The *Chlamydomonas* Cell Wall and Its Constituent Glycoproteins Analyzed by the Quick-Freeze, Deep-Etch Technique

**ABSTRACT** Using the quick-freeze, deep-etch technique, we have analyzed the structure of the intact cell wall of *Chlamydomonas reinhardi*, and have visualized its component glycoproteins after mechanical shearing and after depolymerization induced by perchlorate or by the wall-disrupting agent, autolysin. The intact wall has previously been shown in a thin-section study (Roberts, K., M. Gurney-Smith, and G. J. Hills, 1972, *J. Ultrastruct. Res.* 40:599–613) to consist of a discrete central triplet bisecting a meshwork of fibrils. The deep-etch technique provides additional information about the architecture of each of these layers under several different experimental conditions, and demonstrates that each layer is constructed from a distinct set of components. The innermost layer of the central triplet proves to be a fibrous network which is stable to perchlorate but destabilized by autolysin, disassembling into fibrillar units we designate as “fishbones.” The medial layer of the triplet is a loose assemblage of large granules. The outer layer is a thin, crystalline assembly that is relatively unaffected by autolysin. It depolymerizes into two glycoprotein species, one fibrous and one globular. The wall glycoproteins prove to be structurally similar to two fibrous proteins that associate with the flagellar membrane, namely, the sexual agglutinins and the protomers of a structure we designate a “hammock.” They are also homologous to some of the fibrous components found in the extracellular matrices of multicellular plants and animals. The quick-freeze, deep-etch technique is demonstrated to be a highly informative way to dissect the structure of a fibrous matrix and visualize its component macromolecules.
sional, it should be possible to analyze the rules followed during their construction. Some of these rules may prove to be followed during assembly of the more complex, three-dimensional arrays that usually characterize the extracellular matrices of higher eukaryotes.

MATERIALS AND METHODS

Strains and Culture Conditions: The wild-type strains CC-620 (mt+) and CC-621 (mt−) were both derived from strain 137c (all CC numbers refer to stocks available from the Chlamydomonas Genetics Center, Department of Botany, Duke University, Durham, NC 27706). We also examined wall material from the Norwich wild-type strain kindly provided by Dr. Keith Roberts, John Innes Institute, Norwich, England, and found it to have an identical structure to our wild-type strain. In certain experiments we used bald-2 (CC-478), a basal body-defective mutant (18) which accumulates several layers of cell walls during vegetative growth and lacks the flagella which would otherwise compromise whole cell extracts. Gametes were harvested from Tris-acetate-phosphate (19) plates after 7 days of growth as described (20) and suspended in nitrogen-free high salt medium (21); vegetative cells were grown in constant light in liquid TAP medium for 36–48 h. The Norwich strain was grown in liquid yeast/acetate/peptone medium (22) for 5 days.

Isolation and Mechanical Breakage of Cell Walls: Walls were isolated from vegetative bald-2 cells as previously described (16) except that detergent was not used; the walls were washed free of sucrose and suspended in 10 mM HEPES, pH 7.4, 5 mM MgSO4, 100 mM KCI after purification. Walls were isolated from wild-type gametes by mixing 10^6 cells of each mating type together at a final cell density of 5 x 10^6 and allowing them to agglutinate until, by phase-contrast microscopy, the field was filled with walls released by the action of autolysin (usually 1–2 min). EDTA concentrate (1 M) was then added to a final concentration of 12 mM, in order to quench the activity of autolysin (23). The walls were harvested and purified by centrifugation, taking advantage of the fact that the walls form a loose fluffy layer above any material that pellets at 40,000 g (14); therefore, if most of the supernatant above this layer is carefully pipetted off and the centrifuge tube is gently tapped, the walls become suspended in the residual supernatant and can be pipetted away from the underlying pellet. The fraction is then diluted with fresh 12 mM EDTA/HEPES and the procedure is repeated until no solid pellet forms when the preparation is centrifuged. By phase-contrast microscopy, this material consists of egg-shaped walls torn only at their anterior ends (24), plus large wall segments.

Walls isolated from bald-2 or gametic cells were suspended in 30 mM HEPES, pH 7.4, 100 mM KCl, 5 mM MgSO4, and 0.5 mM EDTA (HKMgE), and disrupted either by ultrasonic disintegration using a Fisher device with a microprobe operating at 70 W for 20-30 s, or by shearing in a Virtis homogenizer operating at top speed for 1–2 min. The samples were then pelleted at 40,000 g for 10 min. Both the pelleted and the supernatant material was mixed with freshly cleaved mica flakes and quick-frozen (10); similar results were obtained with both fractions.

PERCHLORATE SOLUBILIZATION AND FRACTIONATION OF WALLS: Isolated gametic walls were mixed with an equal volume of 2 M perchlorate in distilled water. Phase-contrast microscopy demonstrated that the initially phase-dense walls were converted to barely detectable "inner walls" (14) after 15 min. The inner walls were pelleted by centrifugation at 100,000 g for 30 min. In some experiments the supernatant was loaded on a Sepharose 2B column as described by Catt et al. (5) and eluted with 1 M perchlorate, collecting 2-ml fractions. Each fraction was dialyzed against several changes of distilled water and disrupted either by ultrasonic disintegration using a Fisher device with a microprobe device operating at 70 W for 20-30 s, or by shearing in a Virtis homogenizer operating at top speed for 1–2 min. The samples were then pelleted at 40,000 g for 10 min. Both the pelleted and the supernatant material was mixed with freshly cleaved mica flakes and quick-frozen (10); similar results were obtained with both fractions.

RESULTS

The Cell Wall In Situ

In a previous thin-section study, Roberts et al. (11) concluded that the cell wall of Chlamydomonas reinhardtii is constructed of seven distinct layers; a diagram of their model is reproduced as Fig. 1A. When the wall is quick-frozen and cross-fractured, it is also possible to discern seven layers (Fig. 2). However, it
becomes clear that the domains designated W3 and W5 by Roberts et al. (Fig. 1 A) are in fact spaces, transected by delicate strands, rather than true layers, a conclusion also reached by Dr. K. Roberts (personal communication). When the wall is fractured tangentially, which provides optimal views of the other layers, the W3 and W5 spaces are very difficult to identify. We therefore indicate the W3 and W5 regions when, as in Fig. 2, they can be identified, but in general refer to the wall as possessing five layers—W1, W2, W4, W6, and W7 (Fig. 1 B)—retaining the Roberts numbering system to facilitate comparison between the two studies. We should note that Roberts has concluded that W2 and W6 are identical in substructure (15) whereas our studies indicate that they are distinctly different, a controversy that remains unresolved.

Fig. 3 shows a survey view of the cell and its wall in tangential fracture; Fig. 4 shows the “central triplet” (W2, W4, and W6) at higher magnification; and Figs. 5–11 show stereo views of the individual layers, all from quick-frozen, unfixed cells. In the paragraphs that follow we describe the architectural features of the wall that are revealed by these images, features summarized in the diagram presented as Fig. 1 B.

W1 (Fig. 5): This region extends from the cell membrane to the inner dense layer of the triplet (cf. Fig. 3) and is quite variable in width from one cell to the next. Its constituent fibers, which vary in caliber, radiate outward from the membrane, making numerous side-to-side anastomoses with one another to create a three-dimensional trabeculum. A variable number of relatively large granules (Fig. 5, arrow), many 15–20 nm in diameter, associate with the fibers.

W2 (Fig. 6): This layer also appears to be a network of anastomosing fibers, but the “weave” is much tighter than the underlying W1 layer (cf. Figs. 3 and 5). Relatively thick fibers are interconnected by thinner fibers, the thick fibers tending to lie parallel to the cell surface (Fig. 2).

W4 (Fig. 7): The prominent components of this medial region of the wall are large granules, ~14 nm in diameter, which line up to form layer W4 as viewed in cross section (Figs. 1 A and 2). Above and below the granules are thin strands, not visible in thin section (11), which occupy the electron-transparent layers denoted W3 and W5 by Roberts et al. (11) (see Fig. 1 A); the W3 zone (3) is indicated in Fig. 6, and W5 (5) is shown in Fig. 7. When fractured en face, the W4 granules are found to be loosely aligned into chains that meander in a plane parallel to the cell surface. Often, these anastomosing chains appear to contain amorphous material between the granules, but this may simply represent confluence of adjacent granules.
W6 (FIGS. 8 AND 9): This layer corresponds to the outer dense line of the central triplet (Fig. 1A). When fractured en face it proves to be composed of two discrete sublayers which we will designate W6A and W6B (Fig. 1B). W6A, which lies closer to the cell surface, is very densely woven, almost a mat (cf. Fig. 4), so that its construction can only be discerned when the weave has been pulled apart during fracturing, as in Fig. 8. The layer is then seen to be constructed from stout parallel fibers (running diagonally, 30° from the horizontal, from upper left to lower right in Fig. 8), which are interconnected by delicate cross-fibrils. The overlying sublayer W6B, by contrast, is an open weave (Figs. 6B, 8, and 9) which creates a polygonal lattice (see also Fig. 3). A and B are considered sublayers of W6 for reasons presented in a subsequent section.

W7 (FIGS. 10 AND 11): The constituent fibers of W7 are similar to those in W1: they radiate outward (Fig. 3) and make side-to-side anastomoses to form a trabeculum (Figs. 3 and 10). When adsorbed to mica flakes they retain this branching morphology (Fig. 11) and show no tendency to disassemble into subunits.

The Warp and Weft of the Cell Wall

When vegetative cells are stripped of their walls by the addition of exogenous autolysin, they re-grow new walls over a period of several hours (25). A detailed study of wall regeneration is in progress; here we show images of this process that are relevant to an understanding of wall construction. Immediately after autolysin treatment, the cell membrane is
FIGURES 5–10   Stereo views of individual wall layers from intact cells. (Fig. 5) W1 layer (1) extending from the plasma membrane (at lower right in the uncropped field) upward to the dense W2 layer (2) at left and top. In W1, granules (arrow) are typically associated with the trabecular meshwork. × 120,000. (Fig. 6) W2 layer (2) extending upward to the W3 "space" (3) transected by delicate fibers. × 165,000. (Fig. 7) W4 layer (4) extending upward to the W5 "space" (5) which, in turn, contacts the dense W6 layer (6). × 165,000. (Fig. 8) W6 layer, torn diagonally at right center during sample preparation, with the granular W4 layer at
lower right. The layer is comprised of two sublayers. W6A is constructed from thick strands running parallel to the diagonal tear, which has occurred between two of the strands. These are interconnected by narrow fibrils, a cluster being marked by the arrow. W6B is constructed of an open weave of fibers, seen more clearly in Fig. 9. × 165,000 (Fig. 9) Polygonal weave constituting the W6B sublayer of W6. × 165,000. (Fig. 10) Outer W7 layer. × 120,000.
FIGURE 11 Stereoview of fibers of the W7 layer absorbed to mica (the uncropped micrograph includes an intact cell wall, only the peripheral W7 fibers of which made contact with the mica). x 120,000.

apparently devoid of all surface fibers (contrary to an earlier study from this laboratory [16] in which an incomplete removal was observed). However, after autolysin is washed away, the cell surface quickly acquires a coat of radial fibers (Fig. 12) similar to that seen in our earlier report (16). Discontinuous plaques of laminar material then appear within this trabeculum (Fig. 13, where discontinuities are indicated by arrows). These plaques enlarge in size until they contact to form a continuous layer (Fig. 14). En face views demonstrate that this primary lamina is organized like sublayer W6A (not shown). The medial and inner layers then assemble beneath this primitive W6 layer (Fig. 14, arrow).

We interpret these images to indicate that the W1 and W7 fibers are morphologically continuous with one another and constitute the original "warp" of the wall fabric. The elements of the central triplet are then assembled within this continuum to form the "weft." As the wall matures, the fibers in the W1 and W7 regions may well undergo distinctive chemical modifications and/or associate with distinctive materials (e.g., granules become localized exclusively in W1 [Figs. 3 and 5]). Therefore, W1 and W7 may have quite different biochemical and functional properties in the mature matrix (cf. reference 6).

Structure of the W6 Layer

When intact walls isolated from bald-2 cells are sonicated or homogenized, the W6 layer frequently separates from its underlying layers and forms discrete fragments. Fig. 15 shows such a fragment that has adsorbed to mica by its undersurface (that is, the surface normally facing the cell membrane), where the W6A and W6B domains appear much as in replicas of the intact wall (Figs. 3, 4, 8–10). Fig. 16, by contrast, shows a fragment adsorbed by its upper surface, so that its undersurface is exposed to view. The undersurface, not normally visible in intact walls, displays a prominent lattice of globular units which define a series of parallellograms.

Fig. 17A diagrams the units of this W6 "undersurface" lattice as seen in deep-etch images, where the four major morphological units are designated A–D. Their crystalline spacing defines three major lattice planes, disposed as drawn in Fig. 17B. In Fig. 17C we have redrawn, to the same scale and orientation, an optical reconstruction of negatively stained W6 material obtained by P. Shaw and K. Roberts (15, 26). There is clearly excellent correspondence between the positions of the major foci deduced by the two techniques.

Fig. 17D diagrams the polygonal units that form sublayer W6B (Figs. 8 and 9). The foci of this array are superimposable on the major foci of the W6A crystal (Figs. 17, A–C), leading us to propose that the two arrays represent interacting sublayers of a single wall domain, W6.

In Vitro Assembly and Dissassembly of the W6 Crystal

Catt et al. (27) have demonstrated that when purified cell walls are solubilized in 1 M perchlorate and the extract is subjected to Sepharose 2B chromatography, a fraction designated 2BII is obtained which, when dialyzed against water, assembles into crystals with the same lattice parameters as the W6 layer in situ (27). SDS PAGE of the 2BII fraction reveals the presence of four glycopolypeptides (5). We have found that we can generate apparently identical crystals by a simpler procedure, namely, by extracting living bald-2 cells with 1 M perchlorate, dialyzing and lyophilizing the extract, redissolving the lyophilate in 1 M perchlorate, and allowing this concentrated extract to dialyze (see Materials and Methods of the W6A sublayer).
Figures 12-14. Regrowth of vegetative wall after autolysin treatment of intact vegetative cells. (Fig. 12) 1 h after stripping, showing new radial fibers, X 60,000. (Fig. 13) 2 h after stripping, showing discontinuities (arrows) in the assembling W6 layer, X 55,000. (Fig. 14) 3 h after stripping, showing new W6 layer and onset of W2 accumulation (arrow). Association of W1 fibers with the cell surface is seen to advantage. Note that the polygonal weave has not yet formed in the W6 layer. X 60,000
Figures 15 and 16. Fragments of the W6 layer. (Fig. 15) Upper surface of fragment recovered from a Virtis-homogenized preparation of isolated vegetative bald-2 cell walls. A, W6A layer; B, W6B layer. × 170,000. (Fig. 16) Stereo pair of the undersurface of a W6 fragment from a sonicated bald-2 cell wall preparation. × 165,000.

Figure 17. (A) Diagram of the undersurface of the W6 layer based on camera-lucida drawings of images such as Fig. 16. The fibrous elements B and/or C may represent "inside views" of the W6B system. (B) Lattice planes defined by the major foci (A–D) in Fig. 17A. A nearly upright parallelogram (80° by 100°) and a more obtuse parallelogram (54° by 126°) are evident. (C) Optical reconstruction of the W6 lattice from a negatively stained image, produced by Roberts et al. (15) and Roberts (26) and redrawn here with the same orientation and to the same scale as the other diagrams. (D). Diagram of the W6B polygonal network (Fig. 9) drawn to the same scale as A–C.
for details). At least 50 mg of pure crystals can in this way be prepared from a 5-liter culture. SDS PAGE of these crystals (Fig. 18) reveals the same four major glycopolypeptides that are present in 2BII fractions (5). Deep-etch replicas of the undersurfaces of both the 2BII crystals (Fig. 19) and the bald-2 crystals (Fig. 20) show the same lattice organization found in native W6 fragments (Figs. 16 and 17).

Particularly instructive is the tendency of these crystals to disassemble as they absorb to mica, permitting views of their component glycoproteins. A detailed analysis of such images is underway; here we report two initial major findings.

First, it is clear from viewing the intact crystal that the W6A undersurface (Fig. 19) must be constructed from glycoproteins having discrete globular domains, corresponding to domains A and D in Fig. 17. In the periphery of disassembling crystals we find elongated globular elements—presumably glycoproteins—indicated by black arrowheads in Fig. 19 and, more clearly, in Fig. 20. They vary considerably in length, from ~50 to 70 nm depending on how distended they are on the mica. The caliber of their globular domains is sufficiently similar to those in the crystal to suggest that they interact to form the W6A sublayer (cf. Fig. 1B), but we have not yet deduced how these interactions take place.

It is also clear from viewing the intact crystal that its upper layer (W6B) must be constructed from glycoproteins that are predominantly fibrous, creating the open polygonal meshwork shown in Figs. 9, 15, and 17D. In Figure 19, the arrows

**Figure 18** SDS PAGE of crystals harvested from a dialyzed bald-2 perchlorate extract. The same gel was stained first by the periodic acid-Schiff procedure (A) and then by silver stain (B). The four major glycopolypeptides of the crystal (13) are labeled 1–4. Three minor glycopolypeptides migrate between bands 2 and 3. A few minor bands migrating below band 4 stain only with silver. Gel system and periodic acid–Schiff staining as in reference 16; silver staining was performed according to the procedure of Morrissey (44).
FIGURE 20  W6 crystal (right), polymerized in vitro from a bald-2 extract, disassembling as it contacts the mica surface into two species of glycoprotein, one fibrous and one globular. The 100-nm fibers are aligned horizontally; each carries a head (h) at one end and may kink (arrows) along its shaft. Chains of globular domains (arrowheads), aligned vertically, resemble the globular components of the crystal. × 350,000.

denote regions where the globular glycoproteins are absent and an underlying system of fibers is exposed. The fibers are seen to interact with one another to form a network, leading us to propose that they constitute the polygonal meshwork. Individual fibers of this class are seen at F in Fig. 19 and are prominent in Fig. 20, where they align along the horizontal axis of the field (note that the globular glycoproteins [arrowheads] align along the vertical axis, suggesting that the two species respond to opposite properties of the mica crystal). Each fiber measures 102 ± 7 nm (n = 55) and carries a head at one end; some are straight, some are curved, and some make distinct kinks along their shaft (arrows). These glycoproteins are presumably equivalent to the 100-nm fibers visualized by Roberts (26) in 2BII fractions. We are currently seeking to understand how the fibers interact to form the W6B layer and how they align with components of the underlying W6A layer; we are also asking which of the four glycopolypeptides in the crystal (Fig. 18) forms which domains of the crystal glycoproteins.

The W2 Layer

When isolated cell walls are treated with 1 M perchlorate or other chaotropes, an insoluble, phase-transparent, wall-shaped residue remains which Hills et al. (14) interpreted to be W1. Repeating these experiments with isolated cell walls (Fig. 21) and with intact vegetative cells (Fig. 22), we conclude on the basis of morphology that the bulk of this insoluble material derives from the W2 layer, although a variable amount of the W1-W7 fiber system is also present.

FIGURES 21 and 22  Perchlorate-treated cell walls. (Fig. 21) Wall shed by the Norwich wild-type strain and treated with 1 M perchlorate. The residual layer corresponds to W2 in morphology. The flagellar collars (cf. reference 16), which are also insoluble in perchlorate (14), are labeled C. The W1/W2 system is absent, as is often the case for shed walls of the Norwich strain even before perchlorate extraction (data not shown). × 28,000. (Fig. 22) Vegetative bald-2 cell incubated in 1 M perchlorate for 60 min. The W1, W2, and W7 layers, insoluble in perchlorate, continue to surround the cell, but the other wall layers have been removed. CM, cell membrane, P fracture face. × 56,000.
FIGURES 23 and 24 Cell walls shed during a Chlamydomonas mating and frozen after 20 min. (Fig. 23) The polygonal network of the W6B sublayer (cf. Fig. 9). (Fig. 24) The orthogonal crystalline lattice of the W6A undersurface (cf. Figs. 16 and 17). Fibrous trabeculae extend from both the upper and undersurfaces of the W6 layer, presumably deriving from the W1/W7 system. x 80,000.

Three experimental observations indicate that this W2 layer is disassembled by autolysin, the wall-disrupting agent released during the mating reaction between Chlamydomonas gametes. (a) If perchlorate-generated “ghosts” (Fig. 21) are exposed to autolysin, the ghosts dissolve. (b) Perchlorate-treated bald-2 cells (Fig. 22) lose their surrounding W2 layer when exposed to autolysin. (c) Cell walls released into the medium during a normal mating reaction and frozen after 20 min display both the W6B (Fig. 23) and the W6A (Fig. 24) sublayers of W6 and retain trabeculae of W1 and W7 fibers; however, the W2 layer is absent. We conclude, therefore, that perchlorate and autolysin have reciprocal effects: perchlorate solubilizes W6 but not W2 (Figs. 21 and 22), whereas autolysin solubilizes W2 but fails, at least initially, to solubilize W6 (Figs. 23 and 24). Fragments such as those illustrated in Figures 23 and 24, we should note, are not stable: with time, they break up into smaller pieces and presumably disintegrate completely. Therefore, autolysin may have a slower-acting effect on W6. It is also important to note that when autolysin is secreted during the mating reaction, the cell wall is quickly released from the cell, much like an egg shell from an egg, its anterior end alone carrying a large tear (see reference 24). Therefore, the association between the W1 fibers and the plasma membrane must also be unstable to the presence of autolysin.

It follows from the above that the supernatant from a mixture of mating gametes should initially become filled with the products of W2 disintegration. Fig. 25 shows that such “mating supernatants” contain an abundance of fibrous units shaped like fishbones, with a central “spine” and “ribs” ra-
diating from either side. Fishbones are also released when perchlorate-treated wall "ghosts" (Fig. 21) are exposed to a partially purified preparation of autolysin (not shown). Thus we conclude that the W2 layer is constructed from fishbones, that these normally associate to form an insoluble matrix around the cell, and that the associations are sensitive to autolysin.

Figs. 26-29 illustrate features of fishbone construction. The central spine, when naked (Figs. 28 and 29, arrows), is a meandering unit, 127 ± 7 nm (n = 10) in length. When associated with ribs, the spine appears more acutely twisted (Figs. 26 and 27), suggesting that rib attachment causes it to deform, and it measures 137 ± 14 nm (n = 44) in length, the large standard deviation reflecting one's impression that some spines appear far more stretched out than others. There is considerable variability in the number of ribs attached per spine, but the maximum thus far encountered is 20, meaning that potential binding sites exist at least every 6 nm along the spine. The ribs are of two lengths: 195 ± 7 nm (n = 62) and 102 ± 8 nm (n = 152). It is perhaps significant that the long ribs are roughly twice the length of the short. Short ribs are more common than long, but no obvious pattern is followed in their attachment to the spine: some spines have no long ribs, some alternate long and short ribs, some have clusters of long ribs, and so on.

In situ, the W2 layer consists of stout and narrow fibers (Figs. 4, 6, 21, 22), suggesting that the spines may associate end-to-end to form the stout phase of the meshwork and that the ribs may reach out and bind to several spines to form the narrow interconnecting elements. We have not yet developed, however, a clear picture of how fishbones interact with one another.

Fig. 30 compares the SDS PAGE profile of intact cell walls (A) with perchlorate-insoluble wall residues (B). The major glycopolyptides of the W6 layer (arrowheads) (see also Fig. 18) are depleted (but not absent) from the perchlorate-insoluble material (B) while other bands become more prominent. Imam et al. (29) recently reported that when isolated cell walls are treated with Sarkosyl-urea, they are converted to very thin walls that are degraded by autolysin. They report that these thin walls are not well solubilized for SDS PAGE in the normal SDS lysis buffer but only after boiling in lysis buffer containing concentrated (400 mM) dithiothreitol. Repeating their protocols, we find that three of the bands present in perchlorate-insoluble wall preparations (lane B) become far more prominent after boiling in dithiothreitol (lane C, arrows). The doublet band migrating at the 100 kD position in lane C may correspond to the polypeptide designated "mantelin" by Imam et al. (29). Taken together, it seems likely that the Sarkosyl-insoluble and perchlorate-insoluble wall layers are equivalent, and that both can be solubilized either by strong reducing conditions or by autolysin.

The Medial (W3-W5) Layer

If, as we proposed earlier, the W1/W7 fibers form the "warp" of the cell wall, then many of the slender fibers seen coursing through the medial layer (Figs. 2-4, 6, 7) can be presumed to derive from the W1/W7 system. In addition, the W4 layer contains a loose meshwork of associated granules,
FIGURES 26–29  Fishbones. Fig. 27 is a stereo pair; Figs. 28 and 29 show disassembling units. Arrows, naked spines. (Fig. 26) × 280,000; (Fig. 27) × 165,000; (Figs. 28 and 29) × 280,000.
seen to advantage in Figs. 4 and 7. The granules are not visible in perchlorate-extracted walls (Figs. 21-22) nor in autolysin-generated outer layers (Figs. 23-24), but it is not known whether they are directly affected by perchlorate and autolysin or whether they simply “fall off” once the upper and lower layers separate.

Fibers Associated with the Flagellar Membrane

The flagellar membrane of C. reinhardtii carries fibrous elements with sufficient structural homologies to the wall fibers to suggest that they are specified by a similar family of genes. Fig. 31 shows three sexual agglutinin “canes” and two “short canes,” both species being displayed on the surface of gametic flagella (references 30-32). Included also in the plate are two fishbone ribs (Fig. 31) and a field of 100-nm fibrils from a Sepharose 2BII fraction (Fig. 32). Although dissimilar in length, the agglutinin cane and the 100-nm fibril are otherwise quite similar in morphology: both are equivalent in width, carry prominent heads, and display distinct kinks and bends along their shafts. The long and short ribs are similar in length to the long and short canes, respectively, but they are distinctly thinner, lack prominent heads, and never kink or bend. Collectively, therefore, the fishbones appear by morphology to be the more distantly related species.

Yet another element with structural homologies to the canes and the wall fibers is a woven array which we designate as a “hammock.” A fully extended hammock is shown in Fig. 33, and a single “thread” of the net is shown in Fig. 34. Hammocks are encountered in purified flagellar preparations from both vegetative and gametic cells (reference 32); they are also encountered in mating supernatants, but not in cell wall preparations. They usually lie free on the mica, as in Figs. 33 and 34, but occasionally associate with the flagellar membrane in the fashion of a mastigoneme (references 33 and 34). Unlike the “ordinary” mastigonemes, however, which are composed of globular subunits (33), the hammocks have the distinctive fibrous structure described below.

The basic unit of the hammock is a check-shaped fibril, 55 ± 5 nm (n = 18) in length, with an invariant morphology: the first 80% of its length is a straight rod; it then makes a right-angle turn so that the final 20% is ~90° to the first. These fibrils interact in two planes: end-to-end and side-to-side. The end-to-end association creates long single threads (Fig. 34). A knob-like protuberance is present where the two ends join. Although we suspect that this represents a globular “head” like that on the 100-nm W6 fibril or the agglutinin, it may instead be created by the overlap of the fibrils; these alternatives cannot be distinguished until a means is found to dissociate the hammock into monomers. The side-to-side association, which is less stable to mica adsorption, involves an interaction between the knobs on one thread and the “right angles” on the adjacent thread. The right angles, we should note, are reminiscent of those formed by many of the disassociated 100-nm fibrils from the W6 layer (Figs. 19, 20, and 32), and the overall weave is reminiscent of the weave detected in the W6 crystals (Fig. 19) and in the W6B polygonal network (Fig. 9).

DISCUSSION

Chlamydomonas Wall Construction

Several levels of organization can be described for the cell wall of Chlamydomonas. The central domain is differentiated into three layers: an outer crystalline layer (W6), a medial granular layer (W4), and an inner dense layer (W2). Roberts and his collaborators have shown (5, 14, 27) that some of these layers can be solubilized by 1 M perchlorate and that they reassemble as the perchlorate is removed by dialysis; therefore, at least some of the layering must be established by hierarchies of protein–protein interactions and not, for example, solely by the sequential secretion of proteins during wall assembly. It follows that there must exist interactions between proteins in the same layer that hold it together, and also interactions between one layer and the next so that the whole assemblage holds together as a uniform 50-nm triplet.

A common mechanism used to ensure that elements in a given domain of the extracellular matrix remain together is to cross-link them after they co-assemble. Such cross-linking is used, for example, in the formation of elastin fibers (35), basal lamina (36, 37), and the primary wall of higher plants (37, 38). The W2 layer of the Chlamydomonas wall (Figs. 3, 4, and 6), if not covalently linked, is at least sufficiently bonded to resist dissociation by perchlorate or SDS (11), but is sensitive to the action of autolysin (present report). Imam et al. (29) have suggested that the wall possesses a “framework” held together by strong disulfide linkages and that autolysin dissociates these linkages. If, as we propose, the autolysin-sensitive framework is layer W2, and this layer is formed from fishbone units (Figs. 25-29), then disulfide linkages may hold ribs and/or spines together.
FIGURES 31 and 32  Purified flagellar surface and cell wall proteins. C, three sexual agglutinins, mt⁺; S, two short canes associated by their termini; R, two ribs, one 100 nm and the other 200 nm, derived from fishbone units (Figs. 25–29). Fig. 32 shows 100-nm fibrils from the W6 layer purified by Sepharose chromatography; arrows designate examples of proteins that make kinks or bends in the fashion of agglutinin canes. Note that proteins with globular domains (cf. Fig. 20) are also present in this 2BI fraction. × 205,000.

The W6 and W4 layers, by contrast, are apparently not stabilized by covalent interactions, since they disassemble in the presence of certain chaotropes (e.g., sodium perchlorate) or upon adsorption to mica surfaces. We predict, therefore, that these layers maintain their integrity via electrostatic and/or hydrophobic interactions amongst their constituent glycoproteins.

Studies of wall assembly, which are still in progress, define one of perhaps several systems which serve to integrate the various layers into a coherent unit. As shown in Figs. 12–14, radially disposed fibers initially extend from the cell membrane to the extremity of the W7 layer, and the three major layers of the central triplet then assemble within these fibers. Since the W6 layer can assemble in vitro in the absence of these fibers, they are clearly not essential for W6 polymerization. They may, however, contain a domain along their length which provides a nidus highly favorable to W6 polymerization, thereby assuring that the discontinuous W6 foci
(Fig. 13) merge to form a continuous layer. Certain Chlamydomonas mutants have been described that are unable to organize a wall around themselves but secrete wall proteins that polymerize normally in the medium (22); possibly some of these possess a defective W1/W7 system that fails to induce local W6 polymerization.

**Fiber-to-Fiber Interactions in Chlamydomonas**

In the animal basal lamina, the fibrous protein collagen IV has been shown to interact in both a head-to-head and a head-to-tail fashion to form extensive networks (39). We demonstrate here that much of the wall is formed of fibers that also interact to form networks. Whether by direct or parallel evolution, therefore, Chlamydomonas and animals appear to have come up with strikingly similar solutions to the construction of an extracellular matrix.

The hammock of Chlamydomonas (Figs. 33 and 34), although not a cell wall component per se, appears to be constructed from a wall-type of fibrous protein, and its mode of interaction can be directly visualized on the mica surface. The knobbed end of each hammock fiber proves to carry two binding sites, one with an affinity for the tail of another fiber, the other with an affinity for a bent domain along the shaft of another fiber. This bivalency permits both end-to-end and side-to-side interactions and hence the creation of a two-dimensional array.

When W6 crystals, polymerized in vitro, dissociate upon contact with mica, their upper surfaces are found to be composed of a fibrillar system wherein the fibers align in parallel rows and cross over to associate with fibers in adjacent rows (Fig. 19), highly reminiscent of the pattern found for the hammock array. The unit glycoprotein of this system appears to be a 100-nm fiber with a globular head (Figs. 20 and 32), and we propose that the system corresponds to the W6B network visualized in situ (Figs. 3, 9, and 17D).

Stafstrom and Staehelin (40) have recently reported that extensin, the major structural protein of higher plant cell walls, is an 81-nm fibrous protein that is “kinked” in several places. The presence of analogous kinks along the length of the 100-nm fibril (Figs. 20 and 32) suggests that such discontinuities may play an important role in wall construction throughout the plant kingdom.

The W2 layer is more complex than the hammock or W6B lattices in that it is composed of two distinctly different classes of fibrous proteins, spines and ribs. Moreover, the ribs are either 100 nm or 200 nm in length. Most importantly, the layer is clearly a three-dimensional network (Fig. 2), whereas the hammock and W6B are fundamentally two-dimensional. We imagine that the fishbone unit looks rather like a bottle brush in solution, with ribs radiating out in all directions, and that it contacts other such radiating units to create a three-dimensional array in situ.

In collaboration with Dr. David Kirk, we have used freeze-etching to examine the matrix surrounding the cells of Volvox carteri, whose cell wall is directly related to C. reinhardtii (13). We find its W2 layer is indistinguishable from the Chlamydomonas equivalent. In Volvox, this W2 material increases greatly in thickness during expansion of the adult colony to accommodate the growing gonidia within the spheroid (41). The ability to create an expandable, three-dimensional W2 network would thus appear to have been critical to the evolution of the colonial mode in the Volvocales.

The final class of fibrous protein considered in this report is the sexual agglutinin of Chlamydomonas. We have previously shown this glycoprotein to be rich in hydroxyproline (31), and although the amino-acid composition of individual wall glycopolypeptides has not yet been determined, the glycoproteins as a whole are also rich in hydroxyproline (5, 14). Moreover, Adair (42) and Musgrave et al. (43) have found considerable antigenic cross-reactivity between the agglutinin and the wall glycoproteins, and the morphological similarities between the fibrous wall glycoproteins, the agglutinin, and the hammock units indicate that all probably belong to a similar biochemical family.

We are therefore led to propose the following evolutionary sequence. Fibrous hydroxyproline-rich glycoproteins were initially designed to self-assemble, after secretion, into an extracellular matrix designed to give structural support to the cell. Certain of these glycoproteins then came to be controlled by mating-type loci such that one version of an interacting dyad came to be displayed only on the surface of plus gametic flagella and the other on the surface of minus flagella, so that gametic recognition and adhesion become formally equivalent to cell wall assembly (31). At least some of the interactions between metazoan cells may follow a similar principle: cells destined to form a compound epithelium, for example, may display complementary collagen-like proteins which first recognize each other and then link the cells together by co-assembly.

**Figures 33 and 34** Hammock. Fig. 34 shows a single strand. Arrow, portion of a “regular” mastigoneme (33, 34). x 205,000.
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Note Added in Proof: Y. Matsuda et al. (J. Biol. Chem., 1985, 260:6373–6377) have independently concluded that the perchlorate-insoluble portion of the Chlamydomonas wall is the target of autotolysin activity.

REFERENCES