Protoplasma (1994) 181: 245–258

PROTOPLASMA

© Springer-Verlag 1994
Printed in Austria

Volvocine cell walls and their constituent glycoproteins: an evolutionary perspective

J. P. Woessner* and **Ursula W. Goodenough**

Department of Biology, Washington University, St. Louis, Missouri

Received August 5, 1993
Accepted November 8, 1993

Summary. Similarities in the composition of the extracellular matrix suggest that only some species of the unicellular *Chlamydomonas* are closely related to the colonial and multicellular flagellated members of the family Volvocaceae. The cell walls from all of the algae in this volvocine group contain a crystalline layer. This lattice structure can be used as a phylogenetic marker to divide *Chlamydomonas* species into distinct classes, only one of which includes the volvocacean algae. Similarly, not all species of *Chlamydomonas* are sensitive to each other's cell wall lytic enzymes, implying divergence of the enzyme's inner wall substrate. Interspecific reconstitution of the crystalline layer is possible between *C. reinhardtii* and the multicellular *Volvox carteri*, but not between *C. reinhardtii* and *C. eugametos*. The hydroxyproline-rich glycoproteins (HRGPs) which make up the crystalline layer in genera which have a similar crystal structure exhibit many homologies. Interestingly, the evolutionarily distant cell walls of *C. reinhardtii* and *C. eugametos* also contain some HRGPs displaying a few morphological and amino acid sequence homologies. The morphological similarities between the flagellar agglutinins (HRGPs responsible for sexual recognition and adhesion during the mating reaction) and the cell wall HRGPs leads to the proposal of a superfamily from which novel HRGPs (designed for self-assembly/recognition) can constantly evolve. Just as variations in the wall HRGPs can lead to unique wall structures, new agglutinins facilitate sexual isolation of new species. Thus, the HRGPs could emerge as valuable phylogenetic markers.

Keywords: Evolution; Hydroxyproline-rich glycoprotein; Cell wall; Phylogenetics; *Chlamydomonas*; Volvocales.

Abbreviations: GLE gametic lytic enzyme; GP glycoprotein; HRGP hydroxyproline-rich glycoprotein; SDS PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis; VLE vegetative lytic enzyme; VSP vegetative serine/proline-rich; WP wall protein; ZSP zygotic serine/proline-rich.

Introduction

Within the order Volvocales, the volvocine line connects the unicellular green algae in the genus *Chlamydomonas* with the colonial and multicellular green algae comprising the family Volvocaceae. It has been proposed that the conceptual ordering of the volvocine genera (e.g., from *Chlamydomonas*, through *Gonium, Pandorina, Eudorina,* and *Pleodorina*, to *Volvox*), based on traits such as cell number, organismic size, type of sexual reproduction and ratio of somatic to reproductive cells, represents the directional evolutionary history in the group (Schmitt et al. 1992). Indeed, common patterns of subcellular organization, cell wall structure, and asexual and sexual reproduction are found in *Chlamydomonas reinhardtii* and all members of the family Volvocaceae. The colonial members (*Gonium, Pandorina, Pleodorina,* and *Eudorina*) each contain a species-specific number of identical *Chlamydomonas*-like cells, while the multicellular *Volvox* are comprised of non-motile germ cells and flagellated *Chlamydomonas*-like somatic cells. In all these volvocaceans, the cells are held together in a recognizable pattern by a non-cellulosic extracellular matrix with an abundance of hydroxyproline-rich glycoproteins (HRGPs). The similarities and differences between *C. reinhardtii* and volvocacean algae have been recently reviewed in Schmitt et al. (1992).

With an eye towards phylogenetic and evolutionary interpretations, this review compares the various volvocine cell walls, considering first the intact matrices, then in vitro wall assembly, then the isolated wall proteins, and finally the genes encoding the structural components. While marked differences ar observed among the assembled walls of various *Chlamydomonas* spp., some highly conserved amino acid domains in the wall proteins yield HRGP building blocks with common morphological structures (e.g., fibrous rods with glob-

* Correspondence and reprints: Department of Biology, Box 1229, Washington University, St. Louis, MO 63130, U.S.A.

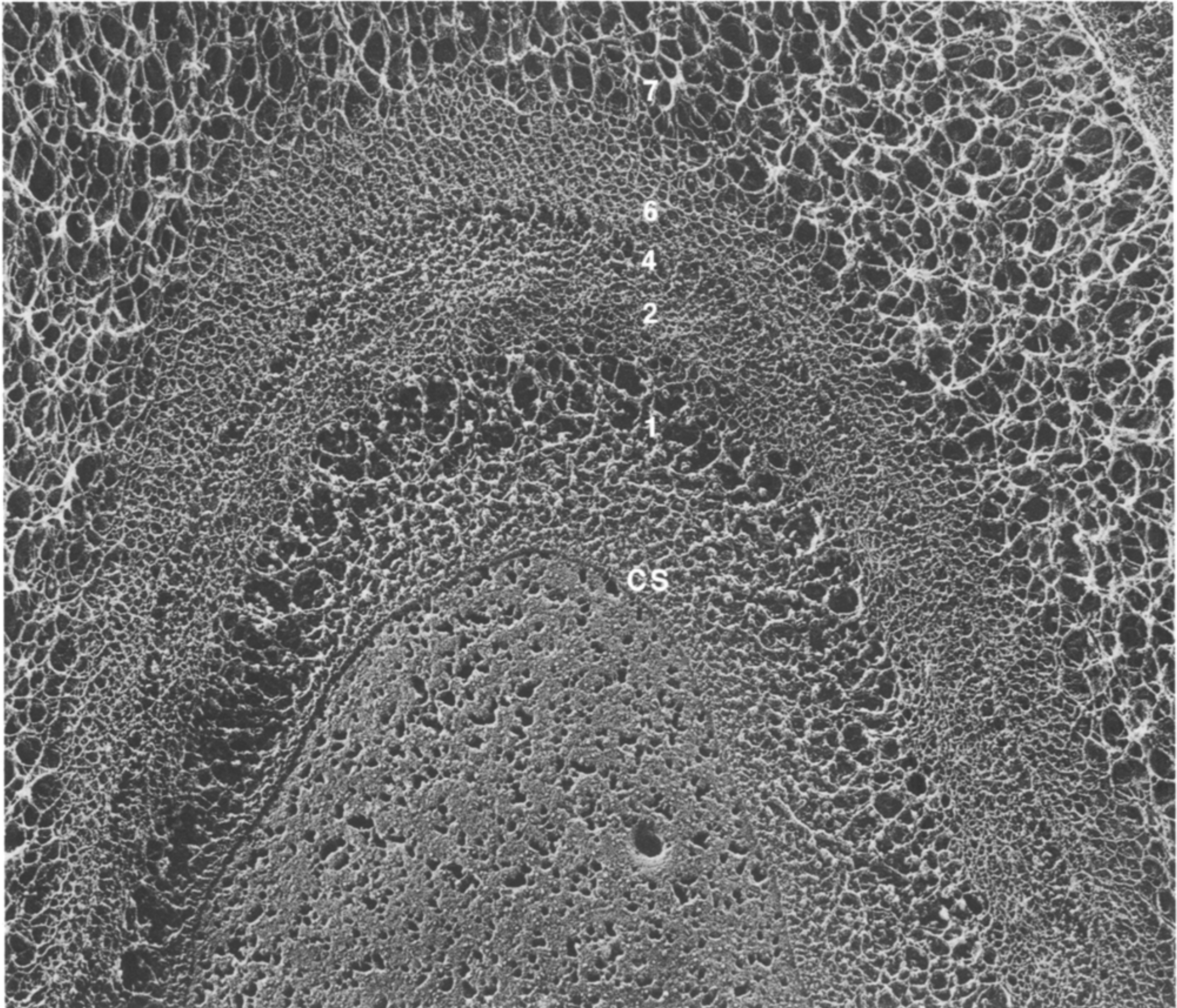

Fig. 1. Cell wall of vegetative *Chlamydomonas reinhardtii* in tangential fracture. *CS* Cell surface; *1, 2, 4, 6,* and *7* major wall layers. × 65,000. This and all of the following micrographs were prepared by John Heuser, Washington University Medical School, St. Louis, MO. Reprinted from Goodenough and Heuser (1985)

ular heads). These assemble into matrices wherein the globular domains are interconnected by the fibrous domains (Roberts et al. 1985 a, Goodenough and Heuser 1988 b). Variability in the assembled matrices comes from differences in two interrelated aspects of the HRGPs, conformation and post-translational modifications. If all of the different cell wall HRGPs derive from a single gene family, as has been proposed (Goodenough 1985), then it should be possible to use these proteins as phylogenetic markers within the volvocine line. An obvious conclusion from the studies presented in this review, reached by other researchers as well, is that the genus *Chlamydomonas* needs to be reorganized. Specifically, it is clear that *C. reinhardtii* has more traits in common with the colonial and multicellular Volvocaceae than with most other *Chlamydomonas* spp., which fall in a group with *C. eugametos*. The closing section will review some molecular phylogenetic studies, involving statistical analysis of aligned rRNA sequences from the Volvocales, which support the monophyletic grouping of *C. reinhardtii* with the Volvocaceae, but indicate that the evolutionary progression from the unicellular *Chlamydomonas* to the multicellular *Volvox* is not unidirectional and monophyletic.

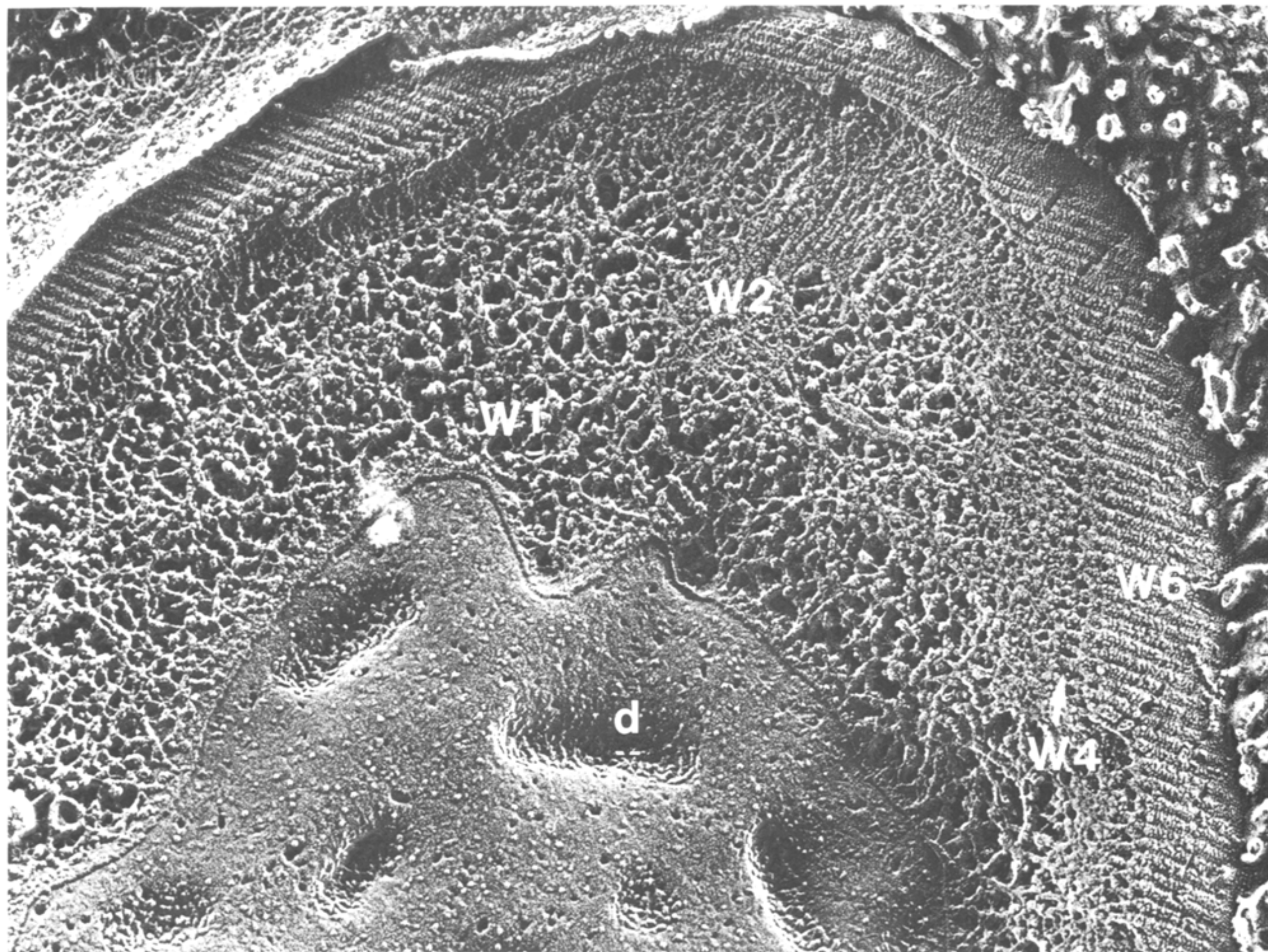

Fig. 2. Cell wall of vegetative *Chlamydomonas eugametos*. The etched face of the plasmalemma shows oblong depressions (*d*) that are absent in *C. reinhardtii*. *W1*, *W2*, *W4*, and *W6* Wall layers. × 80,000. Reprinted from Goodenough and Heuser (1988 b)

Characterization of volvocine cell walls by electron microscopy

Among other morphological traits, the thickness of the cell wall and the overall body shape (which is determined by the cell wall framework; Hills 1973, Imam et al. 1988) were used by Ettl (1976) to define various species of *Chlamydomonas*. The 450 described species of *Chlamydomonas* display a wide array of cell body shapes and sizes. *Chlamydomonas reinhardtii* is the designated type species and has received the most intensive scientific scrutiny (see Harris 1989). The non-cellulosic cell wall of vegetative *C. reinhardtii* cells is constructed from 17–30 types of glycoproteins (Adair and Snell 1990), many of which are HRGPs (Roberts 1974). Each wall is composed of five recognized layers (Goodenough and Heuser 1985, Roberts et al. 1985 a), each of which carries a characteristic set of distinct glycoproteins (Fig. 1). The innermost layer, W1, has a loose network of fibers radiating from the plasma membrane to the central triplet. The central triplet consists of three layers: W2, a tight weave of thick fibers running parallel to the cell surface; W4, a layer of large granules lined up in chains parallel to the cell surface; and the crystalline W6 layer. The outermost layer, W7, also has loose radiating fibers which may or may not be related to those in W1 (see Fig. 3).

The central triplet with its crystalline W6 layer is the most conserved feature of volvocalean cell walls (Roberts 1974). Roberts et al. (1982), using optical diffraction analysis of electron micrographs of cell walls from many different Volvocales, defined four distinct classes of crystalline structure. Class I contains *C. reinhardtii, C. angulosa, C. cribrum, C. inepta,* and the colonial and multicellular Volvocaceae. Class II, considered by Roberts (1974) to be the most primitive wall from which the other types have evolved, is the most predominant type in the unicellular Volvocales, including the ma-

jority of *Chlamydomonas* examined. *C. asymmetrica* is the only representative of Class III, while *Vitreochlamys incisa* and *Lobomonas piriformis* make up Class IV. The large number of species which fall into Class II, and the grouping of several *Chlamydomonas* spp. with Volvocaceae indicates that outer-wall ultrastructure has been highly conserved in the Class II lineage and that the colonial and multicellular Volvocaceae probably evolved from a Class I ancestor. Using crystal structure as a phylogenetic marker, therefore, the genus *Chlamydomonas* can be divided into at least three different lineages.

In their quick-freeze deep-etch electron microscopy analysis of the Class II *C. eugametos* wall, Goodenough and Heuser (1988 b) defined W1, W2, W4, and W6 layers (Fig. 2). While W1 and W2 appear structurally similar to the comparable layers found in *C. reinhardtii*, W4 is not granular in *C. eugametos* but probably a subdomain of W2. W6 is a dense, "chainmail" crystal which is radically different from the open W6 weave in *C. reinhardtii*. Interestingly, the Class II version of W6 closely resembles the crystalline pattern of the flagellar collar in the *C. reinhardtii* and *V. carteri* cell walls (Goodenough and Heuser 1988 b, Imam and Snell, 1988, Kirk et al. 1986). Flagellar collars are specialized domains at the cell anterior through which the flagella traverse the wall. In *C. eugametos* and *Chlorogonium elongatum* (both Class II), the flagellar collar appears as a simple infolding of the W6 crystal; whereas in *C. reinhardtii* and *V. carteri* the collar is quite distinct from the other layers of the cell wall. Therefore, expanding on Roberts' (1974) scheme, it has been proposed that the primordial Volvocales produced Class II cell walls from which a line of unicells arose that kept Class II crystals for its flagellar collars, but evolved Class I structure for its cell walls (Goodenough and Heuser 1988 b). This line then gave rise to the colonial and multicellular Volvocaceae.

The colonial and multicellular Volvocaceae have a more complex cell wall than the unicellular green algae. Kirk et al. (1986) have identified four major zones in the *Volvox* wall – the flagellar zone includes the flagellum and the periflagellar region; the boundary zone (with central triplet found in all Volvocales) surrounds the entire colony; the cellular zone encompasses individual cells in the organism; and the deep zone designates the central space of the colony. Both the cellular and deep zones are unique to the Volvocaceae, and these zones make up the majority of the extracellular matrix. Kirk et al. (1986) point out that while comparisons of the *Volvox* boundary zone with the *C. reinhardtii* crystalline layer emphasize the relatedness of these two organisms, the inner zones are what define species-specific extracellular matrix patterns in the family. Extensive modification and elaboration of the amorphous W2 layer of a unicellular ancestor like *C. reinhardtii* may have led to the diversity found in the colonial and multicellular Volvocaceae (Schmitt et al. 1992).

Enzymatic digestion of volvocalean cell walls

Work by Schlösser (1976, 1984) and Matsuda et al. (1987) on species-specific sensitivity to lytic enzymes among the Volvocales lends support to the idea that phylogenetic diversity is reflected by inner wall composition. The vegetative lytic enzyme (VLE) specifically digests the mother cell wall, releasing the daughter cells after mitosis (Schlösser 1966), while the gametic lytic enzyme (GLE) is produced by mating gametes to digest the cell wall prior to zygote formation (Claes 1971). GLE will digest the vegetative or gametic cell wall at any time during the life cycle and has been shown to act specifically on the flagellar collar and within the W2 layer of *C. reinhardtii* (Goodenough and Heuser 1985, Imam and Snell 1988, Monk 1988, Waffenschmidt et al. 1988). Schlösser (1976, 1984) classified 45 species of *Chlamydomonas* into 15 groups based on sensitivity to VLE. There is not enough overlap in the species examined by Schlösser and by Roberts et al. (1982) to make definitive statements about how the VLE groupings match the crystal lattice classes. Nevertheless, *C. dysosmos* and *C eugametos* which have Class II walls, fall into Schlösser's groups 7 and 12, respectively, and *C. reinhardtii, C. angulosa,* and *C. inepta*, which share Class I walls, belong to groups 1, 2 and 2, respectively. The latter two groups must be closely related because the VLE from group 2 members can digest the walls of all group 1 cells; this action, however, is not reciprocal (Schlösser 1976).

GLE from *C. reinhardtii* can digest the vegetative cell walls of all of Schlösser's group 1 strains as well as *C. cribrum* (not grouped by Schlösser), and partially affects the wall of one group 2 member, *C. komma* (Matsuda et al. 1987). No other *Chlamydomonas* spp. showed any sensitivity to this GLE. Hence, *C. reinhardtii* GLE and VLE both exhibit similar group-specific activity. Matsuda et al. (1987) also tested the ability of this GLE to digest the walls of colonial and multicellular Volvocales, and found that six species of *Gonium* and two species of *Astrephomene* were sensitive to GLE but not to VLE; other colonial or multicellular

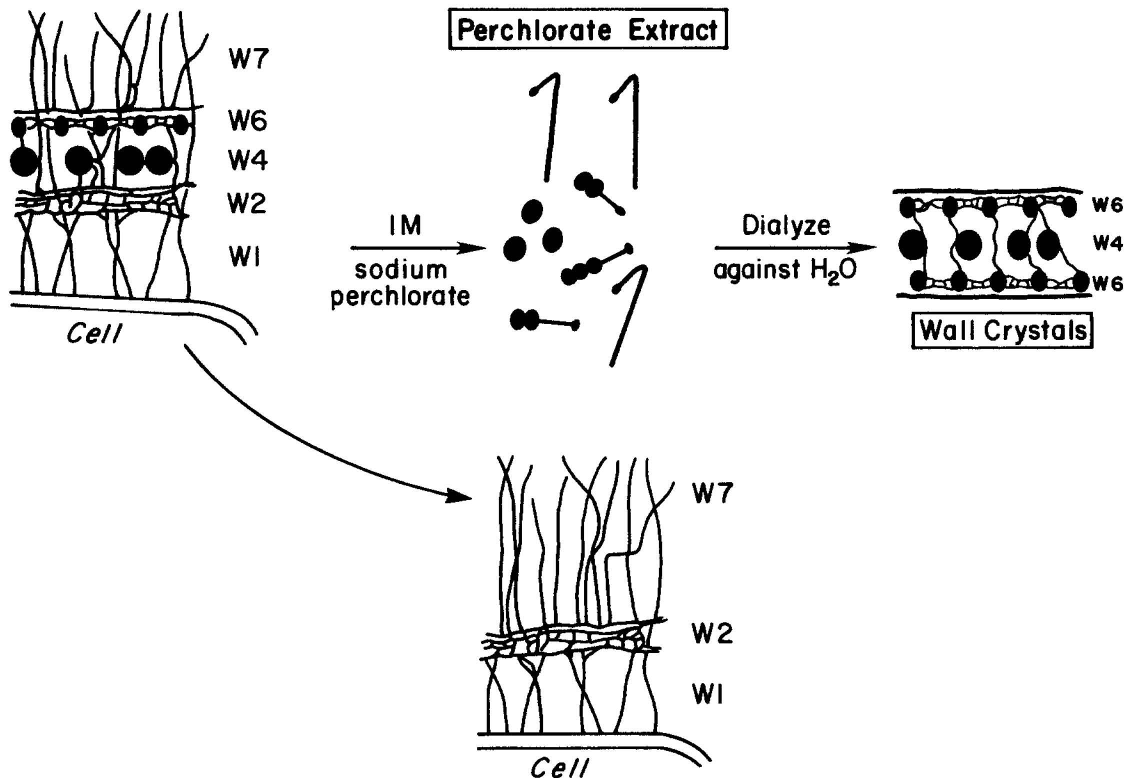

Fig. 3. Proposed sequence of events for *C. reinhardtii* wall crystal formation in vitro. Reprinted from Goodenough et al. (1986 a)

volvocalean algae were not susceptible. Representatives from all four classes of crystalline walls were tested by Matsuda et al. (1987) and only members of Class I showed any sensitivity to GLE from *C. reinhardtii*. While some of the colonial Volvocaceae (Class I) exhibited sensitivity, supporting Roberts' classification, a number of other colonial and multicellular Volvocales (including Class I members) were resistant to this GLE. These observations indicate that there is variation in the inner wall/collar composition such that there can be a common crystalline outer wall (Class I) and a genus-specific inner wall. They also provide additional evidence for an evolutionary sequence from an ancestor resembling *C. reinhardtii* to the colonial Volvocaceae.

In vitro reassembly of volvocine cell walls

In 1973, Hills demonstrated that a portion of the cell wall of *C. reinhardtii* is soluble in chaotropes (LiCl or $NaClO_4$) and that when the chaotrope is dialyzed away, the solubilized proteins reassemble in vitro. This initial observation has now been documented and analyzed in many laboratories (see Adair and Snell 1990). The *C. reinhardtii* cell wall can be divided into two major domains – the salt-soluble outer layers including W4, W6, and W7 (accounting for > 90% of the cell wall mass), and the highly insoluble, covalently cross-linked inner layers W1 and W2 (see Fig. 1). Extraction with chaotropes disassembles the crystalline layer into its component glycoproteins, and if these components are dialyzed in the absence of the insoluble layers they will self-assemble into a crystal structure composed of a W4 layer sandwiched between two W6 layers (Fig. 3) (Goodenough et al. 1986 a). While the crystal structure of the W6 layers in this in vitro sandwich is indistinguishable from the W6 lattice found in situ (Goodenough and Heuser 1988 a), it is only when the chaotrope-extracted components are dialyzed in the presence of the insoluble layers (either wall "shells" (Hills et al. 1975) or salt-extracted cells (Adair et al. 1987)) that the in vitro reassembly creates a single W6 layer as is found in situ. The salt-extracted cells and "shells" retain the characteristic shape of the organism, demonstrating that the inner layers of the wall generate the wide range of cell shapes found in *Chlamydomonas* spp.
With the knowledge that the crystalline layers of *C. reinhardtii, Volvox carteri,* and *Gonium pectorale* are all in Class I, and that the W2 layer (boundary and cellular zones of Kirk et al. 1986) of *V. carteri* resembles that of *C. reinhardtii* (Goodenough and Heuser 1985),

Adair et al. (1987) undertook interspecific wall reconstitution analyses. First they demonstrated that the crystalline layers of *V. carteri* and *C. eugametos* could be solubilized in sodium perchlorate and reassembled in vitro. Next, they took the soluble components from each species and attempted to reconstitute a wall on the insoluble layers of another species that had been salt-extracted. The result was that both *C. reinhardtii* and *V. carteri* can nucleate assembly of each other's crystalline components, while no reconstitution was observed between *C. reinhardtii* and *C. eugametos*. Later experiments showed that *C. reinhardtii* and *Gonium pectorale* can each nucleate assembly of the other's outer wall (Adair and Snell 1990). Therefore, association of the outer wall glycoproteins with the W2 layer components is not species-specific and indicates a high degree of functional conservation in Class I cell walls. No wall reconstitution is observed when cell wall-less mutants of *C. reinhardtii* lacking the W2 layer are mixed with extracted crystalline components from a wild-type cell (Hills 1973, Adair et al. 1987), suggesting that the insoluble inner layers are critical to nucleate assembly of the outer wall monomers, at least in vitro. While the insensitivity of *V. carteri* to the *C. reinhardtii* GLE (Matsuda et al. 1987) implies differences in the inner wall layers between these two organisms, the interspecific reassembly data document the persistence of domains in the *Volvox* W2 layer that are homologous to the nucleation sites in the *C. reinhardtii* W2 layer. The inability of *C. reinhardtii* and *C. eugametos* to reconstitute each other's outer wall provides further proof of the evolutionary distance between these two species and between algae with Class I or Class II walls.

Volvocine structural HRGPs

Since the Class I volvocine algae have a similar cell wall crystal structure, are capable of interspecific wall assembly, and exhibit some cross-sensitivity to one another's autolysins, it is reasonable to expect homologies among the proteins which make up their cell walls. Four main glycoproteins are extracted from the outer wall of *C. reinhardtii* (Roberts 1974, Goodenough et al. 1986 a); three are HRGPs (GP 1, GP 2, and GP 3), and one is glycine-rich (GP 1.5). Detailed electron microscopy studies on in vitro crystal assembly using purified glycoproteins have shown that the W6 crystalline layer is actually a bilayer, with the inner layer (W6A) formed by coassembly of GP 2 and GP 3 monomers and the outer layer (W6B) constructed by self-assembly of GP 1 monomers on the GP 2/GP 3 template (Goodenough and Heuser 1988 a). GP 1.5 was shown to correspond to the granules that comprise the W4 layer (Goodenough and Heuser 1985). When extracted components of the crystalline layers of different Volvocales are compared by SDS PAGE, it becomes clear that only a few glycoproteins are involved in outer wall assembly (Roberts 1974, Adair and Appel 1989). A comparison of apparent molecular weights for the crystalline glycoproteins from *V. carteri*, *G. pectorale*, and *C. reinhardtii* shows that the colonial and multicellular Volvocaceae contain components which migrate in the gel similarly to GP 2 (Goodenough and Heuser 1988 b, Adair and Appel 1989). In addition, Goodenough and Heuser (1988 a) have shown that one of the perchlorate-soluble glycoproteins from *V. carteri* is morphologically similar to GP 2 (Fig. 4). There is no GP 1-like component in either *Gonium* or *Volvox*, and neither organism displays a W6B sublayer (Goodenough and Heuser 1988 a, Adair and Appel 1989).

The issue of GP 2 conservation in the Volvocaceae has also been addressed using a polyclonal antibody raised against GP 2 from *C. reinhardtii* (Roberts et al. 1985 b, Matsuda et al. 1987). This antibody proves to recognize sugar side-chain epitopes on the GP 2 glycoprotein, and by immunofluorescence, cross-reacts with the whole

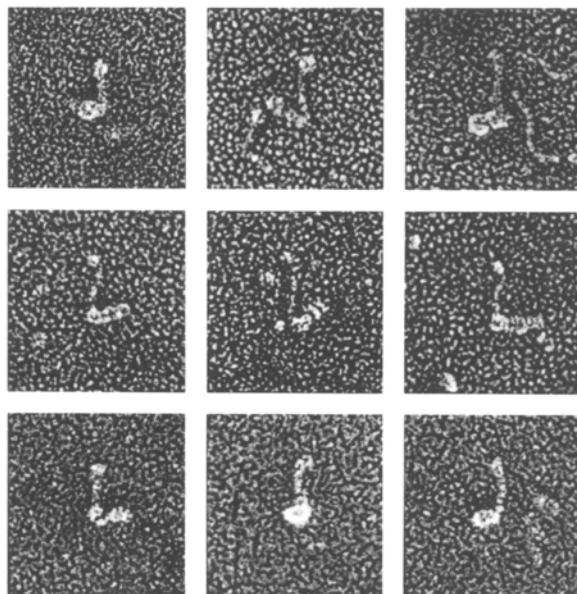

Fig. 4. The top two rows of micrographs show images of the GP 2-like protein found in perchlorate extracts of *Volvox carteri* cell wall crystals. The bottom row shows GP 2 purified from *C. reinhardtii* wall crystals. × 250,000. Reprinted from Goodenough and Heuser (1988 a)

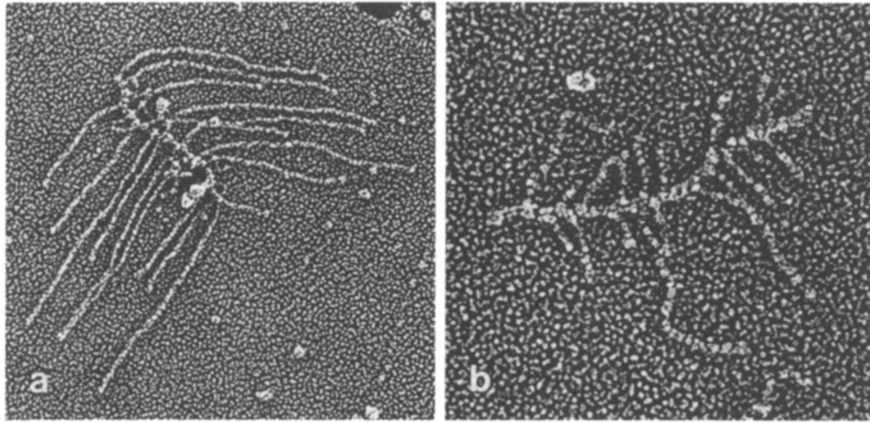

Fig. 5. a W2 fishbone unit from *C. reinhardtii*. × 165,000. Reprinted from Goodenough and Heuser (1985). **b** W2 fishbone unit from *C. eugametos*. × 274,000. Reprinted from Goodenough and Heuser (1988 b)

cell wall, flagella, and flagellar collars of organisms in Roberts' Class I, but not Class II and III. Roberts et al. (1985 b) found cross-reactivity of the antibody to *Lobomonas piriformis* in Class IV, but Matsuda et al. (1987) could not replicate this result. These antibody data prove that sugar side-chain epitopes are conserved in Class I walls, but reveal nothing about protein backbone conservation among crystalline HRGPs. Adair and Appel (1989) used off-diagonal peptide mapping, amino acid analysis, and Western blots with a polyclonal antibody to deglycosylated GP 2 to demonstrate that there are indeed GP 2 homologs in both *V. carteri* and *G. pectorale*, but not in *C. eugametos* (Class II). The Class II W6 crystal of *C. eugametos* is composed of two closely migrating glycopolypeptides, designated GP 265 and GP 245 (Goodenough and Heuser 1988 b). Amino acid analysis of this material indicates a preponderance of hydroxyproline, proline and serine, as with the HRGPs which make up the W6 crystal of *C. reinhardtii* (Adair and Appel 1989, Goodenough et al. 1986 a). These *C. eugametos* HRGPs are morphologically dissimilar to GP 2 or GP 3 and, as noted above, assemble into a very different lattice (compare Figs. 1 and 2) (Goodenough and Heuser 1988 b).

The covalent crosslinking of the inner wall layers has confounded the analysis of the individual HRGPs which make up these layers. Boiling mechanically isolated/salt-extracted cell walls in the presence of SDS and β-mercaptoethanol does not solubilize any inner wall components (other than the 225 kDa flagellar collar polypeptide) as determined by SDS PAGE (Imam and Snell 1988). Only when walls released by GLE treatment of *C. reinhardtii* cells are boiled in SDS and β-mercaptoethanol is any solubilization of the inner wall components observed. This solubilization, however, is incomplete and only one distinct band has been identified by SDS PAGE (Adair and Snell 1990). Electron microscopy has been used to compare the solubilized wall components released into the media by *C. eugametos* cells (Goodenough and Heuser 1988 b) and *C. reinhardtii* cells (Goodenough and Heuser 1985). In both cases, fishbone-like units are found, bearing central "spines" and radiating "ribs" (Fig. 5). The main difference between the fishbones from the two species is that the lengths of the long and short ribs in the *C. eugametos* structure are half that of the *C. reinhardtii* ribs. The *C. reinhardtii* fishbones derive from the solubilization of the W2 layer due to the action of GLE released during mating, and while no autolysin-releasing system has yet been identified in *C. eugametos*, the fishbones in the media are proof of its existence. In both species, the fishbone units are believed to interact with the crystalline layer via their spines and thus, they probably serve as the W2-layer nucleation sites for crystal formation.

The presence of similar fishbone structures in these two species of *Chlamydomonas* has some intriguing implications. By the aforementioned classification schemes (Ettl 1976, Roberts et al. 1982, Schlösser 1984) these two species of *Chlamydomonas* are quite distinct, yet both types of walls have radiating and densely packed fibers making up well-defined W1 and W2 layers, both walls have a crystalline lattice which disassembles in perchlorate and reassembles upon dialysis, and some of the components which compose the inner and outer layers of each wall have comparable morphology. This leads to a concept which originated from research on the crystal lattices: there is a common plan for making

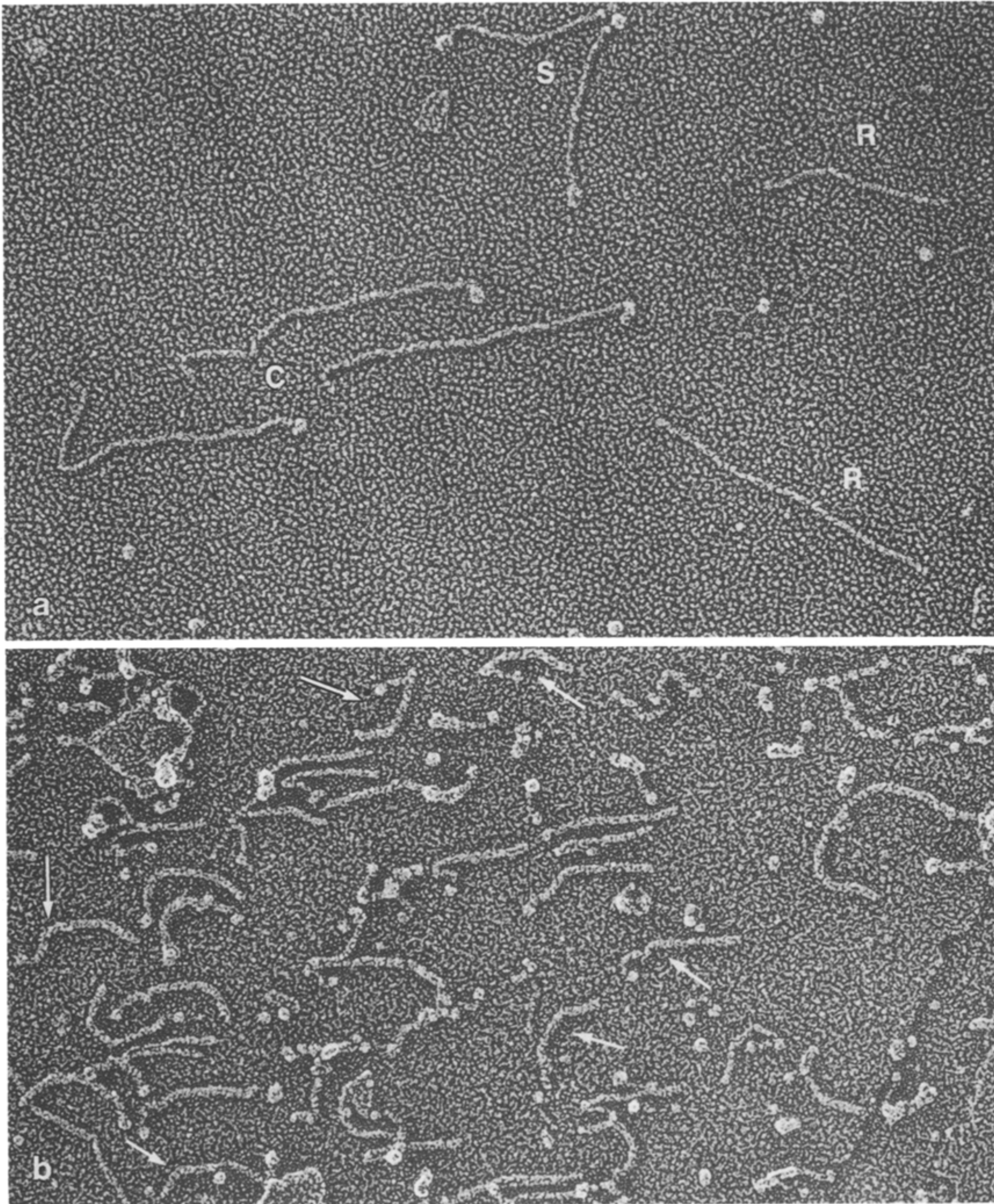

Fig. 6. a A field with three *C. reinhardtii* sexual agglutinins (*C*), two short canes (*S*) that are also found on gametic flagella, and a long (200 nm) and short (100 nm) rib (*R*) which come from the W 2 fishbones. **b** GP 1 monomers (100 nm) with arrows indicating proteins with kinks or bends reminiscent of those seen in agglutinins. × 205,000. Reprinted from Goodenough and Heuser (1985)

a volvocalean cell wall, and modifications in the building blocks led to the diversity exhibited by the assembled matrices (Roberts et al. 1985 a, Goodenough and Heuser 1988 b).

Both Class I and Class II crystals consist of globular and fibrous domains (Goodenough and Heuser 1988 a, b). By transmission electron microscopy, GP 2 (see Fig. 4) and GP 3 from *C. reinhardtii* each carries several globular domains (knobs) interconnected by rods (Goodenough et al. 1986 a). The distant Class II homologs of GP 2 and GP 3, GP 265, and GP 245, are less well differentiated into domains (Goodenough and Heuser 1988 b). The GP 1 monomer of *C. reinhardtii* has the most extreme differentiation (Fig. 6): it carries

a terminal knob and a single long rod with two distinct bends (Roberts 1981, Goodenough et al. 1986 a). Models of the assembly suggest that the knobs, rods, and bends all participate in lattice formation (Goodenough and Heuser 1988 a, b).

Chlamydomonas flagellar agglutinins

Studies of the cell wall HRGPs have been complemented by work on the *Chlamydomonas* sexual agglutinins. Agglutinins are the molecules distributed along the flagellar surface of mating type plus and minus gametes that mediate sexual recognition and adhesion during the mating reaction (Adair 1985). The plus and minus agglutinins from both *C. reinhardtii* and *C. eugametos* are HRGPs with a morphology (long fibrous rods with knobs and curves) and amino acid content remarkably similar to that of cell wall HRGPs (see Fig. 6) (Musgrave et al. 1981, Cooper et al. 1983, Samson et al. 1987, van den Ende et al. 1988, Goodenough 1991). When the *C. reinhardtii* and *C. eugametos* agglutinins are compared, they prove to differ in length: the agglutinins in *C. reinhardtii* are both 225 nm, whereas in *C. eugametos* the mating type plus agglutinin is 276 nm and mating type minus molecule is 336 nm. Recall that the long and short ribs of the *C. eugametos* W2 fishbones are half the length of their counterparts in *C. reinhardtii* fishbones. These ribs, like the GP 1 molecule of *C. reinhardtii*, have a basic structural homology to the agglutinin molecules (see Fig. 6). Goodenough and Heuser (1985) proposed that agglutinins originally evolved from a pair of coassembling wall HRGPs that came under control of the mating type locus such that one monomer of the pair was localized only in minus gametic flagella and the other found only on plus flagella. This would imply that agglutination and cell wall assembly are similar processes. To carry this analogy one step further, one can speculate that just as modifications in cell wall HRGPs can lead to unique wall structures, changes in agglutinin HRGPs lead to novel adhesion specificities and hence serve to sexually isolate incipient species (Goodenough 1985, 1991). Thus, the HRGPs could prove to be useful phylogenetic markers.

Volvocine wall genes

The large number of taxonomic groups in the genus *Chlamydomonas* alone implies that there has been tremendous variation in both wall and agglutinin HRGPs. What makes these HRGPs so mutable? In higher plants, where many of the cell wall HRGPs have been sequenced (Showalter 1993), the genes encode proteins with repetitive amino acid motifs (e.g., Ser(Pro)$_4$TyrLys and ProProValTyrLys). It has been proposed, and later demonstrated, that such repetitive DNA sequences are exceptionally prone to rearrangements (e.g., unequal crossover events and homologous recombination; Goodenough 1985, Raz et al. 1992). Changes in the DNA sequences encoding the repetitive amino acid domains can result in significant alterations in the length, rigidity, post-translational modifications and ultimately, the function of the HRGPs (Kieliszewski and Lamport 1994). The repeating prolines, which are variably hydroxylated and glycosylated depending on the presence of specific neighboring amino acids, contribute to the rigidity of the molecule by adopting an extended polyproline-II helix conformation (Lamport 1977, Kieliszewski et al. 1990). The serines, which can be galactosylated, and the tyrosines, which can form intra- or intermolecular isodityrosine crosslinks, appear only in certain amino acid sequence contexts (Epstein and Lamport 1984, Fry 1986, Kieliszewski and Lamport 1994). Moreover, many of the repeat units are part of peptide palindromes which have been proposed to serve as self-assembly nucleation sites (Kieliszewski et al. 1992). Molecular characterization of volvocine wall genes is just underway and yet there are already some common themes emerging from these studies (repeating amino acid motifs, hydroxylation and glycosylation of prolines, and crosslinking) which mesh neatly with results of work on higher plant cell wall HRGPs. These are reviewed below.

During its life cycle, *C. reinhardtii* erects two biochemically and morphologically distinct cell walls: the vegetative/gametic wall, which has been the focus of this review thus far, and the zygote wall, which is assembled after GLE removes the cell wall from mating gametes. The zygote wall has no obvious crystalline layer, and no perchlorate-soluble components. In deep-etch replicas, the zygote wall appears as an apparently random mass of interconnecting thick fibers (Woessner et al. unpubl.); in thin-sectioned material, several layers can be recognized (Cavalier-Smith 1976, Minami and Goodenough 1978, Grief et al. 1987). Minami and Goodenough (1978) showed that a novel set of glycoproteins is synthesized and secreted by developing zygotes. Ferris and Goodenough (1987) went on to isolate a set of zygote-specific cDNAs whose transcripts were detectable shortly after zygote formation, but absent from gametes or vegetative cells. DNA sequence analysis has demonstrated that at least two of these cDNAs encode wall proteins (Woessner and Good-

1 MRKVSAFGALVAAVAVMSMLTMPTAALAARTSLLATANATATD**APPP**DFSF
52 **EPPP**HEAV**EPPP**AKGAAAF**TPPP**QDLST**EPPP**AEFEQT**SPPP**SASR**SP**
100 **SPSSSASPSPSP**ALVPIPNVGNTA**SPNPSSSPSPSPSPSPSP**AAT**SS**
147 **PSPIASPPP**LPATLTN**QPPP**SAVDQLGGNTQAGSGAAARAGLLLLSCVAA
197 WAFAVL

Fig. 7. Derived amino acid sequence of ZSP-1. The putative signal sequence is underlined and the X(Pro)$_3$ and (SerPro)$_\times$ repeats are in bold face

1 MKSSHRALWASAVLAMAVLACVVPTADAAKK**SPPP**AD**APPP**DSGQ**YPPP**T
51 DWVT**WPPP**VEEHGHH**SPSPSPEDSPSSTPIPTPDILENRDLPSPSPE**
98 **AEDSPSPSPDLEDSPSHSPEPEVESPSPETETESPSPSPEPEVEDS**
144 **ASPSPEPEVEDSPSPSPEPMDESPAPEPSPEPVPSPETPSPAP**YYG
190 A**SPSPSP**YYGGYGNYGPSVPTYG**SSPSPAPSP**YGGYGGYGGYGGYGGYGS
240 YGDDDEEEEDPMSAARRLMLDVPNQGAGRQLSGDAVDLLAGGEEPESEERST
292 RVSRRLLRI

Fig. 8. Derived amino acid sequence of VSP-1. The putative signal sequence is underlined and the X(Pro)$_3$ and (SerPro)$_\times$ repeats are in bold face

enough 1989, 1992). ZSP-1 (originally termed Class IV), which resembles the class of higher plant structural HRGPs called extensins, has a signal sequence, eight X(Pro)$_3$ and two (SerPro)$_\times$ repeats (Fig. 7). The other gene (ZSP-2, originally Class VI) has some similarities to solanaceous lectins (see Showalter 1993) with four lectin-like domains intermixed with two (SerPro)$_\times$ domains (Woessner and Goodenough 1989, and unpubl.). A polyclonal antibody to a ZSP-1/β-galactosidase fusion protein immunoprecipitated 5–10 polypeptides from an in vitro translation of zygote RNA, but detected nothing in immunoprecipitates of secreted zygote proteins from in vivo labelled cells (Woessner and Goodenough 1989, 1992). This result was interpreted to mean that there are several zygote wall components with epitopes similar to those in ZSP-1 but that hydroxylation/glycosylation prevents detection of these proteins in vivo.

To test whether (SerPro)$_\times$ repeats were a common epitope among zygote wall proteins, a polyclonal antibody raised agaist a (SerPro)$_{10}$ oligopeptide was found to immunoprecipitate the identical set of polypeptides from in vitro translations of zygote RNA. In addition, both antibodies were found to recognize a set of five vegetative wall glycopolypeptides from in vitro translations of vegetative RNA, one of which corresponds to the deglycosylated form of GP 2 (Adair and Apt 1990, Woessner and Goodenough 1992). Using clones described by Adair and Apt (1990), we have also isolated several GP 1 cDNAs (none of which is full-length) and determined a partial DNA sequence which encodes a 3′ proline-poor domain preceded by a long domain of (SerPro)$_\times$ (Woessner and Goodenough unpubl.). Therefore, (SerPro)$_\times$ repeats appear to be diagnostic for *C. reinhardtii* wall proteins in much the same way that Ser(Pro)$_4$ repeats indicate extensins in higher plants.

A cDNA expression library made from GLE-treated *C. reinhardtii* vegetative cells, which have high levels of wall-specific messages (Su et al. 1990, Adair and Apt 1990), was screened with the (SerPro)$_{10}$ antibody and five different groups of cDNAs were isolated (VSP-1 to VSP-5; Waffenschmidt et al. 1993). A full-length cDNA for VSP-1 was sequenced and found to encode a signal sequence, four X(Pro)$_3$ repeats, long stretches of (SerPro)$_\times$, a TyrGlyGly domain and an acidic C-terminus lacking both serine and proline (Fig. 8). The amino acid content and abundance of tyrosine distinguish this molecule from any of the outer wall components (Goodenough et al. 1986 a) and thus, VSP-1 is proposed to be an inner wall HRGP. The tyrosines suggested that tyrosine crosslinking may be involved in insolubilization of the inner wall layer, and Waffenschmidt et al. (1993) demonstrated that tyrosine crosslinks in fact mediate wall insolubilization in both vegetative and zygotic cell walls.

Woessner and Goodenough (1992) predicted that (SerPro)$_\times$ motifs might be diagnostic of wall proteins throughout the Volvocales, and a collaborative project with Dr. van den Ende's laboratory supports this proposition. The (SerPro)$_{10}$ antibody was found to immunoprecipitate novel sets of polypeptides from in vitro translations of *C. eugametos* vegetative and zygote RNA and the antibody was used to isolate a vegetative wall gene (WP 6) from a λgt 11 expression library of *C. eugametos* genomic DNA (Woessner et al. in prep.). The DNA sequence of WP 6 has been determined and shown to encode a signal sequence, a 5′ domain rich in cysteine but poor in serine and proline, and a 3′ domain rich in serine and proline with a number of (SerPro)$_\times$ repeats. Interestingly, the (SerPro)$_\times$ repeats are frequently found in a (SerPro)$_3$LysAla motif, a motif repeated as well in *C. reinhardtii* VSP-3, which also has a signal sequence, a 5′ domain rich in cysteine but poor in proline, and a 3′ domain dominated by (SerPro)$_\times$ repeats (Woessner et al. in prep.). Once again, as was pointed out when comparing the fishbone units from these two species, there are homologies in

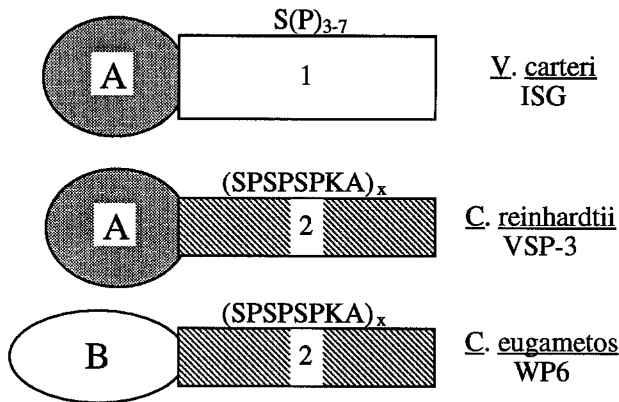

Fig. 9. Domain conservation in volvocine wall proteins. Each protein is represented in cartoon fashion with globular 5′ domain and a rod-like 3′ domain. Homologous 5′ and 3′ domains are shaded and the characteristic amino acid repeat units are presented for each protein

the cell wall structural building blocks, despite the evolutionary distance between *C. eugametos* and *C. reinhardtii.* It is important to point out that the other domains in WP 6 and VSP-3 are quite distinctive, only the (SerPro)$_x$ and (SerPro)$_3$LysAla motifs have been conserved.

Only two other cell wall genes in the Volvocales have been sequenced, both from *Volvox carteri* (Ertl et al. 1989, 1992). SSG 185 is a highly sulfated HRGP which becomes insolubilized in the inner wall layer (cellular zone). There are no extensive (SerPro)$_x$ repeats in this molecule, but an 80 amino acid central domain is almost entirely proline. These prolines are hydroxylated and arabinosylated and give the molecule a rod-like shape as revealed by electron microscopy (Ertl et al. 1989). Covalent cross-linking of this molecule is due to phosphodiester bridges between side chains rather than between peptide chains (Holst et al. 1989). ISG is another sulfated HRGP, expressed only briefly in inverting embryos and sperm cell packets. This glycoprotein localizes to the boundary zone (containing the central triplet) and has two distinct amino acid domains. The 5′ half of the molecule is poor in proline, while the 3′ half is rich in serine and proline, occurring in Ser(Pro)$_{3-7}$ repeats (Ertl et al. 1992). Again, the prolines are both extensively hydroxylated and arabinosylated. Electron microscopy shows ISG to be a rod-like structure (encoded by the 3′ domain) with a globular head (from the 5′ domain). The monomers can aggregate into star-like particles attaching by their knobs with rods radiating outwards (Ertl et al. 1992).

Only a small number of volvocine wall genes have been examined to date, but there is already a striking example of HRGP domain conservation involving *C. reinhardtii, C. eugametos* and *V. carteri* (Fig. 9). The 3′ domains of VSP-3 and WP 6 (which encode the rod-like portion of each protein) are quite similar (88%

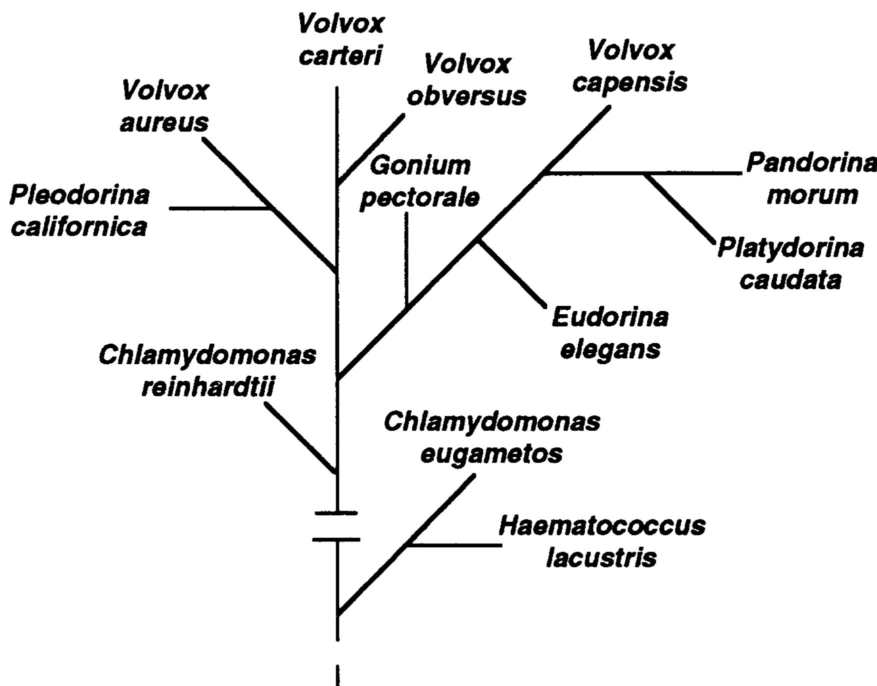

Fig. 10. Phylogenetic tree indicating sister-taxon relationships, as deduced by maximum parsimony analysis of nucleotide sequence from the variable regions of the large and small subunits of nuclear-encoded rRNAs. Reprinted from Schmitt et al. (1992)

similarity over 73 amino acids) while their 5′ domains (encoding the globular portion of each protein) are non-homologous, but the 5′ domains of VSP-3 and ISG (encoding the globular portions of each molecule) are related (52% similarity over 228 amino acids). With a larger pool of characterized volvocine HRGPs it should be possible to define ancestral rod and knob domains and determine the best way to use these proteins as phylogenetic markers.

Conclusions

Recently several groups have constructed consensus trees based on cladistic analyses of aligned nuclear 18 S and 26 S rRNA sequences. Rausch et al. (1989) showed that the volvocine radiation (*Chlamydomonas* to *Volvox*) is recent, probably within the past 50–75 million years. They also demonstrated that the higher plants and the chlorophytes (green algae) last shared a common ancestor about 700 million years ago. In this respect, it is not hard to see why the relatively simple *Chlamydomonas* cell wall, constructed almost exclusively of proteins, is so different from the complex network of numerous proteins and matrix polysaccharides (cellulose, hemicelluloses, lignins, pectins, and waxes) that constitutes the higher plant cell wall (Varner and Lin 1989). Buchheim et al. (1990) compared the results of their cladistic analysis of 18 S rRNA sequences from *Chlamydomonas* spp. with the classification schemes of Ettl (1976), Schlösser (1984), and Roberts et al. (1982). With a few exceptions, all four methods are in close agreement, and all lead to the conclusion that there is no support for a monophyletic or natural genus *Chlamydomonas*. Cladistic analyses also confirm that *C. reinhardtii* is more closely allied with the colonial and multicellular Volvocaceae than with *C. eugametos* (Buchheim and Chapman 1991, Larson et al. 1992) and that there is not a linear evolutionary progression from simple *Chlamydomonas* cells to complex multicellular *Volvox* organisms (Fig. 10). In fact, species of *Volvox* appear on two separate phylogenetic branches of the consensus tree for Volvocales.

In spite of differences seen in crystal structure, lytic enzyme sensitivity, and reassembly of the *C. eugametos* and *C. reinhardtii* cell walls, there are some similarities in the proteins which compose these walls. The accumulation of molecular data about the HRGPs (cell wall proteins and agglutinins) found in the volvocine algae will lend support to the idea of a gene superfamily from which new and different proteins (all specifically designed for self-assembly/recognition) could constantly evolve. The similarities (repeating proline motifs, hydroxylation and arabinosylation of prolines, galactosylation of serines, crosslinking of tyrosines or oligosaccharide side chains, and rod-like molecules) between the volvocine HRGPs and the various types of higher plant cell wall proteins, and the central role these proteins play in organizing cell and plant morphology has led to the proposal that these proteins should make excellent phylogenetic markers for all plants. "The extensin family (including all of the cell wall HRGPs) should mirror the major divisions of the plant kingdom and hence provide another way to assess long-term evolution which, especially in plants, is obscured by extensive homoplasy: gross structures may converge, but molecules probably do not" (Kieliszewski and Lamport 1994).

Acknowledgements

We thank Dr. Marcia Kieliszewski for sending us a preprint of her paper. Part of the work reviewed here was supported by the U.S. Department of Agriculture (Grant no. 92-373304-7936).

References

Adair WS (1985) Characterization of *Chlamydomonas* sexual agglutinins. J Cell Sci [Suppl] 2: 233–260
– Appel H (1989) Identification of a highly conserved hydroxyproline-rich glycoprotein in the cell walls of *Chlamydomonas reinhardtii* and two other Volvocales. Planta 179: 381–386
– Apt KE (1990) Cell wall regeneration in *Chlamydomonas*: accumulation of mRNAs encoding cell wall HRGPs. Proc Natl Acad Sci USA 87: 7355–7359
– Snell WJ (1990) The *Chlamydomonas reinhardtii* cell wall: structure, biochemistry, and molecular biology. In: Adair WS, Mecham RP (eds) Organization and assembly of plant and animal extracellular matrix. Academic Press, San Diego, pp 15–84
– Steinmetz SA, Mattson DM, Goodenough UW, Heuser JE (1987) Nucleated assembly of *Chlamydomonas* and *Volvox* cell walls. J Cell Biol 105: 2373–2382
Buchheim MA, Chapman RL (1991) Phylogeny of the colonial green flagellates: a study of 18 S and 26 S rRNA sequence data. BioSystems 25: 85–100
– Turmel M, Zimmer EA, Chapman RL (1990) Phylogeny of *Chlamydomonas* (Chlorophyta) based on cladistic analysis of nuclear 18 S rRNA sequence data. J Phycol 26: 689–699
Cavalier-Smith T (1976) Electron microscopy of zygospore formation in *Chlamydomonas reinhardtii*. Protoplasma 87: 297–315
Claes H (1971) Autolyse der Zellwand bei den Gameten von *Chlamydomonas reinhardtii*. Arch Mikrobiol 78: 180–188
Cooper JB, Adair WS, Mecham RP, Heuser JE, Goodenough UW (1983) *Chlamydomonas* agglutinin is a hydroxyproline-rich glycoprotein. Proc Natl Acad Sci USA 80: 5898–5901
Epstein L, Lamport DTA (1984) An intramolecular linkage involving isodityrosine in extensin. Phytochemistry 23: 1241–1246
Ertl H, Mengele R, Wenzl S, Engel J, Sumper M (1989) The extracellular matrix of *Volvox carteri*: molecular structure of cellular compartment. J Cell Biol 109: 3493–3501
– Hallmann A, Wenzl S, Sumper M (1992) A novel extensin that may organize extracellular matrix biogenesis in *Volvox carteri*. EMBO J 11: 2055–2062

Ettl H (1976) Die Gattung *Chlamydomonas* Ehrenberg. Nova Hedwigia 49: 1–1122

Ferris PJ, Goodenough UW (1987) Transcription of novel genes, including a gene linked to the mating-type locus, induced by *Chlamydomonas* fertilization. Mol Cell Biol 7: 2360–2366

Fry SC (1986) Cross-linking of matrix polymers in the growing cell walls of angiosperms. Annu Rev Plant Physiol 37: 165–186

Goodenough UW (1985) An essay on the origins and evolution of eukaryotic sex. In: Halvorson HO, Monroy A (eds) The origin and evolution of sex. AR Liss, New York, pp 123–140

– (1991) *Chlamydomonas* mating interactions. In: Dworkin M (ed) Microbial cell-cell interactions. American Society for Microbiology, Washington, DC, pp 71–112

– Heuser JE (1985) The *Chlamydomonas* cell wall and its constituent glycoproteins analyzed by the quick-freeze deep-etch technique. J Cell Biol 101: 1550–1568

– (1988 a) Molecular organization of cell-wall crystals from *Chlamydomonas reinhardtii* and *Volvox carteri*. J Cell Sci 90: 717–733

– (1988 b) Molecular organization of the cell wall and cell-wall crystals from *Chlamydomonas eugametos*. J Cell Sci 90: 735–750

– Gebhart B, Mecham RP, Heuser JE (1986 a) Crystals of the *Chlamydomonas reinhardtii* cell wall: polymerization, depolymerization, and purification of glycoprotein monomers. J Cell Biol 103: 403–417

– Adair WS, Collin-Osdoby P, Heuser JE (1986 b) *Chlamydomonas* cells in contact. In: Gall E, Edelman GM (eds) Cells in contact. Wiley, New York, pp 111–135

Grief C, O'Neill MA, Shaw PJ (1987) The zygote cell wall of *Chlamydomonas reinhardtii*: a structural, chemical, and immunological approach. Planta 170: 433–445

Harris EH (1989) The *Chlamydomonas* sourcebook. Academic Press, San Diego

Hills GJ (1973) Cell wall assembly in vitro from *Chlamydomonas reinhardtii*. Planta 115: 17–23

– Phillips JM, Gay MR, Roberts K (1975) Self-assembly of a plant cell wall in vitro. J Mol Biol 96: 431–444

Holst O, Christoffel V, Frund R, Moll H, Sumper M (1989) A phosphodiester bridge between two arabinose residues as a structural element of an extracellular glycoprotein of *Volvox carteri*. Eur J Biochem 181: 345–350

Imam SH, Snell WJ (1988) The *Chlamydomonas* cell wall degrading enzyme, lysin, acts on two domains within the framework of the wall. J Cell Biol 106: 2211–2221

Kieliszewski M, Lamport DTA (1994) Extensin: repetitive motifs, functional motifs, post-translational codes and phylogeny. Plant J 5: 157–172

– Leykam JF, Lamport DTA (1990) Structure of the threonine-rich extensin from *Zea mays*. Plant Physiol 92: 316–326

– de Zacks R, Leykam JF, Lamport DTA (1992) A repetitive proline-rich protein from the gymnosperm Douglas Fir is a hydroxyproline-rich glycoprotein. Plant Physiol 98: 919–926

Kirk DL, Birchem R, King N (1986) The extracellular matrix of *Volvox*: a comparative study and proposed system of nomenclature. J Cell Sci 80: 207–231

Lamport DTA (1977) Structure, biosynthesis, and significance of cell wall glycoproteins. Rec Adv Phytochem 11: 79–115

Larson A, Kirk MM, Kirk DL (1992) Molecular phylogeny of the volvocine flagellates. Mol Biol Evol 9: 85–105

Matsuda Y, Musgrave A, van den Ende H, Roberts K (1987) Cell walls of algae in the Volvocales: their sensitivity to a cell wall lytic enzyme and labeling with an anti-cell wall glycopeptide of *Chlamydomonas reinhardtii*. Bot Mag 100: 373–384

Minami S, Goodenough UW (1978) Novel glycopolypeptide synthesis induced by gametic cell fusion in *Chlamydomonas reinhardtii* J Cell Biol 77: 165–181

Monk BC (1988) The cell wall of *Chlamydomonas reinhardtii* gametes: composition, structure and autolysin-mediated shedding and dissolution. Planta 176: 441–450

Musgrave A, van Eijk E, te Welscher R, Broekman R, Lens PF, Homan WL, van den Ende H (1991) Sexual agglutination factor from *Chlamydomonas eugametos*. Planta 153: 362–369

Rausch H, Larsen N, Schmitt R (1989) Phylogenetic relationships of the green alga *Volvox carteri* deduced from small-subunit ribosomal RNA comparisons. J Mol Evol 29: 255–265

Raz R, José M, Moya A, Martínez-Izquierdo JA, Puigdomènech P (1992) Different mechanisms generating sequence variability are revealed in distinct regions of the hydroxyproline-rich glycoprotein gene from maize and related species. Mol Gen Genet 233: 252–259

Roberts K (1974) Crystalline glycoproteins of algae: their structure, composition, and assembly. Philos Trans R Soc Lond [Biol] 268: 129–146

– (1981) Visualizing an insoluble glycoprotein. Micron 12: 185–186

– Hills GJ, Shaw P (1982) Structure of algal cell walls. In: Harris JR (eds) Electron microscopy of proteins, vol 3. Academic Press, New York, pp 1–40

– Grief C, Hills J, Shaw PJ (1985 a) Cell wall glycoproteins: structure and function. J Cell Sci [Suppl] 2: 105–127

– Phillips J, Shaw P, Grief C, Smith E (1985 b) An immunological approach to the plant cell wall. In: Brett CT, Hillman JR (eds) Biochemistry of plant cell walls. Cambridge University Press, New York, pp 125–154

Samson MR, Klis FM, Homan WL, van Egmond P, Musgrave A, van den Ende H (1987) Composition and properties of the sexual agglutinins of the flagellated green algae *Chlamydomonas eugametos*. Planta 170: 314–321

Schlösser UG (1966) Enzymatisch gesteuerte Freisetzung von Zoosporea bei *Chlamydomonas reinhardtii dangeard* in Synchronkultur. Arch Mikrobiol 54: 129–159

– (1976) Entwicklungsstadien- und sippenspezifische Zellwand-Lysine bei der Freisetzung von Fortpflanzungszellen in der Gattung *Chlamydomonas*. Ber Deutsch Bot Ges 89: 1–56

– (1984) Species-specific sporangium autolysins (cell-wall dissolving enzymes) in the genus *Chlamydomonas*. In: Irvine DEG, John D (eds) The systematics of green algae. Academic Press, London, pp 409–418

Schmitt R, Fabry S, Kirk DL (1992) In search of molecular origins of cellular differentiation in *Volvox* and its relatives. Int Rev Cytol 139: 189–265

Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5: 9–23

Su X, Kaska DD, Gibor A (1990) Induction of cytosine-rich poly $(A)^+$ RNAs in *Chlamydomonas reinhardtii* by cell wall removal. Exp Cell Res 187: 54–58

Van den Ende H, Klis FM, Musgrave A (1988) The role of flagella in sexual reproduction of *Chlamydomonas eugametos*. Acta Bot Neerl 37: 327–350

Varner JE, Lin L-S (1989) Plant cell wall architecture. Cell 56: 231–239

Waffenschmidt S, Spessert R, Jaenicke L (1988) Oligosaccharide side

chains are essential for cell wall lysis in *Chlamydomonas reinhardtii*. Planta 175: 513–519

Waffenschmidt S, Woessner JP, Beer K, Goodenough UW (1993) Isodityrosine cross-linking mediates cell wall insolubilization in *Chlamydomonas*. Plant Cell 5: 809–820

Woessner JP, Goodenough UW (1989) Molecular characterization of a zygote wall protein: an extensin-like molecule in *Chlamydomonas reinhardtii*. Plant Cell 1: 901–911

– – (1992) Zygote and vegetative cell wall proteins in *Chlamydomonas reinhardtii* share a common epitope, $(SerPro)_x$. Plant Sci 83: 65–76