Plasma Membrane Coat and a Coated Vesicle-associated Reticulum of Membranes: Their Structure and Possible Interrelationship in Chara corallina

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ABSTRACT
Primary fixation with buffered glutaraldehyde plus 2.0 mM CaCl₂ and 0.1% tannic acid results in the preservation of certain portions of the plasma membrane coat of Chara when seen with the electron microscope. Such a coat is not observable after fixation with glutaraldehyde alone. The coat appears to be present on all the above ground, vegetative cells of the male plant. Within complex invaginations of the plasma membrane, which are known as charasomes, the coat has two structural components, a central core that is either tubular or solid and a fibrous or granular peripheral region that surrounds the core. The coat material appears to be at least partially derived, via exocytosis, from the contents of single membrane-bound organelles known as glycosomes. Glycosomes seem to originate from within an assemblage of membranes and coated vesicles that can be described, in purely structural terms, as a partially coated reticulum. Such a reticulum is distinguishable from Golgi stacks because the reticulum (a) is not composed of stacked membranes, (b) is extensively involved with large, clearly detailed coated vesicles and coated invaginations, (c) is closely associated with glycosomes, and (d) is only slightly stained by the zinc-iodide-osmium tetroxide reagent.

Chara corallina is a member of the Characeae, a family of large-celled algae that have been studied by many investigators with regard to processes such as ion transport (1), cyclosis (2), and cell wall growth (3). In an earlier series of articles from our laboratory (4-6), the ultrastructure of Chara was examined with special attention being given to studying the development of the charasomes. Barton originated the term charasome (7) to denote a multi-tiered complex of anastomosing tubules that are part of the plasma membrane of Chara. Periplasm is an ancillary term that refers to the apoplastic space that is present within the charasome. Instead of being evenly distributed over the entire cell surface, charasomes are located in a series of circumferential bands along the length of these cylindrical cells (4). Given the proper conditions (8), such bands will acidify the surrounding solution; between bands, the solution that is adjacent to the cell will become alkaline. The precise function of charasomes is not presently understood. However, it has been shown that enzymes that are capable of using ATP are present in the charasome membrane (9) and it has been suggested that proteins that are involved with chloride transport may be present as well (9).

One of the questions regarding the charasome concerns its ability to maintain an anastomosing tubular form while being compressed by a turgor pressure of ~0.7 mPa. While the plant cell wall can resist turgor pressure along the "smooth" portions of the plasma membrane, it is doubtful that a conventional cell wall is present in the periplasm of the charasome (6). Preliminary evidence that the periplasm of Chara does contain some structural components has been presented (6). Such work did not consider the influence of divalent cations on the structure of the charasome, nor was the structure of other portions of the plasma membrane considered. In animal cells, proteoglycans in the extracellular matrix are associated with the plasma membrane and absorb compressive loads (10). However, to our knowledge, little information is available concerning the existence of a plasma membrane associated extracellular matrix (which we shall refer to as a "plasma membrane coat" to distinguish it from the plant cell wall) of plant cells. Robards (11), Levering and Thomson (12), and Olesen (13) have published micrographs of plant cell plasma membranes that have either dense particles or linings that are associated with the membrane to some degree. Robards (11) and Olesen (13) hypothesized that such particles were cellulose synthesizing enzymes. Levering and Thomson (12) showed an extensive lining with a circular substructure that was located within invaginations of the plasma membrane and
that was heavily stained when compared to the cell wall. Such a paucity of information may have been brought about by the existence of factors similar to those that made visualization of the lamina rara of animal cells difficult, until the proper fixation techniques were discovered (14).

In animal cells, proteoglycans of the extracellular matrix are synthesized within the endoplasmic reticulum and the Golgi complex, and are then further processed after exocytosis (10). Supportive evidence for the existence of a generally similar delivery system, in other plant cells, comes from studies that show that exocytosis of Golgi vesicles that contain wall scales (15) and mucilage (16) can occur. It should be noted that some ambiguity is present in contemporary scientific literature concerning the implication of the phrase “Golgi vesicles.” Such ambiguity mainly stems from the studies of Novikoff et al. (17), who have interposed the concept of a GERL into previously constructed schemes of endomembrane flow. The GERL contains an assemblage of vesicles and lamellae that are structurally similar to, but which sometimes differ in their enzyme content from, the immediately adjacent vesicles and lamellae of the Golgi complex. While usage of the term GERL is becoming common in the scientific literature, some workers still prefer to think of the GERL as being part of the Golgi complex (18).

Here we describe the effect of fixative composition on the complex structure of the plasma membrane coat of Chara. In addition, we present micrographs that support the contention that at least some of the material of the plasma membrane coat is derived from a partially coated reticulum of membranes rather than being directly derived from the Golgi stacks. Finally, we describe the distinctive structure and staining characteristics of the partially coated reticulum and show its close association with both coated vesicles and glycosomes.

**MATERIALS AND METHODS**

**Material and Growing Conditions:** Plants of Chara corallina Klein ex Wild., em. R.D.W. (= C. australis R. Br.), obtained from subcultures that had been maintained in our laboratory for more than 5.0 years, were grown in the laboratory under fluorescent lamps at 21.0-23.0°C in 120-l containers in solutions containing 3.0 mM Na*, 0.2 mM K*, 0.1-0.2 mM Ca*, 1.5 mM Cl-, and ~2.0 HCO₃ (pH 8.5-9.5). Only male plants were used. In all cases, cells were exised from the parent plants during the afternoon, incubated in culture media for 1.0 h and then were transferred to our laboratory's standard physiological testing solution that was composed of 1.0 mM NaCl, 0.2 mM KCl, 0.2 mM CaSO₄, and 1.0 mM NaHCO₃ (pH 8.0). Extensive testing by one of us (W. J. Lucas) has shown that Chara cells remain viable when exposed to pH values ranging from pH 5.5 to 9.5 (19). The incubation period after excision was necessary to allow recovery of the cells from both the action potential and the cessation of cytokinesis that occur following excision from the parent plant. Primary fixation for electron microscopy was begun during the afternoon of the next day.

**Techniques for Electron Microscopy:** Cells were immersed in a very slightly hypotonic fixative solution composed of 1.25% glutaraldehyde, 0.03 M sodium cacodylate, 2.0 mM CaCl₂, and 0.1% tannic acid. The temperature of the primary fixative was initially 21-23°C. After the cells had been immersed in the fixative for 5.0 min, they were then transferred to a refrigerator at 4°C. The pH of the primary fixative and the buffer washes was adjusted to pH 7.2 by using HCl or NaOH. The use of more alkaline fixatives was not possible because of the tendency of calcium and tannic acid to precipitate in alkaline solutions. After 12-16 h, the cells were removed from the refrigerator, washed three times in buffer, and then placed in 1.0% O₃O₄ in 25.0 mM sodium cacodylate buffer, pH 7.2, for 2.0 h at 21-23°C. Next, the cells were washed in buffer and then dehydrated with acetone and embedded in Spurr's resin. Serial sections for transmission electron microscopy were prepared on formvar coated, single-slot rhodium grids.

1 **Abbreviations used in this paper:** PCR, partially coated reticulum; PMC, plasma membrane coat; ZIO, zinc-iodide-osmium.

**RESULTS**

**Preservation of the Plasma Membrane Coat**

While primary fixation with glutaraldehyde alone results in the absence of visible material within the periplasm (4-6), the inclusion of tannic acid and calcium chloride in the fixative results in the preservation of the plasma membrane coat (PMC) both within the periplasm and on the external surface of the plasma membrane outside the charasome (Fig. 1). Because the PMC has been preserved, the periplasmic tubules appear to be denser than the symplasmic tubules; this is the reverse of the image that is seen after fixation with glutaraldehyde alone.

Two structural components, a core and a matrix that surrounds the core, comprise the PMC (Fig. 1). In some instances, the core appears as a discrete rod or tube with a diameter of ~130.0-180.0 Å. Frequently, the core is associated with radial extensions that bridge the space between the core and the plasma membrane. Matrix materials of variable structure always surround the core. Discrete particles are evident in some areas of the matrix. In other cases, the matrix is simply a dense, amorphous material that in some places obscures the structure of the core.

Outside the charasome, the matrix appears as a dense fibrogranular matrix that is attached to the external surface of the plasma membrane; core material is only occasionally observed (Fig. 1). In areas where charasomes are not present and the plasma membrane is closely appressed to the cell wall, it is difficult to perceive any structural distinction between the PMC and the wall. But, when the cell is intentionally plasmolyzed during fixation, the presence of the PMC and its attachment to the plasma membrane are evident (Fig. 2). Such a PMC is present on all the cell types of vegetative, male Chara plants (with the possible exception of rhizoids, which we have not studied).

We found that the preservation of PMC core material is quite sensitive to the presence of calcium in the fixative. Concentrations of calcium as low as 0.2 mM, without any tannic acid being added, are sufficient to preserve small, but faintly discernable amounts of core material (micrographs not shown). Also, we noted that if 4.0 mM NaCl is substituted for 2.0 mM CaCl₂ in the fixative, no core material is present. Such a result indicates that the chloride in the fixative is not responsible for the preservation of the core. The addition of 4.0 mM sodium was assumed to have a negligible effect on the fixation process because 30.0 mM sodium was already present in the buffer.

Chara corallina is a long-lived, multi-cellular alga and so it is possible to harvest cells from the same populations over periods of many months. In old cultures of Chara, the growth rate of the alga slows because of depletion of bicarbonate and other nutrients from the culture solution. We observed that when cells from young, rapidly growing cultures are fixed, they always respond to the presence or absence of calcium and tannic acid in the manner that we have indicated. However, in either young or old cells taken from old cultures, in
FIGURE 1  Plasma membrane (PM). Cell wall (W). Periplasm (P) is quite dense compared to symplast (S). Core (unlabeled arrows) is present throughout the periplasm and also along some areas of the plasma membrane outside the charasome. When cut in cross-section, the core appears either as a discrete rod (R) surrounded by a particulate matrix or as a rod with radial extensions attached to it (unlabeled darts). Although a slight plasmolysis has occurred during specimen preparation, the PMC outside the charasome is still continuous (C) with the PMC in the periplasm and also remains attached to the external surface of the plasma membrane. \( \times 112,500 \).

FIGURE 2  Smooth portion of plasma membrane. Space between plasma membrane and wall is artifact of plasmolysis. Unlabeled arrows indicate PMC still attached to portions of plasma membrane that have moved away from the wall as a result of plasmolysis. Wall (W). \( \times 53,500 \).

FIGURE 3  Glycosome (GL) adjacent to charasome with glycosome-like dense body (unlabeled arrow) within the periplasm. \( \times 64,500 \).

which the starch grains are small or absent, the density of the PMC is markedly diminished.

Transport of PMC Materials to the Plasma Membrane

Several types of vesicles appear to physically interact with the plasma membrane of Chara. Frequently, coated pits were observed on portions of the plasma membrane of mature and immature cells. Presumably such coated pits are the result of some type of interaction between coated vesicles and the plasma membrane. The possible relationship of coated pits to the transport of PMC material will become evident in the following section that concerns coated vesicles in the cytoplasm.

At least one other type of vesicle, the glycosome, seems to interact with the plasma membrane. Glycosomes are polysaccharide containing, single membrane bound organelles (21) that occur in all cells of Chara that we studied. The diameter of glycosomes varies within the range of 0.1 \( \mu \)m to 1.4 \( \mu \)m (Figs. 3, 4, and 10). Characteristically, each glycosome contains a central mass of dense material that is separated from the membrane by a nonstaining space. Structures which appear to be similar to glycosomes occur in the periplasm of charasomes (Fig. 3). In most cases, the presumptive former contents of the glycosome partially resemble the PMC in
form. We note that the apparent fusion of glycosomes with the plasma membrane occurs more frequently in some cells than in others, irrespective of either the cell size or cell type. Also, the observed frequency of apparent fusions of glycosomes with the plasma membrane is generally lower than that observed for coated vesicles.

**Coated Vesicles and Glycosomes in the Cytoplasm**

Both glycosomes and coated vesicles with large, clearly detailed coats are frequently observed in the cytoplasm (Figs. 4 and 5). Often, coated vesicles and glycosomes are in close association with a loosely organized reticulum of membranes which we will refer to as a partially coated reticulum (PCR) (Figs. 4–6). Initially, we believed that the PCR was part of a Golgi stack. However, we have never observed the Golgi stack membranes to contain structures that resemble the contents of glycosomes, nor have we observed clearly detailed coated invaginations of the Golgi stack membranes. The only vesicles with which the Golgi stacks of Chara are closely associated are either smooth vesicles, which do not resemble glycosomes, or vesicles with small coats that are so faintly detailed as to be only tentatively identifiable. Even when the PCR and the Golgi stacks are laterally adjacent to each other in the same section (Fig. 6), the PCR is structurally distinguishable from the Golgi stacks. Such structural dissimilarities are further emphasized by the results of serial sectioning (Figs. 7–10). While the PCR has overall dimensions which are similar to those of the Golgi stack, the membranes of the PCR do not overlay each other in the stacked configuration that is characteristic of the Golgi complex. Instead, the PCR is an assemblage of interconnected membranes having no apparent structural symmetry.

Occasionally, some degree of association between the PCR and the Golgi is evident. Figs. 7–10 are serial thin sections that show an area in which one of the PCRs may connect with a Golgi; but it is also possible that these figures represent an instance in which the membranes are located near each other but are not physically connected. Indeed, we examined serial sections of a number of other PCRs in Chara and have not found either a physical connection or even a spatial association with the Golgi. It seemed possible that shear forces within the streaming cytoplasm of Chara might separate the PCR from the Golgi. We obtained evidence against such an hypothesis by noting that, in very young cell types in which cyclosis does not occur (according to our observations), the PCR and Golgi stacks are no more closely related to each other (Fig. 6) than they are in streaming cells.

The general morphological asymmetry of the PCR is accentuated by the variety of structures that compose it. Tubules, lamellae, fenestrated lamellae, smooth vesicles, coated vesicles, and coated invaginations are all frequently part of, or are located very near, the PCR (Figs. 4–10). We did not observe the presence of ribosomes on any PCR membranes. Coated vesicles are more frequently associated with PCR than with any other structure in the cell except the plasma membrane. In some sections (Fig. 7), we observed few coated vesicles or coated invaginations either near or on the PCR. However, serial sections have always shown that coated vesicles or invaginations are present near or on at least some portion of the PCR (Figs. 8–10). Glycosome-like structures occur within the PCR more frequently in some cells than in others, irrespective of either cell size or cell type. Thus it appears that the processes that control the formation of glycosomes have the same intermittent temporal characteristics as do the processes that control the exocytosis of glycosomes.
**FIGURE 6** Two Golgi complexes (G) that are laterally adjacent to a partially coated reticulum (PCR) in a young, nonstreaming cell of the vegetative apex. Note that while the Golgi are surrounded by many apparent vesicles, the only vesicles with clearly defined coats (CV) are located next to the PCR. x 67,500.

**Endomembrane Histochemistry**

The chemical nature of the PCR and the other membranes in young cells was examined by using both osmium impregnation and ZIO. After osmium impregnation, the only cellular structures that showed increased density when compared with controls were some cisternae of the endoplasmic reticulum (micrographs not shown). In contrast, after ZIO many components of the cell, including the endoplasmic reticulum, Golgi, and nuclear envelope, showed greatly increased density (Figs. 11-13). It appeared that much of the Golgi complex was stained and often membranes that were similar in form to those designated as GERL in cotyledon cells (22) were stained (Fig. 11). In no case was an unstained Golgi stack observed, but in some instances a few cisternae of the Golgi or endoplasmic reticulum were unreactive. Additionally, in a few cells an apparent overreaction occurred and the entire cytoplasm of such cells was filled with dense precipitates. However, in no case in which specific staining of cell components was observed were any of the PCR or glycosome membranes stained with much intensity.

Many attempts were made to localize acid phosphatase activity in Chara by using techniques that were similar to those of Novikoff and Novikoff (23). However, in no case were we able to observe an indication that acid phosphatase activity was present either in or near the Golgi complex. Instead, we observed that the membranes of the Golgi complexes of both the controls (incubated without substrate) and the tests (incubated with either nitrophenyl phosphate or β-glycerol phosphate at pH 5.0-5.5) showed numerous lead deposits (micrographs not shown).

**DISCUSSION**

**Plasma Membrane Coat**

The precise structure of the plasma membrane coat is far from clear at present. It is probable that the core within the periplasm of the charasome is a continuous tubule and that the instances of solid, rod-like cores are artifacts caused by either the thickness of, or plane of, the section. However, it may be that the core is both tubular and solid, depending on the location of the core within the anastomosing tubules of the charasomes. From transverse sections, it appears that the core is always surrounded by radial arrays of particulate and fibrous structures. These structures may represent images of helices wound around the core. To either confirm or refute such an hypothesis it will be necessary to understand the precise details of the distribution of these structures both around and along the core.

In an earlier study on Chara (6), it was proposed that the periplasmic space was filled with “small distinct particles . . ., as opposed to a structurally continuous matrix.” In contrast, the results of the present report support the concept that the periplasm contains a complex array of interconnected structures that are also connected to the plasma membrane. Our results do not necessarily preclude the existence of distinct particles in the periplasm. However, we now believe that such particles may have resulted from the relatively extended period of tannic acid application that was used in previous fixations (6).

We have also shown that the external surface of the plasma membrane outside the charasome is connected to a substantial
FIGURES 7–10  Serial sections. Chloroplast (CL), Golgi complex (G), glycosome (GL), and partially coated reticulum (PCR). Figs. 7–9 show an apparent Golgi complex located in the central portion of the figure. Two PCRs are present near the lateral margins of the Golgi at the top and bottom of each figure. The uppermost PCR could be connected to the Golgi (unlabeled arrows; Fig. 7) while the lower PCR does not appear to be in close contact with the Golgi (unlabeled arrows, Fig. 8). It is possible that the membranes of the upper PCR merely overlay the Golgi without actually merging. × 58,000.
FIGURES 11-13  Results of ZIO staining technique. Golgi (G), partially coated reticulum (PCR), endoplasmic reticulum (ER), glycosome (GL), and mitochondria (M). Fig. 11: A few cisternae of the Golgi are unreactive (unlabeled darts) as are the PCR membranes and the glycosomes. The extended, fenestrated sheet of Golgi membranes (S) resembles the GERL in cotyledon cells (23). x 24,000. Fig. 12: Pore in nuclear envelope (unlabeled dart) is evident as are stained Golgi and unstained PCR. Lack of poststaining makes coated regions on PCR difficult to discern (unlabeled arrows) at this relatively low magnification. x 24,000. Fig. 13: Coated regions (unlabeled darts) on PCR are evident as is small amount of ZIO staining within PCR tubules (unlabeled arrow). In contrast, ER and mitochondrion are almost completely stained. x 55,000.
extracellular matrix. Such an observation is at variance with the conventional ultrastructural image of plant plasma membranes as being smooth, tripartite structures with little material being connected to the external surface. The literature already contains some recent information regarding the existence of projections on the internal surface of plant plasma membranes in association with microtubules (24). Thus, it seems that while the complex structure of the plant plasma membrane is becoming more thoroughly understood, its limits are becoming less clearly defined.

Franceschi and Lucas (6) have advanced the hypothesis that the electron-dense material within the periplasm serves to stabilize the structure of the charasome against turgor pressure. Our results support their hypothesis by showing that in the periplasm there exists an abundance of structures that could conceivably form a continuous internal scaffolding to support the charasome membrane against turgor pressure. It also seems possible that the structural continuum of the plasma membrane coat might support the plasma membrane against turgor pressure during a coated vesicle-mediated endocytotic event. The presence of a plasma membrane coat on areas of the plasma membrane other than the charasome suggests that a compressive cushion may function around the entire periphery of the protoplast.

Composition

Commercial tannic acid is a heterogeneous mixture, thus it is difficult to fully understand the nature of its reaction with the plasma membrane coat. However, tannic acid has been used to enhance the contrast and/or preservation of many structures such as microfilaments (25), microtubules (26), the cytoplasmic matrix (27), and the extracellular matrix of animal cells (28). The common attribute of these structures is that they are proteinaceous in nature. Calcium is known to decrease the amount of lipid that is lost during fixation (29). This could stabilize proteins which are linked to lipids in the plasma membrane and PMC. Calcium may also be binding to acidic polysaccharides in the PMC in a manner similar to the binding of calcium to pectins, a class of carbohydrates present in the plant cell wall. It seems reasonable to propose that the PMC is composed of proteoglycans similar to those which are known to be present in the extracellular matrix of animal cells (30).

Formation

The plasma membrane coat is present in all the cell types of *Chara* that we investigated. However, images both of apparent fusions of glycosomes with the plasma membrane and of glycosome production in the PCR are not visible in all cells. It seems probable that either the production and exocytosis of glycosomes is a periodic phenomenon, or that variations in the metabolic rates of particular cells allow us to observe events that occur either more rapidly or slowly in other cells. A further complication is that at present we have not determined which, if any, of the other types of smooth or coated vesicles in *Chara* may be capable of, or necessary for, the formation of the plasma membrane coat. The frequent and close association of coated vesicles with the PCR and nascent glycosomes may indicate that the vesicles are involved in the processing of glycosome-like material. Speculatively, it is suggested that either coated vesicles or small, smooth vesicles that are derived from coated vesicles may represent the most frequently used route for the transfer of coat materials to the plasma membrane.

The Partially Coated Reticulum

The PCR has been previously identified as being a part of a dictyosome or Golgi complex (21). Such an identification is understandable because of the fenestrations that are present in membranes of both the PCR and the Golgi complex. However, it should be noted that the fenestrated configuration is not restricted to Golgi membranes. Recently, Harris and Oparka (22) have shown the existence of fenestrated endoplasmic reticulum membranes in cotyledonous cells. They have also observed membranes having a fenestrated morphology that they believe may be a GERL. The GERL in cotyledons is connected to the Golgi stack by extended tubules; this may be the case in *Chara* as well. In the future we intend to search for the existence of such tubules in *Chara* by examining the cytoplasm with high-voltage electron microscopy. However, the results of the present investigation lead us to conclude that in most cases the PCR in *Chara* possesses a high degree of structural autonomy from the Golgi complex.

It is somewhat difficult to understand why, to our knowledge, a PCR has not been observed in other organisms. In this regard it is interesting to note that, in many cases, coated vesicles or coated invaginations having large, clearly detailed coats are frequently observed to be associated with membranes that are located near Golgi stacks (18 and 31) rather than being directly attached to the lamellae of the Golgi stacks. We propose that the PCR structure in *Chara* has undergone hyperplasic development; in other organisms, the PCR may be composed only of a few coated vesicles and a relatively small amount of membrane. An additional distinction is that in many cases, in *Chara*, the PCR does not appear to be specifically associated with the Golgi stacks.

The results of our cytochemical tests clearly differentiate the PCR and the glycosome from both the Golgi and much of the ER. Given the many instances in the literature of a close association between the Golgi complex of plant cells and coated vesicles (31), we were surprised that the PCR differed so much from the Golgi. To our knowledge, no previous studies of plant cells have documented the reaction of the ZIO reagents with membranes that contain coated regions. As far as the other membranes of the cell are concerned, our results were quite consistent with those obtained for cotyledon cells by Harris and Oparka (22). While in many cases we observed structures that were quite similar to those identified morphologically as the GERL in cotyledon cells (22), our inability to demonstrate acid phosphatase activity in the Golgi makes the use of the term GERL questionable in the case of *Chara*.

The lack of ZIO staining in both the glycosomes and the PCR strengthens our earlier assumption that the glycosomes are directly derived from the PCR. Additionally, such results offer little support for the existence of either membranous connections or membrane flow from the Golgi to the PCR. Instead, it seems more plausible to postulate that the PCR functions as part of a plasma membrane retrieval system using coated or smooth vesicles in a manner similar to that reported by Farquhar (32) for somatotrophs. As such, by monitoring the rate and condition of retrieved membranes, the PCR may apportion plasma membrane coat material into either a storage form (glycosomes) or a form that is immediately used (coated or smooth vesicles). The relationship of the
PCR to the Golgi in such a scheme is unclear, although the evidence does not preclude the eventual passage of membrane from the PCR to the Golgi.

This work was supported by National Science Foundation Grant PCM 81-17721.

Received for publication 20 June 1983, and in revised form 16 January 1984.

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