particle arrays coexist within a small sector of the C. reinhardtii cell flagellar membrane. Of these, three are newly described in this report. (a) Flagellar bracelets, which encircle the flagellar bases, appear to be intrinsically ordered strands of particles of unknown function. (b) Strut arrays, representing nine sites where the basal body attaches to the membrane, appear to serve a mechanical function. (c) Contractile vacuole arrays, which develop into circular plaques of particles, appear to serve as “membrane gates” through which water is discharged from the cell.

The intramembranous particles revealed by freeze-fracture microscopy are believed to represent intercalated proteins (3, 18, 31) and are usually randomly distributed within the fracture plane (4, 6, 15). Occasionally, however, they assume a recognizable pattern, a phenomenon termed “array formation” by Satir and Satir (27). Well-studied particle arrays include those found in intercellular junctions (reviewed in reference 8), in cilia and flagellar membranes (1, 2, 9, 19, 20, 29, 32, 36), and in certain secretory membranes (16, 20, 28, 30).

In the course of a freeze-fracture study of cell fusion in Chlamydomonas reinhardtii (33), we discovered that a small portion of the anterior plasma membrane carries a diverse collection of novel particle arrays. Because these arrays serve as important membrane landmarks in identifying specialized cell-fusion sites of C. reinhardtii gametes, their description is necessary to the accompanying paper (33). More importantly, however, their consideration has led us to some general interpretations of membrane particle patterning and to a specific proposal as to how the contractile vacuole may function.

MATERIALS AND METHODS

Cells of Chlamydomonas reinhardtii strain 137c-H were grown vegetatively or as plate gametes (14) and harvested and frozen as previously described (1), except that in some cases the wash with distilled water was eliminated with no noticeable effect. Preliminary experiments revealed that glutaraldehyde fixation of the cells before freezing tended to cause the fracture plane to leave the interior of the plasma membrane when it came to a flagellum, resulting in the loss of much structural information on the flagellar region. The replicas in this and the accompanying paper therefore derive from cells frozen in the living state, without the addition of glycerol.

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RESULTS

Flagellar Necklaces

Figs. 1-3 show the two flagella of a C. reinhardtii cell as seen in freeze-fractured, thin-sectioned, and freeze-etched preparations, respectively. The membrane associated with the transition region (Fig. 2, tr) between the flagellum proper and the basal body is seen in Fig. 1 to be festooned with three rows of particles that are indistinguishable in appearance and location from the ciliary necklace first described by Gilula and Satir (9) (Fig. 1, n); the presence of three necklace strands contrasts with Ojakian's report (cited in reference 9) of a two-stranded C. reinhardtii necklace.

In longitudinal thin sections of the transition region, two discrete zones of dense material extend between the axoneme and the flagellar membrane (Fig. 2, arrows; see also references 5 and 22); each of these zones is seen in cross section to be made up of nine discrete “connectives” between the nine doublets and the membrane (5, 22). Gilula and Satir (9) suggest that similar connectives generate the necklace arrays in cilia and propose that array-producing interactions occur between the connectives and intramembranous particles. If such a model is applied to C. reinhardtii, however, a two-stranded (or perhaps a four-stranded) necklace would be predicted and not a three-stranded C. reinhardtii necklace.

Several features distinguish bracelets from necklaces. (a) Bracelets associate with the base of the transition region whereas necklaces associate with the mid-transition region. (b) Bracelets are invariably present and easily recognized in the C. reinhardtii membrane whereas necklaces are often indistinct or unrecognizable (e.g., Figs. 3 and 4). (c) The component particles of the bracelet are highly uniform in size and are distinctly smaller than the average plasma-membrane particles, whereas the necklace particles are of the same general size and appearance as the average particles found in the flagellar and plasma membranes. (d) The bracelet particles appear to touch one another whereas the necklace particles do not. (e) The bracelet particles are quantitatively retained in the P face, leaving distinct grooves in the E face, whereas the necklace particles partition between the two faces and do not leave grooves (reference 9 and our unpublished observations).

Flagellar Bracelets

Beneath the necklace region is an array of distinct particles for which we propose the name “flagellar bracelet” (Figs. 1 and 3, b). The bracelet can be seen in Fig. 3 to define the point at which the plasma membrane everts to form the flagellar membrane; in thin section, this corresponds to the distal-most portion of the basal body (Fig. 2). When flagella are cross-fractured (by far the most common fracture plane in our material), the bracelets are seen to surround each flagellum on the P (protoplasmic) membrane face (Fig. 4) and to leave complementary grooves on the E (ectoplasmic) membrane face (Fig. 5). Additional micrographs showing flagellar bracelets are found in the accompanying paper (33). Etched external surfaces of bracelet-containing membranes are smooth (our unpublished observations), indicating that the morphological differentiation does not project beyond the outer membrane surface.

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FIGURE 4 Freeze-fractured plasma membrane P face from a *C. reinhardtii* vegetative cell showing the disposition of the bracelet around the perimeter of a flagellum; the second flagellum of the pair lies directly above the one illustrated in the uncropped micrograph. The straight (s) and undulating (u) aspects of the bracelet are indicated. The necklace particles do not lie in identifiable strands. This cell has lost its flagella and membrane has grown over the tops of the "stub," presumably in preparation for flagellar regeneration (24). × 102,000.

FIGURE 5 Freeze-fractured plasma membrane E face from a *C. reinhardtii* gamete showing the disposition of the bracelet grooves (bg) around the flagellar perimeter. × 102,000.

Each continuous row of contiguous bracelet particles can be termed a "bracelet strand." Approximately 150 particles make up a single strand, and from one to three strands are present at various positions around the circle (Fig. 4). The strands begin and end in no apparent pattern. A pattern can, on the other hand, be recognized when the bracelets are considered in relation to the orientation of the flagella. In the P face, the bracelets usually exhibit both a straight and an undulating aspect (Fig. 4, s and u), and the undulating aspect invariably faces away from the flagellar pair while the straight aspect occupies the membrane between the flagellar pair.

**Strut Arrays**

The arrows in Figs. 6 and 7 point to aggregates of particles lying just outside the bracelet grooves in the E faces. A variable number of these aggregates may be found: in some replicas, particularly those of gametes engaged in sexual agglutination (1), nine aggregates can be discerned (e.g., Fig. 7); in other replicas, only three to seven aggregates are seen (e.g., Fig. 6); and in other replicas, particularly those of free-swimming cells, aggregates are inconspicuous (e.g., Fig. 5). The most obvious candidates for creating such arrays are the nine basal body "struts," first described by Ringo (22) and illustrated in thin section in Fig. 8, which extend from the distal-most ends of the basal body triplets to the plasma membrane. The radial distance from the center of the basal body to the end of the struts is roughly 270–300 nm in thin section, which corresponds to the 270–300 nm radius between the center of a basal body-flagellum hole and the particle aggregates as seen in freeze-fracture replicas (compare Figs. 6–8). The ninefold aggregates are therefore interpreted as sites where struts interact with the membrane (see also Discussion) and will hereafter be referred to as "strut arrays." The fact that these arrays partition exclusively to the E face (27) suggests that each strut is strongly associated with the external half of the cell membrane.

In replicas such as Fig. 6, where fewer than nine strut arrays are present, a pattern can invariably
be recognized: the arrays that are present lie lateral to the flagellar pair, whereas the membrane lying between the flagellar pair either is free of arrays or carries "weak" arrays (Figs. 6 and 7, small arrowheads). Moreover, in favorable replicas where the bracelet grooves are heavily shadowed so that their undulations (cf. Fig. 4, u) can be distinguished, strut arrays are often seen to occupy the "valleys" of the undulations.

The Contractile Vacuole

A C. reinhardtii cell normally possesses two contractile vacuoles, which pulsate alternately, at its anterior end; if the cell were viewed end-on, a line joining the two vacuoles would be at right angles to a line joining the flagellar basal bodies (12). Each vacuole system consists of small vesicles and channels which appear to originate in the anterior perinuclear region and become confluent with a large cisterna beneath the cell membrane.

When the P face of the plasma membrane overlying the contractile vacuole region is examined, particle aggregates that we shall call "contractile-vacuole arrays" are often observed. Fig. 9 shows a small aggregate of particles, Fig. 10 a circle of particles with a few central particles, Fig. 11 a circle of particles with a central aggregate, and Fig. 12 a circular plaque of particles. The diameters of these aggregates are usually about 0.1 μm, but occasionally they may be as large as 0.5 μm.

**Figure 6** Freeze-fractured plasma membrane E face from a C. reinhardtii mt+ gamete agglutinating with mt- flagella (see reference 1). Large arrowheads point to three prominent strut arrays, and small arrowheads point to four less prominent arrays around the cross-fractured flagellum. Faint bracelet grooves (bg) associate with each flagellum in the field. × 74,000.

**Figure 7** Freeze-fractured plasma membrane E face from a C. reinhardtii gamete in a mating culture at 4°C (33). Large arrowheads point to seven prominent strut arrays, and small arrowheads point to two less prominent strut arrays. The second flagellum of this cell is located directly beneath the illustrated field in the original micrograph. × 74,000.

**Figure 8** Thin section of a basal body and its nine associated struts in a cell exposed for 10 min to 0.1 ml/ml of dimethylsulfoxide (DMSO) and subsequently fixed as previously described (14). The disrupted cell is illustrated because all nine struts are included in one section whereas the skewed disposition of the struts in an intact cell precludes such a section (see, e.g., Fig. 37 of reference 22). Arrowheads point to three struts where vestiges of the membrane attachment are apparently present. × 74,000.
ters of these arrays, given in the figure legends, increase from 54 nm to 180 nm, and the increase in size is accompanied by the formation of increasingly well-defined particle-free regions surrounding the central arrays. The freeze-fractured array corresponds, we believe, to the event illustrated at the arrow in Fig. 13: a cytoplasmic contractile vacuole cisterna is seen in thin section to make a direct, localized contact with the plasma membrane, the visible contact region measuring in this case about 65 nm.

The circular contractile vacuole arrays can form without any detectable deformation in the contour of the membrane fracture face, as illustrated in Figs. 9-12. The membrane contours are, however, interrupted in other replicas (e.g., Fig. 14). In some cases, an inward puckering is observed at the periphery of the cleared region (Fig. 14, arrows). In other cases (e.g., Fig. 14), the peripheral pucker is present along with a more central ragged hole that is best termed a "crater."

An examination of the craters reveals that a second membrane always lies underneath; a hole into the cytoplasm has never been observed. In several instances, moreover, this underlying membrane bears a circle of particles. The circle seen in Fig. 14, for example, is approximately 160 nm in diameter and is very reminiscent of the plasma membrane circles shown in Figs. 10 and 11.

It should be noted that the center of each circular plaque and the center of each crater lie medial to, and approximately 1 μm from the two flagellar bracelets, as illustrated in Fig. 3 of the accompanying manuscript (33). Similarly, the contractile vacuole contact region in Fig. 13 lies about 1 μm from the flagellar basal bodies. It is by this fixed medial position that we have identified the contractile vacuole region in replicas and have distinguished its associated arrays and craters from adventitious particle clusters or membrane discontinuities. It should also be noted that while perhaps 30% of our replicas depicting this region of the plasma membrane P face exhibit some sort of circular plaque or crater, the remaining 70% of the P face replicas show no differentiations in this region.

DISCUSSION
The Generation and Function of Membrane Arrays

Four ways that membrane particle arrays might be generated can be listed. (a) The particles may be held in place via interactions with cytoplasmic or ectoplasmic components. (b) Cytoplasmic or ectoplasmic components may exclude particles from discrete regions of the membrane, causing particles to accumulate in the nonexclusion regions. (c) An array of particles established in one membrane may exert an array-forming influence on another membrane with which it comes into contact. (d) The particles may represent a discrete class (or classes) of proteins that are built into the membrane, either at the time of or subsequent to membrane biogenesis, and that spontaneously self-assemble into aggregates or linear arrays without any external influences on the final patterning that is achieved. When these four array-forming possibilities are considered collectively, it is clear that the first three classes can be grouped together under the generalization "induced arrays" and distinguished from the fourth class which can be called an "intrinsic array."

Turning next to the functions of arrays, it seems important to keep in mind two possibilities. (a) Cytoplasmic or ectoplasmic structures may make array-forming physical contact with preexisting specialized membrane proteins to create some sort of functional unit. For example, secretory-vesicle membrane proteins may make exclusive, array-forming contact with specific "receptor" proteins in the plasma membrane as a prelude to the secretory event (28). (b) Alternatively, cytoplasmic or ectoplasmic structures may make physical contact with pre-existing nonspecialized membrane proteins and orient them into stable arrays; for example, array-producing contacts could be established between cytoplasmic structures and random membrane proteins to promote the mechanical stability of the cell (see, e.g., reference 7). In the sections that follow we shall apply these concepts to the arrays observed in the C. reinhardtii membrane.

Arrays Associated with Microtubule Connectives

Of the five distinct arrays of particles that can be identified within the C. reinhardtii anterior cell-flagellar membrane, three clearly relate to the presence of underlying microtubule "connectives" and can thus be classed as induced arrays. Thus the longitudinal arrays along the flagellar shaft (1) clearly bear some direct relationship to the underlying flagellar axoneme (see also reference 29); the flagellar necklaces are presumably the consequence of mid-transition region doublet-to-mem-
Figures 9–12 Contractile-vacuole arrays in the P faces of freeze-fractured plasma membranes. Array diameters: 54 nm (Fig. 9), 115 nm (Fig. 10), 150 nm (Fig. 11), and 180 nm (Fig. 12). × 112,000.

Figure 13 Contractile vacuole in thin section. Small vesicles fuse at the periphery of the large central cisterna (c). Arrowhead indicates where the cisterna makes direct, localized contact with the plasma membrane; dense periodic material is seen to interconnect the inner leaflets of the two membranes. A daughter basal body (22) is included in the field; the parent basal bodies lie below it in the uncropped micrograph. Cell was fixed as described in reference 14. × 112,000.
The Flagellar Bracelet

The flagellar bracelet is present at all times in the *C. reinhardtii* membrane, and there is no evidence that basal body struts, underlying membrane (20), or any other cytoplasmic or ectoplasmic structures "maintain" the bracelet arrays. The distinct size of the component bracelet particles further argues against an origin for the bracelet via a patterning of pre-existing membrane components. Therefore, the bracelet represents a fixed and unique component of the plasma membrane P face, with complementary grooves left in the E face, which would appear to qualify as an "intrinsic array" (viz., the first section of this Discussion).

In considering the possible function(s) of the flagellar bracelet, three additional observations are pertinent. (a) The bracelets are in the correct position to influence the elaboration of the two
"tunnels" that serve as flagellar egresses in the cell wall; we have no direct evidence for such a function, however, and the approximately 150 particles of the bracelet bear no obvious relationship to the 56 components of the tunnel (23). It should be noted that bracelets persist in mating gametic cells that have lost their cell walls and tunnels (see, for example, Figs. 12–25 of reference 33) so that wall "constraints" cannot in themselves account for the bracelet patterns. (b) Bracelets are not detected in replicas of bald-2 cells (our unpublished observations) which carry a mutation affecting normal basal body formation and elongation (10). This finding suggests that mature bracelets are built into the membrane only under the aegis of mature basal bodies. (c) The bracelet lies well below the natural "shear point" at which flagella break off C. reinhardtii cells (see, e.g., Fig. 4 and reference 24) and there is therefore no reason to suppose that it plays a role in the shedding of flagella.

Examination of the replicas of Gilula and Satir (9) indicates that structures comparable to the bracelets are not present in the flagella of the biflagellar Tetrahymena nor in the cilia of sea-urchin blastulae; the fracture planes do not include the bracelet region in their replicas of gill and trachea cilia. Arrays that appear analogous to the bracelet are, on the other hand, present in several replicas of Tetrahymena (36) where they are termed necklaces, while Paramecium possesses unique ciliary plaques (19). One is not led by such comparisons to further insight on the function of the bracelet, although the speculations that have been advanced for necklaces and plaques (e.g., as sites of cation translocation [19]) can, of course, be translated downward.

Perhaps the most interesting thesis to emerge from our consideration of bracelet function is the idea that the bracelet might act as a lateral diffusion barrier, restricting flagellar membrane protein to the flagellum and denying any mobile plasma membrane proteins access to the flagellar compartment. While the polypeptide composition of the plasma membrane of C. reinhardtii is not yet known, it is almost certainly more complex than the single glycopolypeptide found in the flagellar membrane (1, 35); moreover, the fuzzy carbohydrate coat seen in thin section to be associated with the flagellar and transition-region membrane is not associated with the subjacent plasma membrane (Fig. 2). Therefore, some mechanism must ensure that these two continuous membranes retain their biochemical identities, and the flagellar bracelet is possibly a structural manifestation of this mechanism.

**Contractile Vacuole Arrays**

The arrays produced in the C. reinhardtii plasma membrane by the contractile vacuole system are initially very reminiscent of the "fusion-rosette" images published by Satir and colleagues (25, 26, 28); a small aggregate of particles forms in the membrane and the surrounding membrane is cleared of particles. Here, however, the similarity ends; whereas in trichocyst and mucocyst secretion the next events are the formation of a "fusion pocket" and then an opening into the interior of the secretory granule (25, 26, 28), no such openings into the interior of the contractile vacuole have been detected. Instead, the aggregates of small particles go on to increase in diameter to form circular plaques without any deformation in the contours of the membrane. A membrane puckering may then ensue, or the membrane's fracture plane may be interrupted by a jagged "crater," but no cytoplasmic openings accompany these changes: instead, a second membrane is found to underlie the crater, often bearing a similar circle or plaque of particles.

The freeze-fracture images suggest the following sequence of events for contractile vacuole discharge. (a) During the early "filling" (diastole) stage of the cycle, which appears with the light microscope to take perhaps 15–30 s, no contractile vacuole arrays exist in the plasma membrane (explaining their absence in many of our replicas). (b) At the time of discharge (systole), circular arrays of particles appear at a discrete site within the contractile vacuole membrane and at a discrete site within the plasma membrane. We have been unable to determine whether these arrays are independently established within their respective membranes (see reference 28) or whether an array first established in one membrane exerts a patterning influence on the second membrane. (c) In either case, the arrays come to make very close contact with one another. The closeness of the postulated contacts may be promoted by the observed membrane puckering or, alternatively, may create the membrane puckering. The craters are also interpreted to indicate the very close association established between the two membranes: the fracture plane readily skips down into the underlying membrane in the contact region.

Since no "hole" is ever observed within the contractile vacuole membrane, the only apparent
avenue for water egress during systole (13) is through the circular zone of membrane contact. Therefore, while we cannot rule out the possibility that a hole forms and seals so rapidly that we have not detected it in our replicas, it seems reasonable to postulate that the contractile vacuole-plasma membrane contacts create functionally hydrophilic channels which allow water to pass from the vacuole to the exterior, a flow perhaps aided by "squeezing" forces exerted on the cisternal surface by cytoplasmic contractile proteins (21, 34).

In recent reviews (25, 26), Satir has offered two models to explain how vesicle discharge might occur. In both models, matching arrays of intramembranous particles first establish contact between vesicle and plasma membranes. Discharge is then visualized as occurring either by membrane gate release, wherein low molecular weight material passes directly through a hydrophilic channel created by the matching arrays, or by membrane fusion release, wherein the membranes go on to fuse together to create an opening through which secretory products are ejected. Satir's freeze-fracture studies on mucocyst and trichocyst secretion provide examples of membrane fusion release; the contractile-vacuole system described here provides, to our knowledge, the first morphological example of what may be membrane gate release. It is interesting to speculate, in keeping with the concepts of Satir, that membrane gates were the first to evolve and that secretory organelles and intercellular junctions represent modifications of this basic construct.

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