

Nucleated Assembly of *Chlamydomonas* and *Volvox* Cell Walls

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Abstract. The *Chlamydomonas reinhardtii* cell wall is made up of hydroxyproline-rich glycoproteins, arranged in five distinct layers. The W6 (crystalline) layer contains three major glycoproteins (GP1, GP2, GP3), selectively extractable with chaotropic agents, that self-assemble into crystals in vitro. A system to study W6 assembly in a quantitative fashion was developed that employs perchlorate-extracted *Chlamydomonas* cells as nucleating agents. Wall reconstitution by biotinylated W6 monomers was monitored by FITC-streptavidin fluorescence and quick-freeze/deep-etch electron microscopy. Optimal reconstitution was obtained at monomer concentrations (0.2–0.3 mg/ml) well below those required for nonnucleated assembly. Assembly occurred from multiple nucleation sites, and

faithfully reflected the structure of the intact W6 layer. Specificity of nucleated assembly was demonstrated using two cell-wall mutants (*cw-2* and *cw-15*); neither served as a substrate for assembly of wild-type monomers. In addition, W6 sublayers were assembled from purified components: GP2 and GP3 coassembled to form the inner (W6A) sublayer; this then served as a substrate for self-assembly of GP1 into the outer (W6B) sublayer. Finally, evolutionary relationships between *C. reinhardtii* and two additional members of the Volvocales (*Chlamydomonas eugametos* and *Volvox carteri*) were explored by performing interspecific reconstitutions. Hybrid walls were obtained between *C. reinhardtii* and *Volvox* but not with *C. eugametos*, confirming taxonomic assignments based on structural criteria.

THE structure and assembly of cell walls in the unicellular alga *Chlamydomonas reinhardtii* have been analyzed in vitro (2, 7, 8, 11–13, 19, 22, 23) and in vivo (8). Each wall consists of chaotrope-insoluble (W1, W2) and chaotrope-soluble (W4, W6) layers. The W6 layer is of particular interest in that it displays a crystalline lattice (2, 7, 8, 11–13, 19, 22, 23). Moreover, dialysis of chaotrope-soluble W6 components leads to their in vitro reassembly into native crystalline arrays (2, 7, 13). Recently, each of the three hydroxyproline-rich glycoproteins (GP1, GP2, and GP3) that make up W6 have been purified to homogeneity and characterized biochemically and morphologically (7).

When Hills (11) and Hills et al. (13) initially described this in vitro assembly system, they noted that chaotrope-soluble components produce only small crystalline fragments when dialyzed alone, whereas they form large wall-shaped units in the presence of the chaotrope-insoluble material, which we have shown to consist primarily of W2 (8). While monitoring the process by phase microscopy, they observed that the initially phase-translucent “shells” were gradually transformed into phase-refractile structures as W6 components associated with the W2 surface. Because the buildup of phase refractil-

ity occurred over the entire surface, they concluded that multiple sites of initiation existed on an inner-wall template. Finally, they reported that wild-type shells are capable of nucleating assembly of W6 material recovered from the wall-less mutant *cw-2*, suggesting that this mutant is nucleation-deficient.

The above cited studies utilized a strain of *C. reinhardtii* which, for unknown reasons, fails to break down its walls during vegetative growth, accumulating walls and wall fragments in the growth medium. In a previous study (8), we reported that it is possible to extract W6 components from intact, living cells, obviating the need to isolate cell walls. The present study is based on the realization that chaotrope-extracted cells are, in fact, the structural equivalent of W2 shells, and can be used to study the individual steps of crystal assembly in a far more quantitative fashion than is possible with isolated walls: in contrast to shells, extracted cells are easily quantitated, are of uniform size, and can be easily pelleted and washed.

This article reports results obtained using perchlorate-extracted *C. reinhardtii* cells as nucleating agents for the in vitro assembly of crystalline layer (W6) glycoproteins. We describe optimal conditions for assembly, and document by quick-freeze/deep-etch electron microscopy that a normal crystalline lattice is constructed on the W2 surface from mul-

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multiple nucleation sites. We show that W6 components do not assemble onto two wall-less mutants (*cw-2* and *cw-15*); however, W6 glycoproteins secreted into the medium by *cw-2* are capable of assembly onto wild-type cells, confirming the observations of Hills et al. (13). Using purified W6 glycoproteins, we describe the stepwise assembly of inner (W6A) and outer (W6B) sublayers of the crystal, and show that an intact W6A structure is required for the assembly of W6B. Finally, we demonstrate wall reconstitution for two additional *Volvox* species; the multicellular *Volvox carteri*, and *Chlamydomonas eugametos*, a *Chlamydomonas* species distantly related to *C. reinhardtii*. By performing interspecific reconstitutions, we show that extracted *C. reinhardtii* cells will nucleate the assembly of *V. carteri* crystals and vice versa, whereas no interspecific assembly occurs between *C. reinhardtii* and *C. eugametos*.

Materials and Methods

Isolation and Purification of *C. reinhardtii* Crystal Components

Wall crystals were prepared from the *bald-2* strain of *C. reinhardtii* according to the protocol outlined in detail in Goodenough et al. (7). Crystal glycoproteins (GP1, GP2, and GP3) were purified as described (7), and were quantitated using the procedure of Lowry et al. (16) in the presence of 1 M sodium perchlorate, or from dry weights, assuming a protein content of 60% by weight.

Extraction of Crystal Glycoproteins from *V. carteri* and *C. eugametos*

Volvox carteri spheroids were grown in synchronous liquid culture as described (15). Spheroids were extracted with sodium perchlorate and crystals prepared as described by Goodenough and Heuser (manuscript submitted for publication). For reconstitution studies, crystals were solubilized in 1 M perchlorate.

C. eugametos cells were grown on yeast extract-Tris acetate phosphate/agar medium to stationary phase. Cells were harvested in distilled water, washed once in water, and extracted for 1 h in 4 M sodium perchlorate. Extracted cells were pelleted at 39,000 g for 5 min and the clear supernatants were transferred to dialysis bags and treated essentially as described for *Volvox*, with the lyophilate resolubilized in 3 M perchlorate.

Biotinylation of Crystal Glycoproteins

Crystal glycoproteins were biotinylated by the method of Della-Penna et al. (5). Crystals (5–10 mg) were suspended in 10 ml of 0.1 M sodium carbonate, pH 9.0, and mixed with 0.5–1.0 ml of biotinyl-*N*-hydroxysuccinimide ester (Calbiochem-Behring Corp., San Diego, CA), dissolved in nonaqueous dimethylformamide at 1 mg/ml. The reaction was allowed to proceed overnight at 4°C with end-over-end mixing. The solution was then dialyzed extensively against distilled water and lyophilized to dryness.

Nucleated Assembly

C. reinhardtii (CC-621, *mf*⁻) was grown in constant light for 36–48 h in Tris-acetate-phosphate (9). Vegetative cells extracted with 1 M sodium perchlorate as described above. As documented in a previous report (8), all traces of cell-wall layers W4 and W6 are removed by this treatment. Extracted cells were used immediately or fixed with 2% paraformaldehyde, followed by a 2-h incubation in 25 mM glycine, pH 7.0. Fixed cells retained their nucleation competence for at least 1 wk when stored at 4°C. Cell-wall mutants *cw-2* and *cw-15* were fixed with glutaraldehyde (1% in 15 mM Hepes, pH 7.0) for 1 h, followed by a 2-h incubation in 25 mM glycine, pH 7.0. *Volvox* spheroids, extracted in 2 M perchlorate, and *C. eugametos* cells, extracted in 4 M perchlorate, were reconstituted without fixation.

To initiate nucleated assembly of *C. reinhardtii*, biotinylated soluble glycoproteins (crystal supernatants) were added to extracted cells at a concentration (0.3–0.5 mg/ml) well below that required for in vitro crystal formation (7). Alternatively, extracted cells and biotinylated crystal extracts were

mixed in the presence of 1 M sodium perchlorate, and the mixture was dialyzed against distilled water. *Volvox* crystals, biotinylated as described for *C. reinhardtii*, and unbiotinylated *C. eugametos* crystals were reconstituted by the second procedure. Initial assessment of *C. reinhardtii* and *Volvox* reconstitutions was made by staining an aliquot of each sample with FITC-streptavidin and inspection by fluorescence microscopy.

Iodination of *C. reinhardtii* Crystal Components

150 μ l of *C. reinhardtii* crystal protein, 5 mg/ml in labeling buffer (1 M sodium perchlorate, 0.17 M sodium carbonate, pH 8.5, 0.14 M NaCl) was mixed with 1 mCi carrier-free Na¹²⁵I (Amersham Corp., Arlington Heights, IL) and added to a glass test tube (13 \times 100 mm) coated with 10 μ g of Iodogen (Pierce Chemical Co., Rockford, IL). After a 5-min incubation on ice, the reaction was quenched with 20 μ l of L-tyrosine (0.4 M in water) for 5 min, and labeled proteins were separated from unincorporated iodine by spun-column chromatography through 1.5 ml of Biogel-P6DG (BioRad Laboratories, Richmond, CA), equilibrated with 1 M sodium perchlorate. Labeled proteins were immediately diluted to a final concentration of \sim 0.35 mg/ml with distilled water and dialyzed against 2 liters of the same at 4°C overnight. Before cell incubations, the solution was clarified by centrifugation at 100,000 g for 1 h.

SDS-PAGE/Autoradiography

5×10^7 fixed *C. reinhardtii* cells (perchlorate-extracted and unextracted) were incubated with 5 ml of ¹²⁵I-labeled crystal supernatant (0.3 mg/ml, sp act = 8.8×10^5 dpm/ μ g) for 2 h at room temperature with end-over-end rotation. After six washes with PBS (20 mM sodium phosphate, pH 7.5, 0.15 M NaCl), cell pellets were extracted with 200 μ l of 2 \times SDS-PAGE lysis buffer. 5 μ l of each sample was electrophoresed on a 5–15% gel (7) and subjected to autoradiography at -70°C with intensifying screens (Cronex Lightning Plus, Dupont Co., Wilmington, DE).

Fluorescence Microscopy

Cells reconstituted with biotinylated crystal glycoproteins were washed several times with PBS, and incubated for 15 min in 50 mM Na₂CO₃, 0.15 M NaCl, containing FITC-streptavidin (Bethesda Research Laboratories, Gaithersburg, MD) at 20 μ g/ml. They were then washed several times with PBS, mounted in PBS containing 25% glycerol, and examined by fluorescence microscopy using a Zeiss photomicroscope 1 equipped with a III RS epifluorescence condenser. Where noted, a K550 barrier filter was employed to reduce chlorophyll autofluorescence.

Electron Microscopy

Electron microscopy of extracted and reconstituted cells was performed using the quick-freeze/deep-etch procedure of Heuser (10).

Results

Concentration Dependence of *C. reinhardtii* Nucleated Assembly

To determine optimal conditions for nucleated assembly, a constant number of fixed, extracted cells (10^6) was mixed with increasing concentrations of biotinylated crystal extract (0.003–1.4 mg/ml in 0.5 ml of 2 M perchlorate). To initiate assembly, samples were placed in individual chambers of a multiple dialysis manifold (Bethesda Research Laboratories) and dialyzed against distilled water for 2 h. Cells were then stained with FITC-streptavidin and examined by fluorescence microscopy. At concentrations below 0.02 mg/ml little staining above background was observed (not shown). At higher concentrations, cells were distinctly fluorescent, indicating the presence of biotinylated crystal protein (Fig. 1 b). Staining usually appeared as a distinct halo surrounding the cell body at a position corresponding to the cell wall. Under these conditions, the sharpest fluorescent images were routinely obtained at a protein concentration of 0.2–0.35 mg/ml.

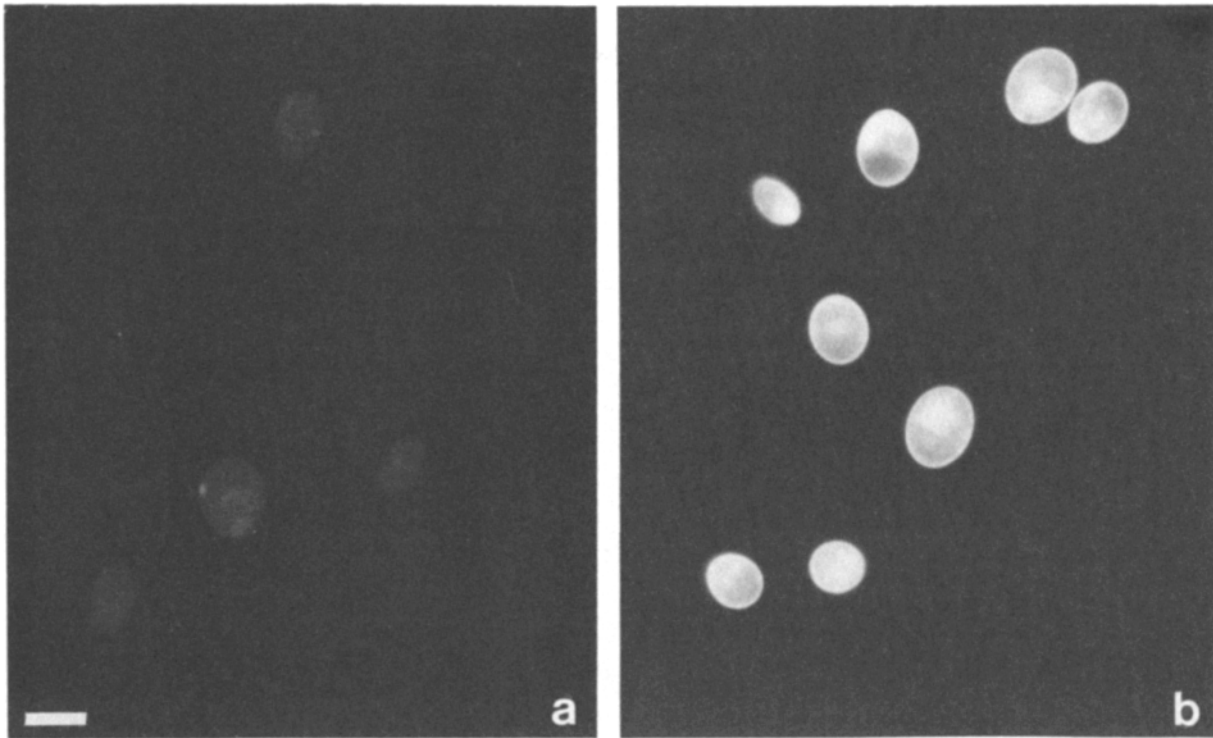


Figure 1. FITC-streptavidin staining of reconstituted *C. reinhardtii* cells. Perchlorate-extracted cells were mixed with biotinylated crystal glycoproteins (0.35 mg/ml) in perchlorate, dialyzed against water for 2 h, and stained with FITC-streptavidin. (a) Nonreconstituted control; (b) reconstituted cells. Bar, 10 μ m.

Polypeptide Composition of Reconstituted *C. reinhardtii* Walls

The identity and stoichiometry of bound crystal components were determined by reconstituting fixed, extracted cells with 125 I-labeled crystal supernatants. After extensive washing, bound material was released with SDS lysis buffer and analyzed by SDS-PAGE/autoradiography. Fig. 2, lane 1 shows the polypeptide profile of 125 I-labeled crystals, where the major glycoproteins (GP1, GP2, GP3) are indicated. Fig. 2, lane 2 documents that these glycoproteins are recovered in the same relative amounts from perchlorate-extracted cells reconstituted with 125 I-labeled crystal supernatants. In contrast, very little labeled material binds to unextracted cells (Fig. 2, lane 3). Quantitation of bound counts by gamma counting indicates that unextracted cells bind <5% as much crystal glycoprotein as extracted cells (not shown).

Structure of Reconstituted Cell Walls

Quick-freeze/deep-etch electron microscopy demonstrates that W6 monomers assemble into an organized crystalline matrix onto the W2 surface. Fig. 3 a shows an unextracted *Chlamydomonas* cell wall that was quick-frozen and cross-fractured. When viewed from this perspective the four distinctive layers of the wall (W1, W2, W4, W6) are apparent. The upper (W6B) sublayer of W6 (Fig. 3 a, arrow), which forms an open polygonal lattice, is made up of a single glycoprotein, GP1 (Goodenough and Heuser, manuscript submitted for publication). Perchlorate extraction (Fig. 3 b) removes the W6 and W4 layers quantitatively, together with a

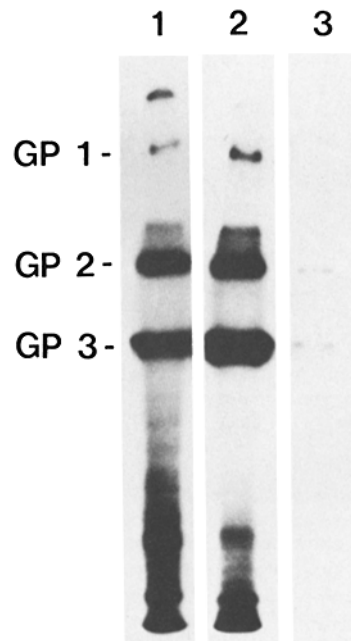


Figure 2. Gel analysis of reextracted polypeptides. Extracted *C. reinhardtii* cells were reconstituted with 125 I-crystal glycoproteins and washed three times, and bound material was solubilized with SDS lysis buffer. Released material was fractionated by SDS-PAGE (5–15% gel), and subjected to autoradiography. Lane 1, 125 I crystals; lane 2, reextracted crystal. Note that GP1 under-labels with the labeling procedure employed (Iodogen).

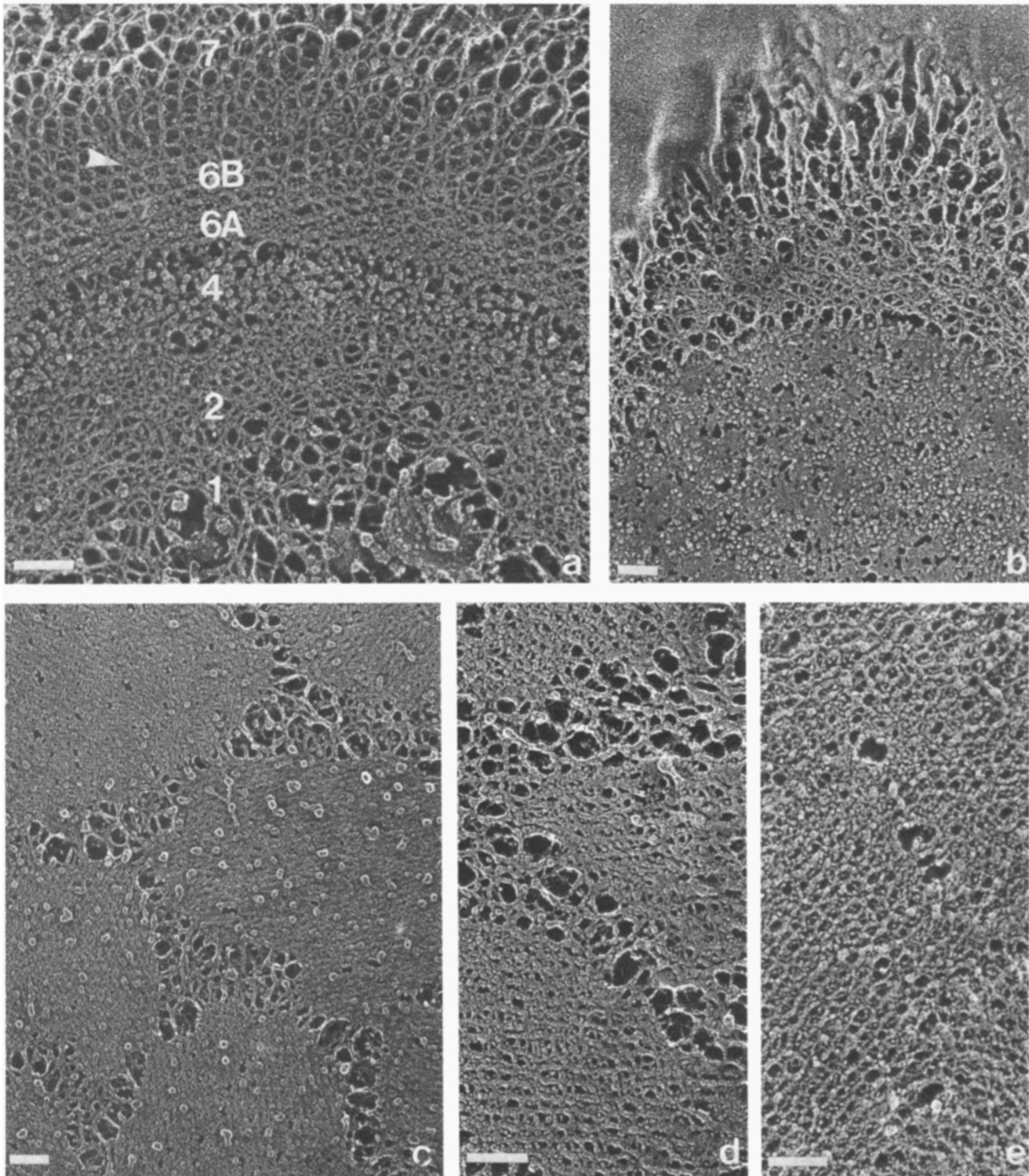


Figure 3. (a) *C. reinhardtii* cell showing the five distinctive layers of the cell wall (W1–W7) and the cell surface. Note the outer (W6B) sublayer of W6, with its diagnostic polygonal weave (arrow). (b) Treatment of the intact *C. reinhardtii* cell with 1 M sodium perchlorate quantitatively removes the W4 and W6 layers of the cell wall, together with a variable amount of W7. Inner (W1, W2) wall layers remain intact. (c) Reconstituted *C. reinhardtii*. Although the structure of reconstituted W6 crystals are identical to that of unextracted cells, they nucleate from multiple sites, leading to a patchwork array. (d) Higher magnification of reconstituted patches. (e) As in d, but weave is better resolved. Compare with a. Bars, 100 nm.

variable amount of large fibers that constitute the W1–W7 “warp” of the wall (8). The remaining perchlorate-insoluble residue contains an intact W2 layer, overlying W1 fibers. Although residual sprigs of W7 fibers often remain, most are

removed; therefore, the notion that W1 and W7 are continuous fibers (8) may be erroneous. Incubation of extracted cells with crystal glycoproteins leads to their deposition as a distinct outer layer (Fig. 3, c–e). In deep-etched whole cells,

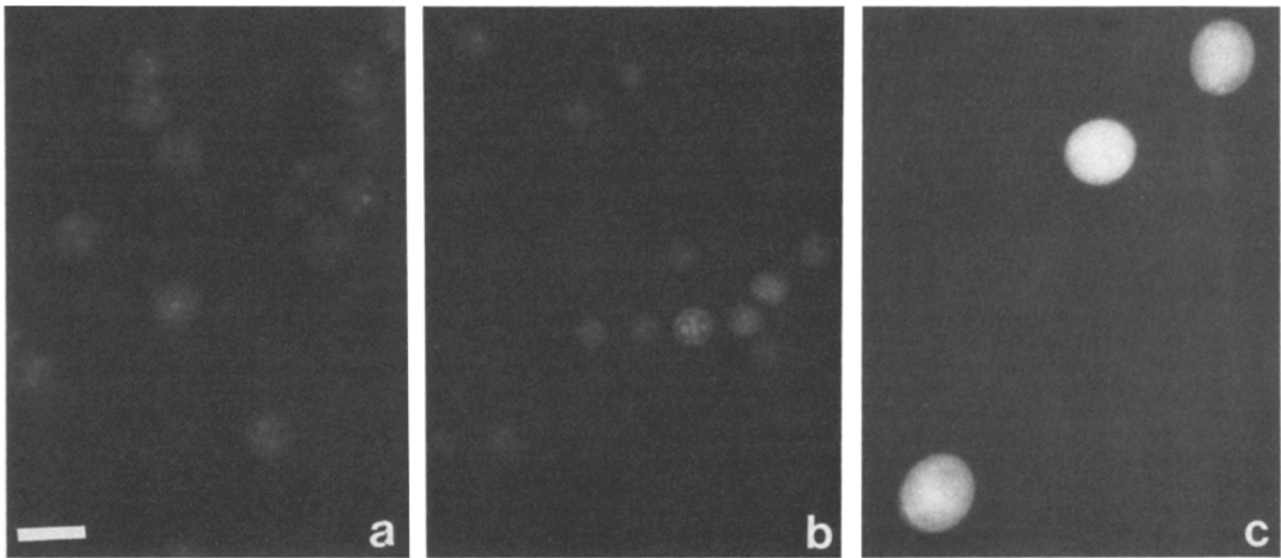


Figure 4. Specificity of nucleated assembly. Cell-wall mutants (a) *cw-2* and (b) *cw-15* were incubated with biotinylated *wt* crystal supernatants, washed, and stained with FITC-streptavidin. Absence of detectable fluorescence indicates that neither mutant is able to nucleate assembly of W6 glycoproteins. (c) By contrast, W6 components secreted into the medium by *cw-2*, assemble onto extracted *wt* cells. Bar, 10 μm .

the only level of organization that can be unambiguously identified is the open weave of W6B (Fig. 3 *d*); in that we have shown that W6B can only assemble on a W6A template, however (see below), the W6B weave must lie above a W6A lattice. The weave is seen to assemble as contiguous patches, $\sim 4 \times 7 \mu\text{m}$, which display an apparently random orientation with respect to one another, indicating that nucleated assembly initiates from multiple sites.

Specificity of Nucleated Assembly

To examine whether nucleated assembly requires the participation of the W2 layer, the reconstitution assay was performed with two mutants, *cw-2* and *cw-15*, which lack this layer (see Monk et al. [17] for *cw-15*, and Fig. 5 for *cw-2*). After incubation with biotinylated extracts, neither mutant stained with FITC-streptavidin (Fig. 4, *a* and *b*). The absence of W2, moreover, apparently explains why *cw-2* cannot assemble a W6 layer, even though it synthesizes all of the constituent glycoproteins: Fig. 4 *c*, shows that extracted wild-type cells are able to nucleate assembly of secreted *cw-2* glycoproteins in an apparently normal fashion. From these observations, and the fact that crystals assemble onto wild-type cells in direct contact with W2, we suggest that nucleated assembly requires specific interaction(s) between crystal components and W2.

Assembly of W6A and W6B Sublayers

The W6 layer is bilaminar, consisting of inner (W6A) and outer (W6B) sublayers, each composed of distinct glycoproteins: whereas W6A is formed by the coassembly of two molecules, GP2 and GP3, W6B is made up entirely of a single species, GP1 (Goodenough and Heuser, manuscript submitted for publication). To examine the individual steps of W6 assembly, each glycoprotein was purified to homogeneity and employed in the stepwise construction of individual W6A and W6B sublayers in vitro. Fig. 6 *b* shows that purified GP2

and GP3 coassemble onto extracted cells in the absence of GP1, forming a matrix reminiscent of the inner (W6A) sublayer (8). By contrast, purified GP1 does not assemble onto W2, nor is any self-assembled GP1 recovered in the medium (not shown). However, when GP1 is added to cells previously

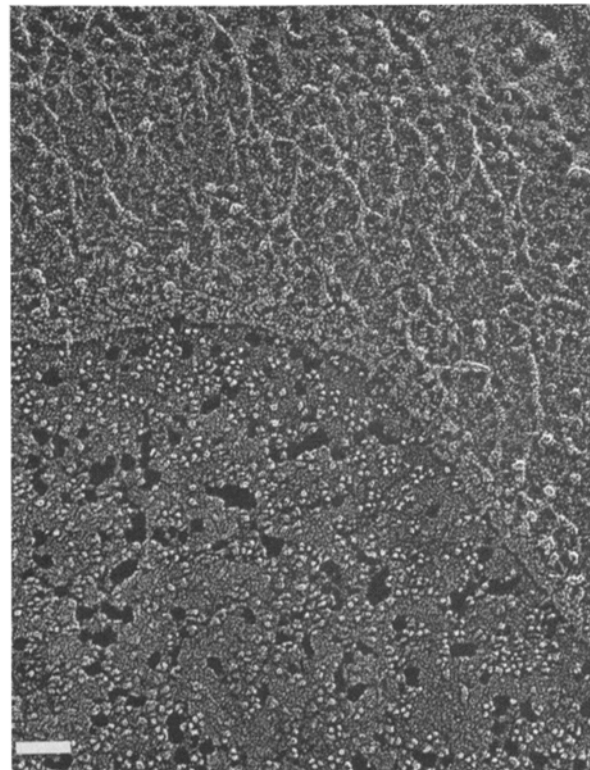


Figure 5. Cell surface of *cw-2* cells carry only occasional, disorganized fibers, overlying an otherwise bare cell membrane. Bar, 100 nm.

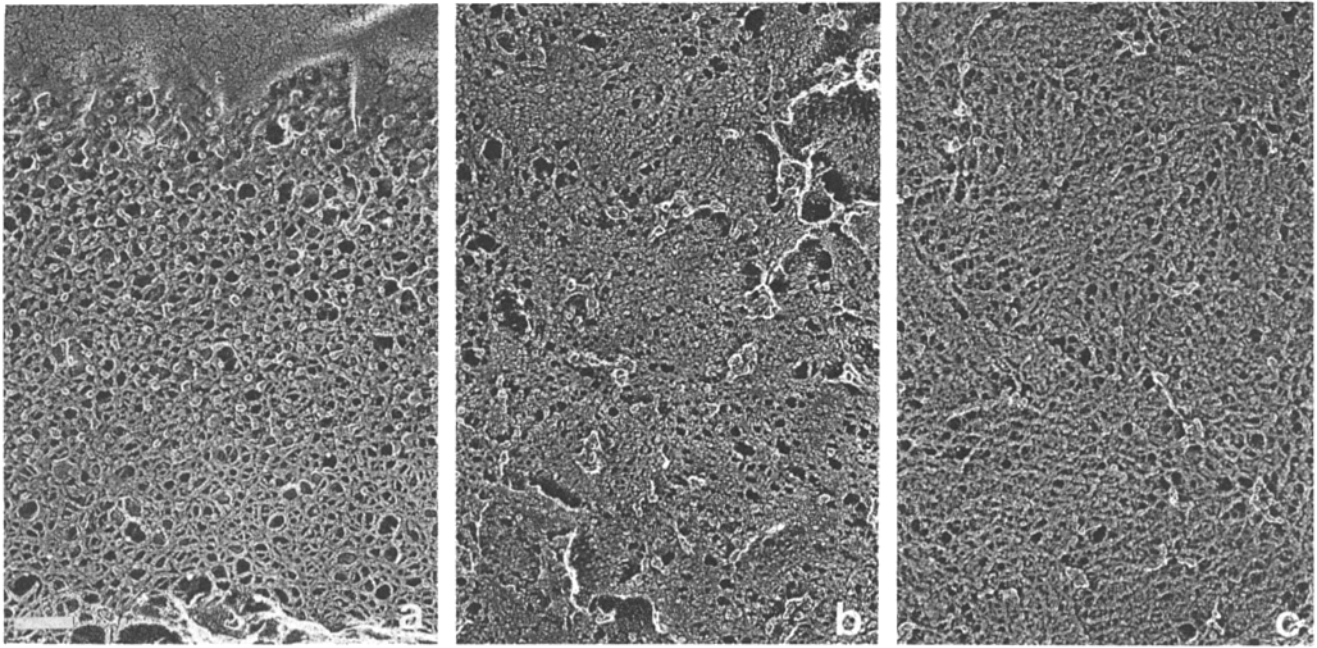


Figure 6. Stepwise assembly of W6 sublayers. (a) Perchlorate-extracted *C. reinhardtii* cells retain intact W1 and W2 layers, but lack W4 and W6. (b) Purified GP2 and GP3 (0.2 mg/ml each) coassemble an inner (W6A) sublayer onto the W2 surface. Note the absence of an open (W6B) weave. (c) Incubation of b with purified GP1, leads its self-assembly into an outer (W6B) crystalline sublayer. Bar, 100 nm.

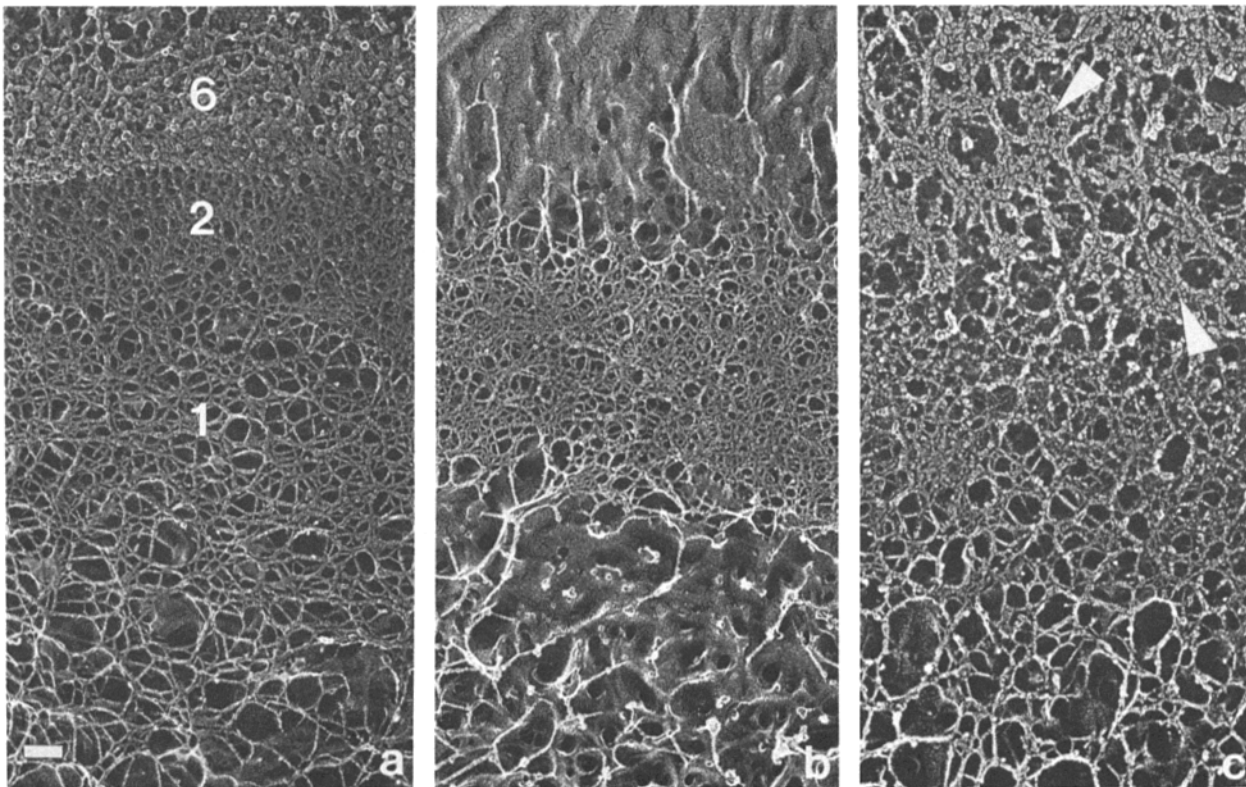


Figure 7. Extraction and reconstitution of *Volvox carteri*. (a) Unextracted spheroid. (b) Perchlorate-extracted spheroid. Note the similarity of the W2 layer to the corresponding *C. reinhardtii* layer (Fig. 6 a). (c) *Volvox* reconstituted with *Volvox* crystal glycoproteins. Note the “patchy” appearance of the reconstituted wall (arrows). Bar, 100 nm.

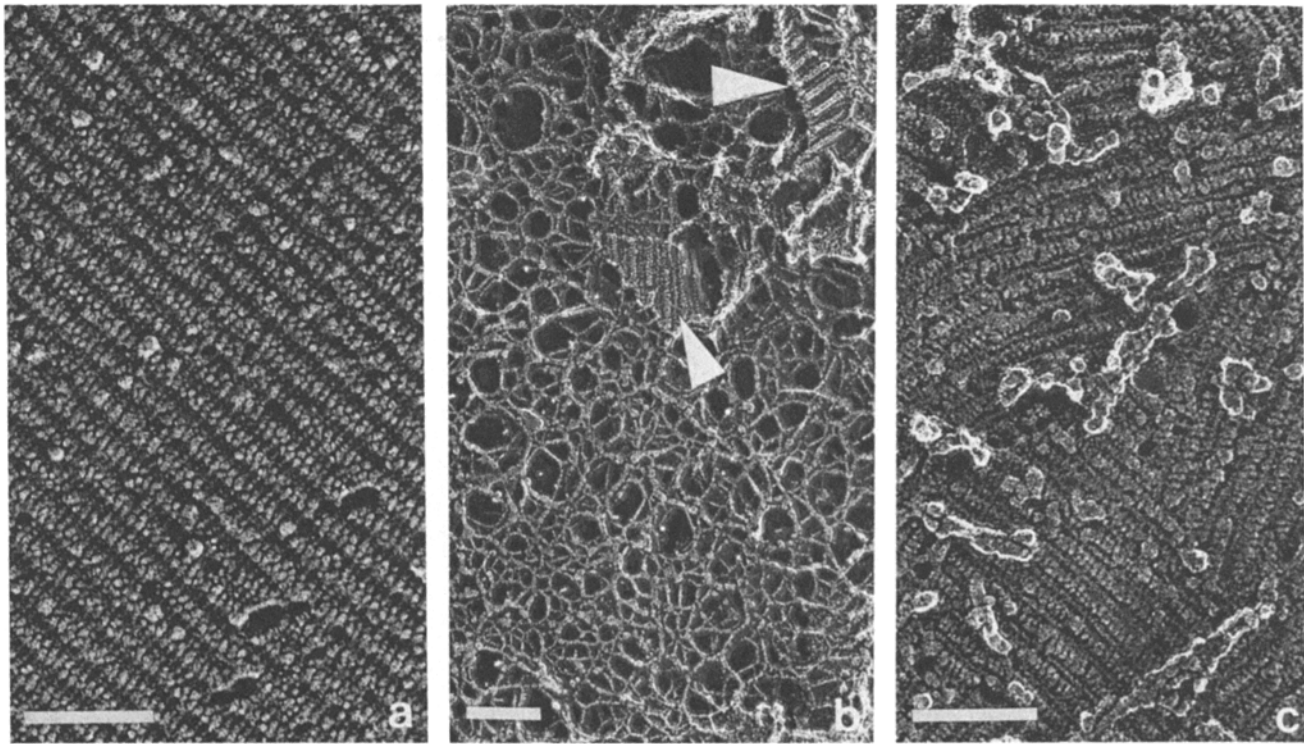


Figure 8. Extraction and reconstitution of *C. eugametos*. (a) The intact *C. eugametos* cell wall. (b) Extracted *C. eugametos*. Note the residual wall fragments (arrows). (c) Reconstituted *C. eugametos*. Note the orientations of individual patches. Bars, 100 nm.

reconstituted with GP2/GP3, a characteristic W6B weave is constructed (Fig. 6 c). From these results we conclude that: (a) GP2 and/or GP3 (but not GP1) has a specific binding site(s) for the underlying matrix; and (b) an intact W6A layer is required for the self-assembly of GP1 into W6B.

Interspecific Reconstitutions

All members of the algal order Volvocales have cell walls containing a crystalline layer overlying an amorphous inner layer (21). From studies of a large number of Volvocales species, Roberts and co-workers concluded that the cell walls of these algae fall into four major structural classes (19, 23). *C. reinhardtii* has a wall structure shared by the multicellular, colonial Volvocales, including *Volvox* (21, 23). Recently *Volvox* was found to have a W2 layer indistinguishable from that of *C. reinhardtii* (8), and a crystalline layer glycoprotein biochemically (Adair and Appel, unpublished observation) and morphologically (Goodenough and Heuser, unpublished observation) similar to *C. reinhardtii* GP2. In contrast, a very different cell wall is made by *C. eugametos*, a distantly related *Chlamydomonas* species. The *C. eugametos* wall, which has the more prototypical Volvocales structure, is

made up of a single major glycoprotein (18) which interestingly, also appears to be a GP2 homologue.

To further explore possible evolutionary relationships between the wall glycoproteins of these species, we have attempted interspecific reconstitutions. These experiments required the development of methods for crystalline-layer extraction and reconstitution of *Volvox* and *C. eugametos*. These methods, which employ harsher extraction conditions than *C. reinhardtii*, are described in detail in Materials and Methods. Figs. 7 and 8 document that both species can be extracted and reconstituted with their own crystal glycoproteins. Note that *Volvox* (Fig. 7) and *C. reinhardtii* (Fig. 3 a) have cell walls structurally similar and very different from *C. eugametos* (Fig. 8). Note also that *Volvox* lacks a sublayer that would correspond to *C. reinhardtii* W6B.

Results of interspecific reconstitution experiments, summarized in Table I and documented below, show that *C. reinhardtii* and *Volvox* are both able to nucleate the assembly of the other's crystalline glycoproteins, producing hybrid cell walls. By contrast, interspecific reconstitution between *C. reinhardtii* and *C. eugametos* is not observed.

Fig. 9 b is a fluorescence micrograph of *C. reinhardtii* cells reconstituted with biotinylated *Volvox* crystal monomers and

Table I. Summary of Cell Wall Reconstitution Experiments

Template	Crystal extract		
	<i>C. reinhardtii</i>	<i>C. eugametos</i>	<i>V. carteri</i>
<i>C. reinhardtii</i>	Reconstitution	No reconstitution	Reconstitution
<i>C. eugametos</i>	No reconstitution	Reconstitution	Not tested
<i>V. carteri</i>	Reconstitution	Not tested	Reconstitution

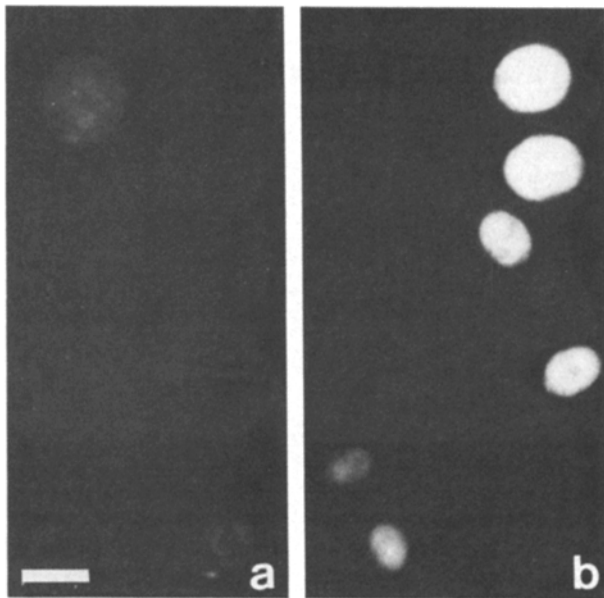


Figure 9. Assembly of *Volvox* crystal components onto *C. reinhardtii*. *C. reinhardtii* cells were perchlorate-extracted and reconstituted with biotinylated *Volvox* crystal glycoproteins. Staining with FITC-streptavidin demonstrates significant binding of *Volvox* components to the *Chlamydomonas* inner wall. (a) Unextracted control. (b) Reconstituted. Bar, 10 μ m.

stained with FITC-streptavidin. The image is indistinguishable from that obtained with *C. reinhardtii* crystal glycoproteins (Fig. 1). That an organized *Volvox* matrix assembles onto the *Chlamydomonas* template is confirmed by electron microscopy (Fig. 10). Figs. 11 and 12 demonstrate that interspecific reconstitution between *C. reinhardtii* and *Volvox* is reciprocal. *Volvox* spheroids incubated with biotinylated *Chlamydomonas* crystal monomers stain brightly with FITC-streptavidin (Fig. 11). Note that staining is localized to the outer spheroid boundary, at a position corresponding to the crystalline layer of this multicellular alga (14). Electron microscopy of *Volvox* spheroids reconstituted with *Chlamydo-*

monas monomers (Fig. 12) documents that *Chlamydomonas* crystal glycoproteins assemble a typical W6 matrix onto the *Volvox* W2 layer. As with conspecific reconstitution (see Fig. 3 d), assembly occurs from multiple nucleation sites.

From these results we conclude that: (a) specificity of nucleated assembly is related to cell-wall type, and is not species-restricted; and (b) *C. reinhardtii* is, in this regard, closely related to the multicellular *Volvox carteri*, but lacks a close functional relationship with the unicellular *C. eugametos*.

Discussion

Nucleated Assembly of W6 Glycoproteins

The *Chlamydomonas reinhardtii* cell wall has five distinct layers (W1, W2, W4, W6, and W7), three of which (W2, W4, and W6) make up the tripartite layer common to all members of the order Volvocales (2). In their original studies of the *C. reinhardtii* wall, Roberts and co-workers demonstrated that treatment of isolated walls with chaotropic agents (e.g., sodium perchlorate) produces salt-soluble and salt-insoluble fractions, which correspond respectively to the inner "amorphous" and outer (crystalline) cell-wall layers observed by electron microscopy (11, 12, 20). Dialysis of crystal glycoproteins alone led to their self-assembly into crystals, while a cell-wall shape was re-formed in the presence of the salt-insoluble material (11, 13). In each case a W6 layer assembled, with lattice coordinates faithfully reflecting those of the in situ wall (11–13, 19). From these early studies two important concepts emerged: (a) all the information required for the assembly of the crystalline layer resides in its molecular constituents; and (b) the insoluble inner layer has specific binding site(s) for crystalline glycoproteins, and is capable of nucleating their assembly.

To study the individual molecular associations involved in W6 assembly in a more quantitative fashion, we have developed a nucleated assembly system employing intact extracted cells as a template. Unlike isolated walls or wall fragments, cells are of uniform size and are easily manipulated experimentally. We have shown that the crystalline cell wall layer of *Chlamydomonas* can be reconstituted in vitro onto

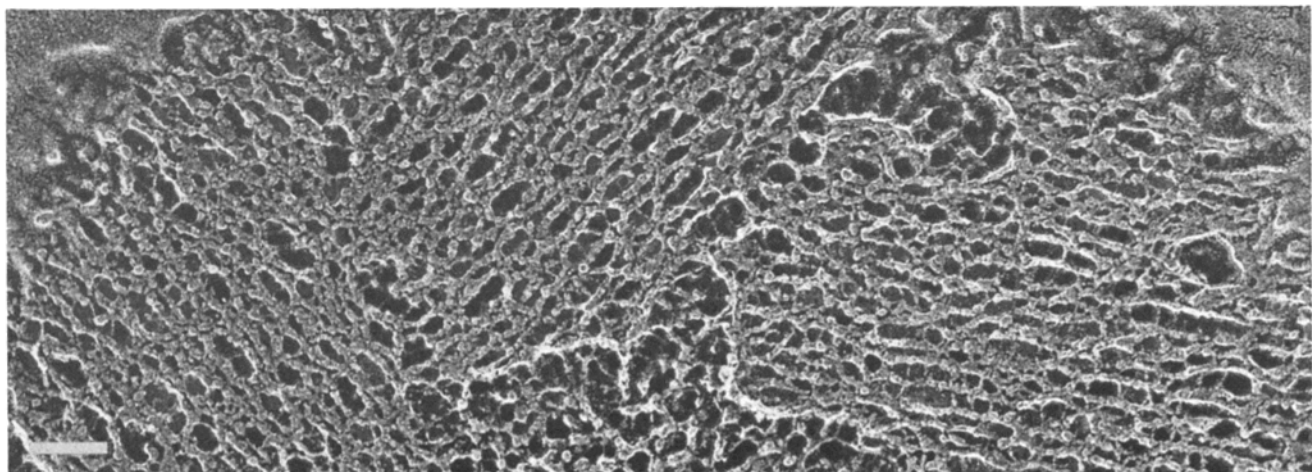


Figure 10. *C. reinhardtii* reconstituted with *Volvox* crystal components. Note that the *Volvox* crystalline layer lacks a W6B equivalent (see also Fig. 7 a). Bar, 100 nm.

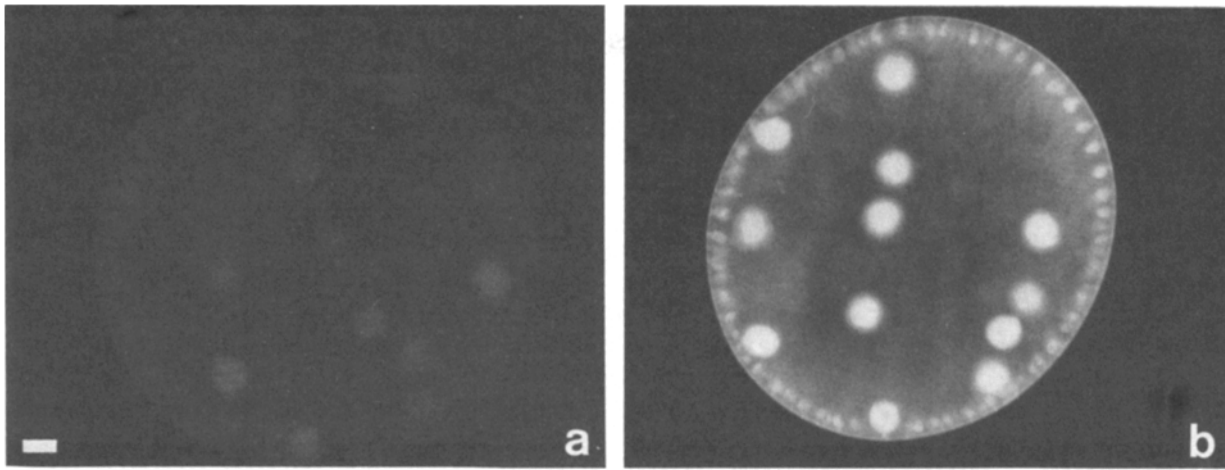


Figure 11. Assembly of *C. reinhardtii* crystal components onto *Volvox*. *Volvox* spheroids were extracted with 2 M perchlorate and reconstituted with biotinylated *C. reinhardtii* crystal components. Presence of a reconstituted crystalline layer is demonstrated by FITC-streptavidin fluorescence. (a) Unextracted control. (b) Reconstituted. Bar, 10 μ m.

perchlorate-extracted cells in a reproducible fashion, at monomer concentrations far below that required for crystal formation in solution.

Electron microscopy using the quick-freeze/deep-etch procedure demonstrates that wall reconstitution occurs from multiple nucleation sites. Although each assembly site displays a characteristic W6 lattice, no obvious relationship exists between neighboring sites; i.e., reconstituted walls have a patchwork-quilt appearance. In that walls assembled in vivo have a uniform structure, a polarizing influence must be operative during in vivo assembly which is absent from the in vitro protocol. Organization in vivo might be imposed by polarized secretion; however, the participation of additional unidentified molecule(s) cannot be ruled out.

Whereas the orientation of reconstituted wall fragments is patchy, failure of cell-wall mutants to nucleate assembly of wild-type monomers indicates that the underlying matrix is indeed important. In vitro assembly of *cw-2* crystal glycoproteins onto a wild-type template, moreover, supports the

notion (4, 11) that this mutant has a lesion in the inner layer(s) that participate in this interaction. Because *cw-15* (which also does not reconstitute) appears to form a W1 layer (17), we suggest that W6 components associate with W2, a conclusion consistent with the relative location of the two layers in the in situ wall.

In a previous study (8) we noted that the W6 layer is bilaminar, having inner (W6A) and outer (W6B) sublayers; a more recent study (Goodenough and Heuser, manuscript submitted for publication) documents that W6A is formed by the coassembly of two glycoproteins (GP2 and GP3), whereas W6B is made up of a single glycoprotein (GP1). In this report we have demonstrated that specific association of W6 with W2 is mediated by component(s) of the inner sublayer (W6A), which is, in turn, capable of nucleating the self-assembly of GP1 into W6B. The molecular nature of W6A components responsible for binding to the W2 layer and mediating GP1 assembly remains to be defined.

The association of W6 glycoproteins with W2, while spe-

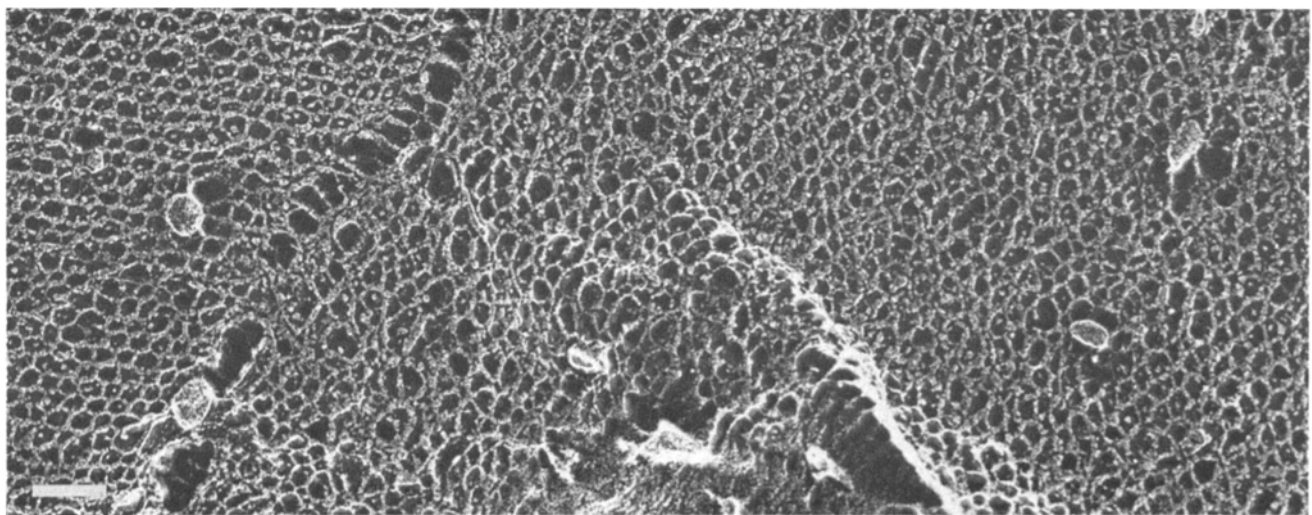


Figure 12. *Volvox* reconstituted with *C. reinhardtii* crystal components. Note the presence of the diagnostic *C. reinhardtii* W6B weave, present in patches, on the *Volvox* W2 layer. Bar, 100 nm.

cific, is not species-restricted, as demonstrated in the present study by in vitro construction of hybrid (*C. reinhardtii/Volvox*) cell walls. That interspecific reconstitution between *C. reinhardtii* and *C. eugametos* does not occur further documents the specificity of nucleated assembly and indicates that cell-wall type is the important factor. Roberts and co-workers have concluded that four major cell-wall types exist in the order Volvocales (19, 23), and have suggested that cell-wall structure be used as a phylogenetic marker (19). *C. eugametos* has a simple wall, characteristic of the majority of these algae (23); the *C. reinhardtii* wall has a crystal structure shared by *Volvox* and the other multicellular, colonial Volvocales (23). Our interspecific reconstitution experiments support the idea (23) that *C. reinhardtii* is only distantly related to *C. eugametos*, and is on the evolutionary line that includes the multicellular Volvocales.

As described previously (1, 3, 6) the agglutination of complementary *plus* and *minus* gametes during mating in *Chlamydomonas* is mediated by hydroxyproline-rich glycoproteins, agglutinins, that closely resemble the major cell wall hydroxyproline-rich glycoproteins. This relationship is particularly apparent for GP1 which, like the agglutinins, is an extended fibrous molecule (8, 20), with a prominent globular domain at one end that has recognition properties (7, 8). Immunotopographic mapping studies (Adair, Goodenough, and Heuser, unpublished data), employing an anti-agglutinin monoclonal antibody (A15), show that the globular domain of GP1 bears a shared carbohydrate epitope that maps to the two ends of the *plus* agglutinin molecule (1), sites which also have recognition/binding functions (6). These relationships have led us to speculate that *Chlamydomonas* sexual agglutinins and cell-wall proteins are members of a family of molecules sharing a common evolutionary heritage (1, 3, 6). The observation that *Chlamydomonas* and *Volvox* cell walls have morphologically indistinguishable W2 layers (8) and the demonstration in this study that hybrid *Chlamydomonas/Volvox* cell walls can be assembled in vitro, suggests that this multigene family extends to other Volvocales species as well. This hypothesis has recently gained experimental support with the finding that a molecule, biochemically and immunologically related to *C. reinhardtii* GP2, is present in the cell walls of *Chlamydomonas eugametos*, *Volvox carteri*, and *Gonium pectorali* (Adair and Appel, unpublished observations). In addition, molecules with morphologies similar to *C. reinhardtii* GP2 have been identified in the walls of *C. eugametos* and *Volvox* (Goodenough and Heuser, unpublished observations). If the *C. eugametos* wall structure represents the closest descendent of an ancestral wall type (21), it is reasonable to consider the possibility that many of the Volvocales hydroxyproline-rich glycoproteins, perhaps including the sexual agglutinins, have evolved from a primordial GP2 gene. Molecular cloning studies in progress are aimed at addressing this issue.

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