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Domain conservation in several volvocalean cell wall proteins

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Abstract

Based on our previous work demonstrating that $(SerPro)_x$ epitopes are common to extensin-like cell wall proteins in *Chlamydomonas reinhardtii*, we looked for similar proteins in the distantly related species *C. eugametos*. Using a polyclonal antiserum against a $(SerPro)_{10}$ oligopeptide, we found distinct sets of stage-specific polypeptides immunoprecipitated from *in vitro* translations of *C. eugametos* RNA. Screening of a *C. eugametos* cDNA expression library with the antiserum led to the isolation of a cDNA (WP6) encoding a $(SerPro)_x$-rich multidomain wall protein. Analysis of a similarly selected cDNA (VSP-3) from a *C. reinhardtii* cDNA expression library revealed that it also coded for a $(SerPro)_x$-rich multidomain wall protein. The C-terminal rod domains of VSP-3 and WP6 are highly homologous, while the N-terminal domains are dissimilar; however, the N-terminal domain of VSP-3 is homologous to the globular domain of a cell wall protein from *Volvox carteri*. Exon shuffling might be responsible for this example of domain conservation over 350 million years of volvocalean cell wall protein evolution.

Introduction

The primary cell wall of higher plants is a highly dynamic structure whose composition varies in response to growth, development, environmental stresses, and infection. In addition to an assortment of polysaccharides, the wall contains numerous lignins, waxes, and proteins [51]. Four groups of cell wall hydroxyproline-rich glycoproteins (HRGPs) have been defined: the extensins, proline-rich proteins, arabinogalactan proteins, and solanaceous lectins [46]. Despite the ever-increasing number of such proteins being isolated and characterized, a precise role for any cell wall protein has yet to be established. Nevertheless, we are beginning to gain a good understanding of the structure, regulated expression, and tissue localization of these proteins.

The four groups of HRGPs all display repeating amino acid motifs, and the amino acid sequence of the repeat unit is frequently used to assign a newly isolated cell wall protein to a particular class. For example, the extensins display $Ser(Pro)_4$ repeats and the proline-rich proteins

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers L29028 (*Chlamydomonas eugametos WP6*) and L29029 (*Chlamydomonas reinhardtii VSP-3*).

carry ProProXYLys repeats. However, as the number of characterized HRGPs has increased, considerable variation has been found in the canonical repeats, and there has been a growing tendency to emphasize the novelty of these variants (e.g., monocot vs. dicot extensins). Recently, Kieliszewski and Lamport [25] suggested that we instead focus on the similarity of the HRGPs because they appear to belong to a common superfamily. If this is the case, it should be possible to trace the evolutionary origin of all HRGPs to a small number of archetypal peptide domains. To date, however, most of the characterized genes and proteins derive from dicots, with the rest coming from monocots and gymnosperms, and there are few examples in the literature where multiple HRGPs have been examined from a single species [46] or from two species in the same genus [36]. An important resource for this kind of evolutionary approach is the green algae, which also produce cell wall HRGPs [58] and whose lineage within the phylum and the plant kingdom has been extensively analyzed [3, 4, 28, 35]. Conserved motifs identified in this phylum may well prove to represent archetypal peptide domains subsequently utilized by the vascular plants.

Vegetative cells and zygotes of the green alga *Chlamydomonas reinhardtii* possess structurally and biochemically distinct types of cell walls, each composed almost entirely of HRGPs. In previous work, we demonstrated that a $(SerPro)_x$ motif is found in both zygote and vegetative cell wall proteins [53, 56, 57] suggesting that $(SerPro)_x$ repeats might be diagnostic for volvocalean cell wall proteins in the same manner that $Ser(Pro)_4$ repeats are diagnostic of dicot extensins [57].

In this paper, we present an analysis of the HRGP family in the distantly related species *Chlamydomonas eugametos*. We show that $(SerPro)_x$ epitopes are present in distinct sets of gamete, vegetative, and zygote-specific polypeptides, and we characterize a *C. eugametos* cDNA which codes for a multi-domain wall protein (WP6). DNA sequencing of a VSP-3 cDNA from *C. reinhardtii* reveals that it also codes for a multi-domain wall protein with a $(SerPro)_x$-rich C-terminal domain highly homologous to that found in WP6. The N-terminal domain of VSP-3, but not WP6, resembles the N-terminal domain of a cell wall HRGP found in a closely related alga, *Volvox carteri* [8]. We discuss the implications of conserved N- and C-terminal domains in HRGPs from different taxa representing an estimated evolutionary distance of ca. 350 million years.

Materials and methods

Strains and culture conditions

Chlamydomonas eugametos strains UTEX 9 (mating type +) and UTEX 10 (mating type −) and *Chlamydomonas yapensis* (UTEX 792) were obtained from the Culture Collection of Algae at the University of Texas at Austin. *Chlamydomonas reinhardtii* strains CC-620 (mating type −) and CC-621 (mating type +) were obtained from the *Chlamydomonas* Genetics Center, Duke University, Durham, NC. *Chlamydomonas moewusii* strains 23.91 and 24.91 were obtained from the Sammlung von Algenkulturen at Goettingen, Germany.

C. eugametos cells were routinely grown on agar plates containing M1 medium [31] at 20 °C in a 12 h light/12 h dark regime; synchronous cultures were obtained by growing cells in liquid M1 medium as described [30]. Gametes were obtained by flooding 2- to 4-week-old agar-plate cultures with sterile deionized water, and zygotes were produced by mixing gametes of UTEX 9 and UTEX 10 in glass Petri plates at the start of a light period; the plates were kept in continuous light for 24 h and then put in the dark.

Vegetative *C. reinhardtii* cells were maintained in continuous light on either TAP [17] agar plates or in TAP medium. Gametes were generated by flooding 7-day-old cultures on TAP plates with HSM medium lacking nitrogen [17]; the plates were kept in the light for at least 1 h to allow for flagellar regeneration. Zygotes were obtained by mixing equal numbers of gametes of each mating type at a density of 5×10^7 cells per ml.

In vitro *translation and immunoprecipitation*

Total RNA from different stages of the *C. eugametos* life cycle was isolated as described in Molendijk *et al.* [30], and poly(A)$^+$ RNA was isolated by two passes over an oligo-dT cellulose column (Pharmacia) as detailed in Jacobsen [22]. Poly(A)$^+$ RNA (1.5 μg) or total RNA (10 μg) was translated *in vitro* in 50 μl reactions of a rabbit reticulocyte lysate system (Promega) with ^{35}S-methionine (Amersham). Incorporation varied from 15% when using total RNA to 30–75% with poly(A)$^+$ RNA. Immunoprecipitation with affinity-purified antiserum against a $(SerPro)_{10}$ oligopeptide (α-$(SerPro)_{10}$) and protein A-sepharose CL-4B beads (Pharmacia) was performed as in Woessner and Goodenough [56]. For each immunoprecipitation assay, about 25 μCi of incorporated label was used from the *C. eugametos* translation reactions and 4 μCi was used from the *C. reinhardtii* translation reaction. Immunoprecipitates (ca. 25 nCi) were analyzed by 5–15% SDS-PAGE. The gels were fluorographed using Amplify (Amersham), dried, and exposed to X-omat AT film (Kodak) for 1–5 days at −70 °C.

Preparation and screening of cDNA libraries

The preparation and screening of the λZAPII cDNA library made from poly(A)$^+$ RNA isolated from *C. reinhardtii* vegetative cells was described in Waffenschmidt *et al.* [53]. The VSP-3 gene presented here corresponds to the VSP-3 group of cDNAs isolated by Waffenschmidt *et al.* [53].

A λgt11 cDNA library was custom-made by Clontech (Palo Alto, CA) using poly(A)$^+$ RNA isolated from synchronous vegetative cultures of *C. eugametos* (UTEX 10). This library was both oligo dT and random primed, and contained 2×10^6 recombinants. Immunological screening of this library was performed as described in a Clontech protocol. Nitrocellulose filter (Sartorius, Goettingen, Germany) lifts were blocked in 3% bovine serum albumin, 20% horse serum, 0.05% Tween-20, PBS for 16 h at 20 °C, and then incubated in a 1:500 dilution of affinity-purified α-$(SerPro)_{10}$ in blocking buffer for 1.5 h. Immunopositive signals were detected using the peroxidase-Vectastain anti-rabbit IgG (Vector Laboratories). The peroxidase color was developed by incubating the filters in 50 mM sodium acetate buffer, pH 4.5, containing 0.5 mM 3-amino-9-ethylcarbazole and 0.03% hydrogen peroxide. After color development, the filters were washed in 50% ethanol. All positive plaques were picked and subjected to two additional rounds of immunoscreening. DNA inserts from all selected λgt11 phage were subcloned into pUC 18 for DNA sequence analysis.

Following the manufacturer's instructions, a λZAPII cDNA library was made from poly(A)$^+$ RNA isolated from synchronous cultures of vegetative UTEX 10 at the end of cell division when 50% of the spores had germinated. This library contained 3×10^6 recombinants, and was screened with DNA probes according to standard procedures [39]. Duplicate plaque lifts were made on nylon filters (Hybond N, Amersham). The final wash of the filters after hybridization was in 1 × SSC, 0.1% SDS at 65 °C for 30 min. The pBlueScript SK− phagemid carrying the cDNA insert was excised from all selected phage following the Stratagene procedure.

DNA sequencing and analysis

Subclones for sequencing were generated by either the unidirectional deletion method [19] or the Erase-a-Base kit (Promega). All clones were sequenced by the dideoxy chain termination method [42] using the Sequenase kit (US Biochemical) or T7 DNA polymerase (Pharmacia). 7-deaza-dGTP or dITP was used to sequence G/C-rich regions of DNA. DNA sequences were assembled and analyzed using either the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin, Madison) for VAX/VMS computers or the DNA Strider 1.1 program.

RNA gel blots

RNA from different life cycle stages in *C. eugametos* was isolated as described above. Total RNA from the different life cycle stages in *C. reinhardtii* was prepared by the method of Kirk and Kirk [27]. The RNA samples for the *C. reinhardtii* vegetative wall regeneration time course were isolated as described in Waffenschmidt *et al.* [53]. RNA samples for the *C. eugametos* flagellar regeneration time course were isolated at various times following detachment of flagella from UTEX 10 gametes by pH shock [55]. RNA samples were either glyoxylated prior to electrophoresis or electrophoresed in formaldehyde gels [39]. 10 μg of total RNA or 1 μg of poly(A)$^+$ RNA was loaded on each lane. Transfer to nitrocellulose or Hybond N, hybridization and washes were done as described in Sambrook *et al.* [39].

Genomic DNA gel blots

Genomic DNA from *C. eugametos*, *C. moewusii*, and *C. yapensis* was isolated from 5×10^8 cells grown in liquid cultures. The cells were pelleted and resuspended in 500 μl of extraction buffer (20 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.1% (w/v) SDS, 0.1% (v/v) Triton X-100, 15 mM DTT, 100 μg/ml proteinase K). 0.5 g of glass beads (500–900 μm diameter) were added and the mixture was vortexed 5 min and incubated at 45 °C for 20 min. 60 μl of 3M sodium acetate and 500 μl of Tris-equilibrated phenol was added. The mixture was again vortexed briefly and spun for 5 min at 13000 rpm in a microcentrifuge. The aqueous phase was recovered and reextracted with phenol/chloroform. This aqueous phase was ethanol precipitated, the pellet was washed twice with 70% ethanol and lyophilized. The pellet was resuspended in 100 μl of deionized water and treated with RNAse A (20 μg/ml) for 30 min at 37 °C. Genomic DNA from *C. reinhardtii* was isolated as described in Ferris and Goodenough [10]. Genomic DNA from *C. monoica* and from *V. carteri* were gifts from Patrick Ferris and David Kirk (Washington University, St. Louis) respectively. Southern blots, hybridizations and washes were done according to standard procedures [39]. ^{32}P-labelled probes were prepared by the random-primed oligolabelling method [9].

Results

Stage-specific immunoreactive polypeptides

To determine whether *C. eugametos* wall proteins contain $(SerPro)_x$ epitopes, affinity-purified antibodies generated against a $(SerPro)_{10}$ oligopeptide (α-$(SerPro)_{10}$), [57]) were used to immunoprecipitate the *in vitro* translation products of poly(A)$^+$ RNA samples from three different stages of the life cycle in which (based on prior studies) cell wall synthesis was expected to be proceeding at a high level. These stages were: (a) young vegetative cells, harvested at the end of the dark period, when they were escaping from the mother cell wall and producing their own walls [30]; (b) young gametes of both mating types that were agglutinating in the presence of 5 mM cysteine (which prevents fusion and zygote formation [41]); and (c) 22-h-old zygotes, in which the gametic wall had been completely replaced by a zygotic wall [32, 49]. In Fig. 1, lanes 2–4 show the total translation products from each RNA sample and lanes 6–8 show the corresponding immunoprecipitated polypeptides. Also shown is a parallel control experiment (Fig. 1, lanes 1 and 5) using total RNA from 1-h-old *C. reinhardtii* zygotes, in which $(SerPro)_x$-rich polypeptides have previously been described [56, 57]. As expected, the 66 kDa Class VI protein [56] is the dominant signal observed in the immunoprecipitate.

Among the *in vitro* translation products of RNA from young *C. eugametos* vegetative cells, four immunoprecipitable polypeptides, of 85, 63, 55 and 39 kDa, were detected (Fig. 1, lanes 2 and 6). The *in vitro* translation products of gametes (Fig. 1, lanes 3 and 7) also contained four major immunoprecipitable polypeptides; two were similar in size to those in vegetative cells (85 and

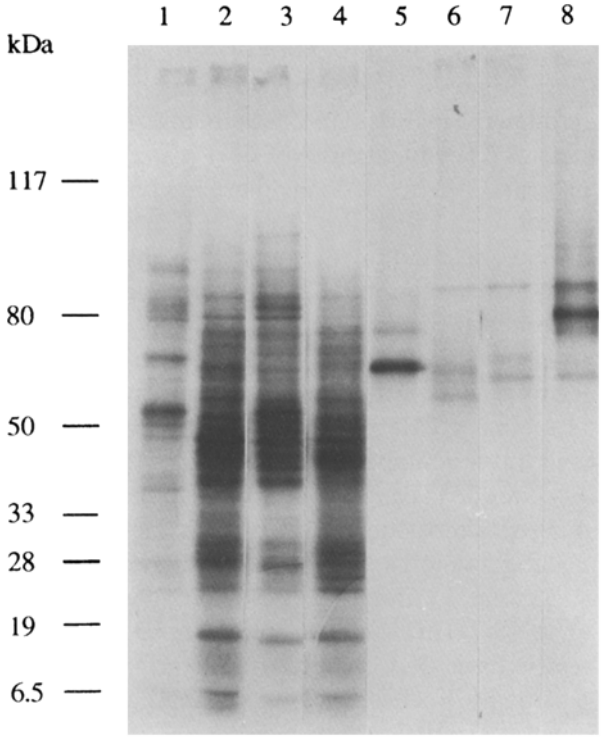

Fig. 1. Immunoprecipitation with α-$(SerPro)_{10}$ from *in vitro* translations of RNA. The total translation products from *in vitro* rabbit reticulocyte translations of each RNA sample are shown in lanes 1–4, and the corresponding α-$(SerPro)_{10}$ immunoprecipitable products are shown in lanes 5–8. Lanes 1 and 5 derive from *C. reinhardtii* zygote RNA (R); lanes 2 and 6 are from *C. eugametos* vegetative RNA (V); lanes 3 and 7 are from *C. eugametos* gamete RNA (G); lanes 4 and 8 are from *C. eugametos* zygote RNA (Z). Positions of the molecular weight standards are indicated at the left.

39 kDa) and two were different (66 and 60 kDa). The *in vitro* translation products of RNA from *C. eugametos* zygotes contained at least six major immunoprecipitable polypeptides, with apparent sizes of 130, 100, 85, 76, 71 and 61 kDa (Fig. 1, lanes 4 and 8). Thus, like *C. reinhardtii*, *C. eugametos* appears to produce different sets of α-$(SerPro)_{10}$-precipitable polypeptides at different stages of the life cycle. Polypeptides of 63 and 55 kDa appear to be vegetative-cell specific, that of 65 kDa appears to be gamete-specific, and those of 130, 100, 76, and 71 kDa appear to be zygote-specific.

cDNA cloning

With evidence that $(SerPro)_x$ epitopes are present in *C. eugametos* proteins, our next goal was to isolate the genes coding for some of these potential wall proteins. We screened 1.5×10^5 recombinants from a λgt11 cDNA library made from RNA of asynchronous *C. eugametos* vegetative cells with α-$(SerPro)_{10}$, selected several positive plaques, and then purified, subcloned and sequenced the one with the strongest signal. This cDNA was 0.43 kb, encoded several stretches of $(SerPro)_x$ repeats and was used as a probe to select a longer (0.72 kb) cDNA from 8×10^5 recombinants of the same library. DNA sequencing and 5′-end PCR analysis [20] revealed that this clone was still not full-length. Moreover, both the 0.43 and 0.72 kb cDNAs were found to have been derived from random priming events and thus lacked a poly(A) tail. We therefore constructed an oligo-dT primed λZAPII library using poly(A)$^+$ RNA from synchronous *C. eugametos* vegetative cells at the end of the dark period (the sample used in the experiment depicted in Fig. 1). When this library was screened with the 0.72 kb cDNA, we obtained 9 positive plaques among 8×10^4 recombinants. One of these clones contained a 1.755 kb insert, and 5′-end PCR experiments on randomly primed first-strand cDNA from the same RNA used to make this library demonstrated that this cDNA was close to full-length (data not shown).

Figure 2 presents the complete DNA sequence for this cDNA, which we have named WP6. The first ATG in the sequence starts an open reading frame of 1053 bp. There is a 129 bp 5′-untranslated region and a 561 bp 3′-untranslated region including the putative polyadenylation signal (TGTAA) 18 bp upstream of the poly(A) tail. The open reading fame could encode a protein of 351 amino acids with a molecular mass of 35.3 kDa. A hydrophobic signal sequence of 31 amino acids, with a putative cleavage site for signal peptidase, was delineated using the guidelines of von Heijne [52]. Thus, the molecular mass of the mature WP6 polypeptide is predicted to be 32.2 kDa. Presumably this corresponds to the 39 kDa

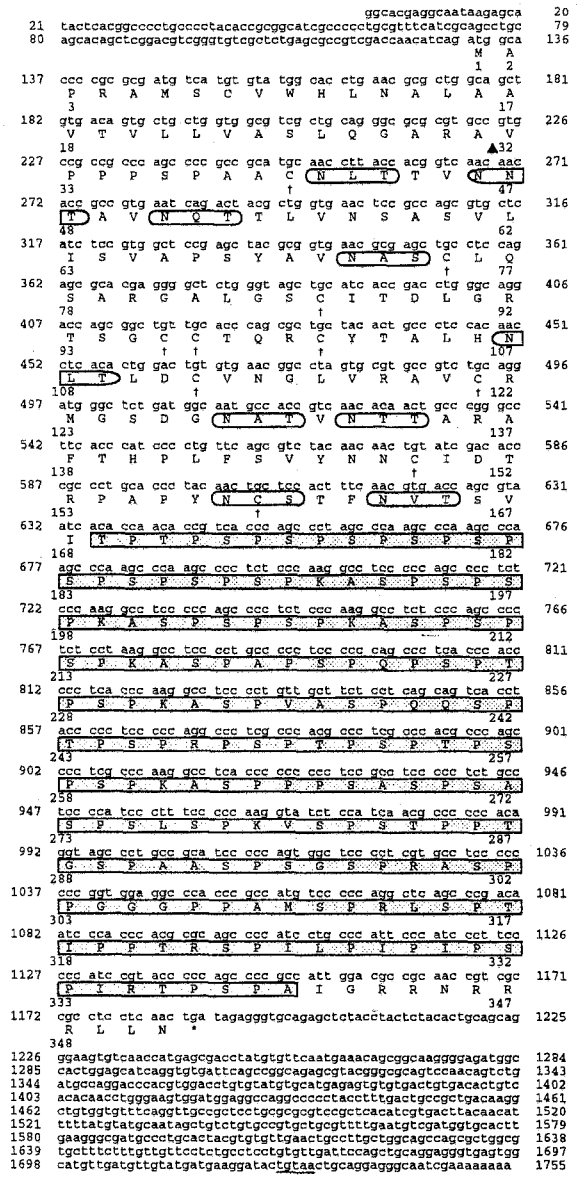

Fig. 2. Nucleotide and derived amino acid sequence for a WP6 cDNA. The sequence of the noncoding DNA strand is presented. The open reading frame is arranged in triplets with the corresponding amino acids shown below. The numbering of the DNA sequence starts at the 5′ end of the cDNA, while numbering below the sequence indicates amino acid position. The cleavage site for the putative signal sequence is marked by an arrowhead and a putative polyadenylation signal is underlined. Potential sites for N-linked glycosylation are circled, cysteines are marked with a †, and the $(SerPro)_x$-rich domain is boxed and shaded.

polypeptide that is immunoprecipitated from the *in vitro* translations of gametic and vegetative RNA (Fig. 1).

The WP6 sequence displays an abundance of serines (19%) and prolines (23%) clustered in a 172 amino acid repetitive domain at the C-terminus. This domain contains many $(SerPro)_x$ repeats resembling those of *C. reinhardtii* cell wall proteins, and occasionally these are part of larger repeat units of $(SerPro)_3LysAla$. If these prolines are post-translationally modified to hydroxyproline, as is common for the volvocalean cell wall proteins, then this domain has many potential sites for O-linked glycosylation. An arginine-rich 9 amino acid unit at the C-terminus is reminiscent of the basic C-terminal peptide found in the cell wall 'inversion-specific glycoprotein' (ISG) from *V. carteri* [8]. The 137 amino acid N-terminal domain is almost devoid of proline, lacks any repeating aa motifs, and contains 10 cysteines and 9 potential sites for N-linked glycosylation (AsnXSer/Thr). Overall, the deduced WP6 protein is basic (net charge +20) like the dicot extensins [46]. Thus, WP6 has many hallmarks of a cell wall HRGP.

The WP6 cDNA of *C. eugametos* is clearly related to the VSP-3 cDNA of *C. reinhardtii*, obtained by α-$(SerPro)_{10}$ immunoscreening of an expression library made with vegetative RNA [53]. The DNA sequence of VSP-3 is presented in Fig. 3. The first ATG begins an open reading frame of 1419 bp. There is a 123 bp 5′-untranslated region and a 470 bp 3′-untranslated region that includes a putative polyadenylation signal (TGTAA) 14 bp upstream of the poly(A) tail. The open reading frame could encode a protein of 473 amino acids with a predicted molecular mass of 47.5 kDa. A signal sequence of 32 amino acids, identified following the guidelines of von Heijne [52], would yield a molecular mass of 44.2 kDa for the mature VSP-3 polypeptide.

VSP-3, like WP6, has an abundance of serines (22%) and prolines (22%) found almost exclusively in a 203 amino acid C-terminal domain. The serines and prolines are arranged in repeating units of $(SerPro)_x$ and in several cases these are part of a larger $(SerPro)_3LysAla$ repeat. There are also several $X(Pro)_{3-4}$ repeats in this domain, repeats which have also been found in zygote and other vegetative cell wall proteins [53, 56]. Once

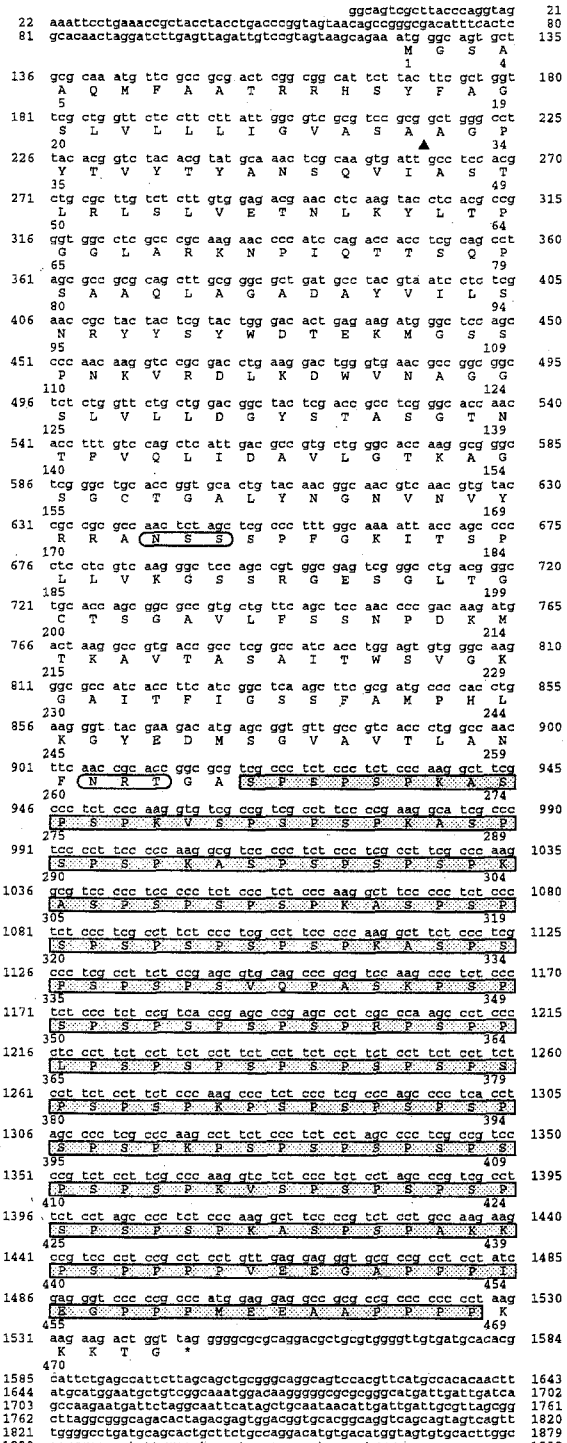

Fig. 3. Nucleotide and derived amino acid sequence for a VSP-3 cDNA. The sequence of the noncoding DNA strand is shown. Numbering for the DNA sequence starts at the 5′ end of the cDNA, while amino acids are numbered below the sequence. An arrowhead marks the cleavage site for the putative signal sequence, and a putative polyadenylation signal is underlined. Potential sites for N-linked glycosylation are circled and the $(SerPro)_x$-rich domain is boxed and shaded.

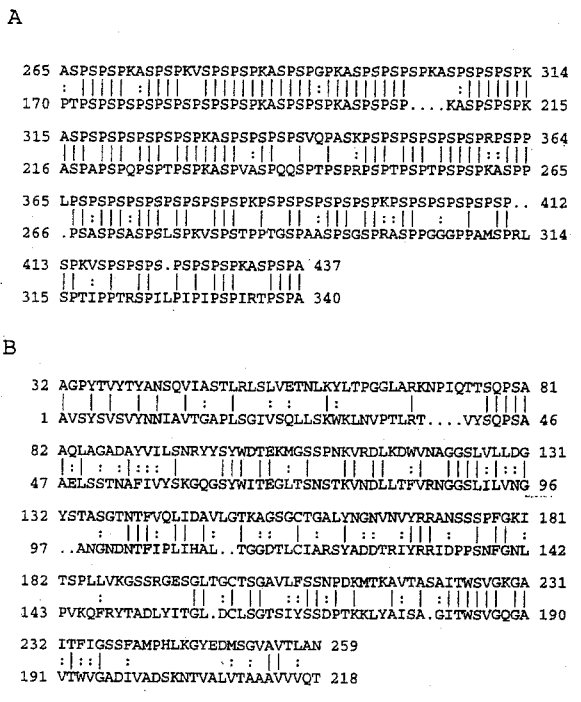

Fig. 4. Comparison of derived amino acid sequences in N- and C-terminal domains. A. The C-terminal domain of VSP-3 (top line) is aligned with the C-terminal domain of WP6 (bottom line). Numbering corresponds to the amino acid position as presented in Figs. 2 and 3. Vertical lines denote exact matches while colons indicate conservative changes. B. The N-terminal domain of VSP-3 (top line) is aligned with the N-terminal domain of ISG (bottom line). Numbering corresponds to the amino acid position as in Fig. 3 or as in Ertl *et al.* [8]. Matches are depicted as described above.

again, these prolines are probably modified to hydroxyproline and serve, along with the serines, as sites for O-linked glycosylation on the mature VSP-3 protein. The 234 amino acid N-terminal domain has a low abundance of proline (3%), no repeating aa motifs, and two potential sites for N-linked glycosylation (AsnXSer/Thr). This protein is a basic molecule with a net charge (+21) quite similar to that of WP6.

The presence of similar repeating units of $(Ser\text{-}Pro)_x$ in the C-terminal domains of both WP6 and

VSP-3 is demonstrated in Fig. 4A where the aa sequences are aligned. These two domains show 66% identity and 75% similarity. When either domain is subjected to a homology search of the peptide sequence databases at the National Center for Biotechnology Information using the BLASTP [2] network service, many matches are found, especially to the higher plant extensins and *C. reinhardtii* cell wall proteins (data not shown). The N-terminal domains of VSP-3 and WP6 show no strong similarities to each other, and a homology search of the databases using the 137 amino acid N-terminal WP6 domain revealed no significant matches. The N-terminal domain of VSP-3 does, however, have a good match to the N-terminal domain of the cell wall ISG of *V. carteri* (Fig. 4B). Here there is 33% identity (52% similarity) and the alignment of the two sequences is good over the entire length of the domain.

Genomic analysis

Although the derived aa-sequence comparison indicates that the VSP-3 and WP6 genes are related, the codons employed are quite different, even in regions of highest homology (Figs. 2 and 3). The DNA gel blots shown in Fig. 5 demonstrate that both genes are species-specific and single copy. The WP6 probe recognizes homologs in interfertile *C. eugametos* and *C. moewusii* strains (Fig. 5A, CE and CM lanes), but does not hybridize to any bands in the incompatible *C. yapensis* (Fig. 5A, CY lanes) and *C. monoica*

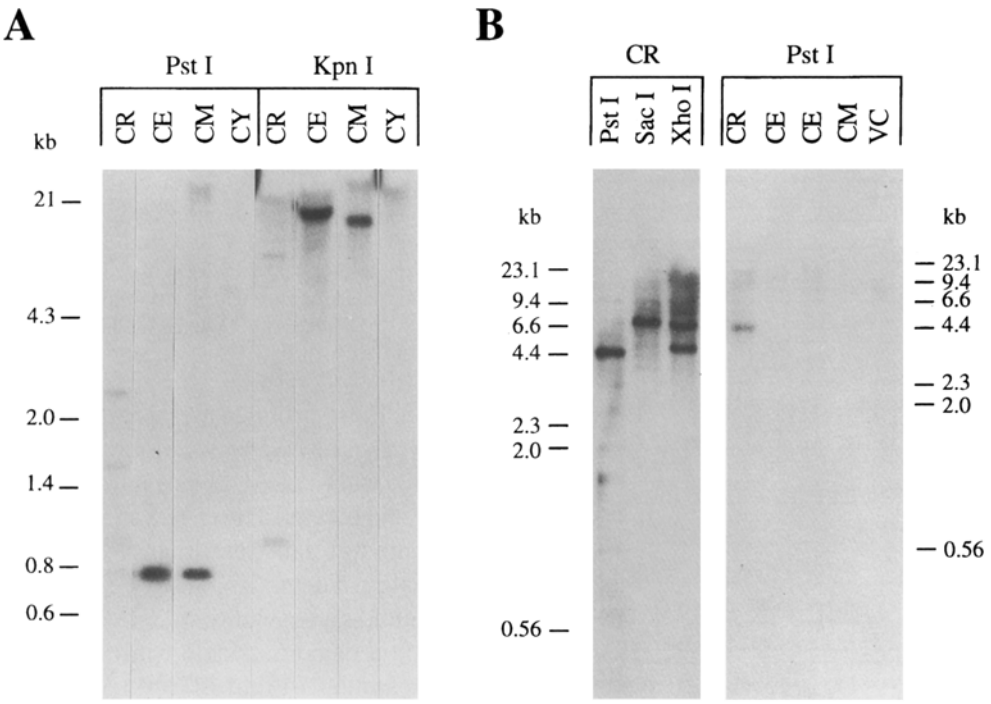

Fig. 5. Genomic DNA gel blots probed with WP6 or VSP-3. A. Genomic DNA from four *Chlamydomonas* species was digested with either *Pst* I or *Kpn* I and probed with radiolabelled WP6 cDNA. CR, *C. reinhardtii*; CE, *C. eugametos*; CM, *C. moewusii*; CY, *C. yapensis*. Hybridization was at 65 °C and washes were at 65 °C with 0.1 × SSC. Size markers are indicated at the left. B. Two DNA gel blots are presented. The blot on the left has genomic DNA from *C. reinhardtii* (CR) digested with *Pst* I, *Sac* I or *Xho* I and probed with radioactively labelled VSP-3 cDNA. Size markers for this blot are shown on the left. The blot on the right shows *Pst* I-digested DNA from *C. reinhardtii* (CR), *C. eugametos* UTEX 9 (CE, second lane) and UTEX 10 (CE, third lane), *C. monoica* (CM), and *V. carteri* (VC) probed with radiolabelled VSP-3 cDNA. Hybridization was at 65 °C and washes were at 65 °C with 0.1 × SSC. Size markers are presented on the right.

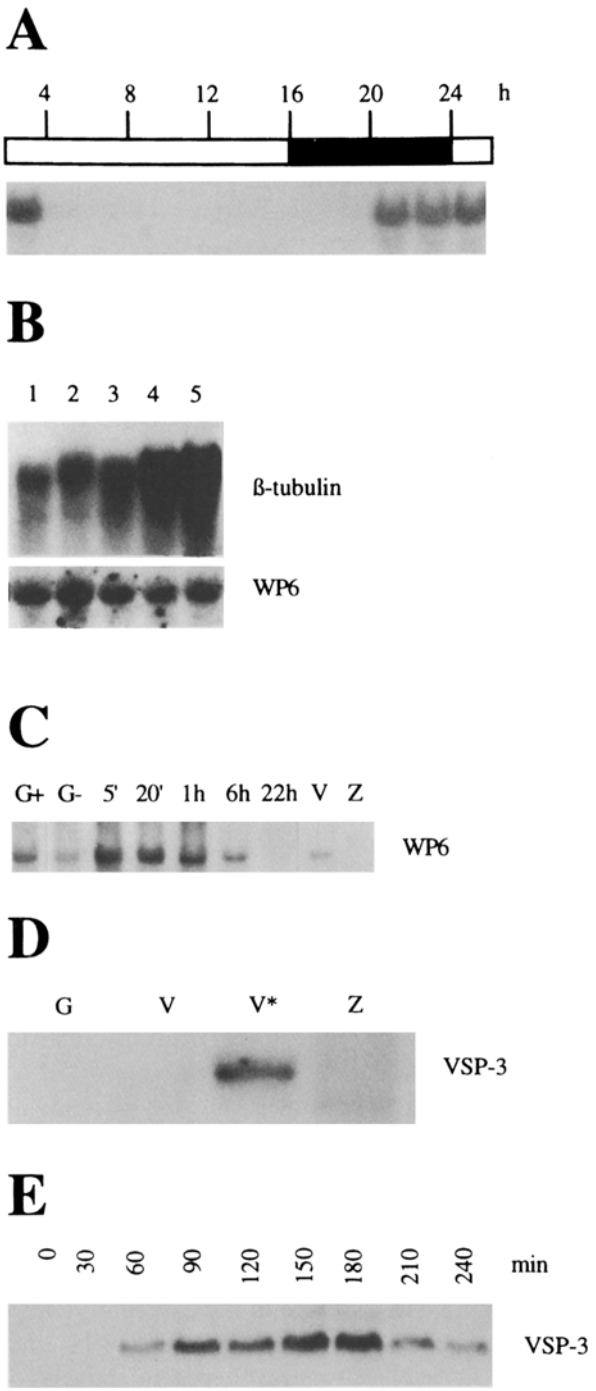

Fig. 6. RNA gel blots probed with WP6 and VSP-3. A. Total RNA samples isolated every two hours from a light/dark synchronized culture of vegetative *C. eugametos* probed with radiolabelled WP6 cDNA. Light and dark periods are indicated by the white and black time bar shown above the blot. B. Total RNA isolated at different times during pH shock induced flagellar regeneration in *C. eugametos* probed with radiolabelled clone of β-tubulin from *C. reinhardtii* (top) or WP6 cDNA (bottom). Lane 1 is an RNA sample isolated prior to pH shock; lane 2 is from 122 min after the shock; lane 3, 19 min; lane 4, 36 min; lane 5, 64 min. C. Samples of total RNA extracted at various times during agglutination and zygote formation in *C. eugametos* probed with radiolabelled WP6 cDNA. Time points after gamete mating are indicated above. G+ is UTEX 9 gamete RNA and G− is UTEX 10 gamete RNA. V is poly(A)$^+$ RNA from asynchronous vegetative cells, and Z is poly(A)$^+$ RNA from 22 h zygotes. D. Total RNA samples isolated from several developmental stages of *C. reinhardtii* probed with radioactively labelled VSP-3 cDNA. G, gamete RNA; V, asynchronous vegetative RNA; V*, GLE-treated vegetative RNA (same as the 60 min lane in Fig. 6E); Z, 1 h zygote RNA. E. Vegetative *C. reinhardtii* cells were treated with GLE to remove their cell walls and then resuspended in fresh media lacking GLE (time 0) to allow V wall regeneration. Total RNA samples prepared every 30 min were probed with radiolabelled VSP-3 cDNA.

(data not shown) strains. The faint signals in *C. reinhardtii* (Fig. 5A, CR lanes) when probed with the WP6 cDNA are due to a presumably non-specific hybridization to chloroplast DNA. The VSP-3 probe hybridizes only to *C. reinhardtii* DNA (Fig. 5B, CR lanes), and not to DNA from *C. eugametos*, *C. monoica*, or *V. carteri* (Fig. 5B, CE, CM, and VC lanes).

Gene expression studies

Studies of gene expression support the proposal that both WP6 and VSP-3 encode wall proteins. Figure 6A displays an RNA gel blot with RNA isolated at 2 h intervals from a light/dark-synchronized culture of vegetative *C. eugametos* that has been probed with a WP6 cDNA. Peak levels of WP6 mRNA abundance correspond to the phase of the cell cycle after mitosis when new walls are being elaborated by the daughter cells. Although these results suggest that WP6 is a structural component of the vegetative cell wall, new flagella are also being generated at this time of the cell cycle, and several flagellar HRGPs have been identified [6, 40]. To test whether WP6 is instead a flagellar protein, vegetative cells were subjected to pH shock, a procedure that removes flagella, initiates flagellar regeneration,

and induces many genes for flagellar components, including the tubulins [29, 43]. Figure 6B presents the autoradiogram from a blot of RNA samples isolated at various times after pH shock, probed with a β-tubulin cDNA and a WP6 cDNA. As controls, lane 1 shows the abundance of each message prior to pH shock, and lane 2 shows the level 122 min afterward when flagellar regeneration is complete. Lanes 3, 4 and 5 are 19, 36 and 64 min after pH shock, respectively. While β-tubulin mRNA clearly increases during flagellar regeneration, WP6 mRNA does not. Thus, WP6 does not appear to be a flagellar component.

We next asked whether WP6 is vegetative-cell-specific or if it is found in the zygote as well. The zygote wall of *C. eugametos* is produced within the gametic cell walls, and while the onset of its production has not been well-defined, it probably occurs 6–8 h after sexual agglutination [32, 33]. Figure 6C shows a blot of RNA samples from different stages of the *C. eugametos* life cycle probed with the WP6 cDNA. There is a strong signal in poly(A)$^+$ RNA from asynchronous vegetative cells (lane V), from agglutinating gametes, and from early stages of zygote formation (5 min, 20 min and 1 h). The level of WP6 mRNA then drops (6 h) and the signal is absent at 22 h in both total and poly(A)$^+$ RNA (lane Z) samples, when the cell is still actively producing a zygote wall. Therefore, WP6 is probably not a zygote wall component.

The analysis of changes in VSP-3 mRNA levels during the *C. reinhardtii* life cycle is more straightforward because there is no wall synthesis in the gametes and zygote wall synthesis and assembly occurs in the absence of any vegetative/gametic wall. RNA samples from various stages of the *C. reinhardtii* life cycle were electrophoresed, blotted and probed with the VSP-3 cDNA. The resulting autoradiogram is shown in Fig. 6D. No VSP-3 mRNA is detected in zygotes. In gametes and asynchronous vegetative cells, levels of wall-specific transcripts are extremely low: gamete lytic enzyme (GLE) treatment of vegetative cells causes an induction of wall-specific mRNAs [1, 48]. Figure 6E shows a time-course analysis of VSP-3 mRNA levels in vegetative cells recovering from GLE treatment. VSP-3 mRNA levels are low in vegetative cells (as in Fig. 6D), increase for the next 2 h, and then decline as wall regeneration is completed, a pattern very similar to that seen for another vegetative wall protein, VSP-1 [53]. So, like WP6, VSP-3 is a vegetative-specific wall protein.

Discussion

In their proposal for a single, but diverse, HRGP superfamily, Kieliszewski and Lamport [25] described some examples of small functional peptide domains that are frequently found as repeating motifs, often within the context of larger repeat units. These include: (X)HypHyp(Hyp)$_n$ and ProProValTyrLys, which impart rigidity and an extended conformation to the molecule; ValTyrLys, TyrLysTyrLys and TyrTyrTyrLys, which are potential sites for inter- and intramolecular isodityrosine crosslinking; and finally, hydroxyproline, serine and threonine, all hydroxyamino acids that can serve as sites for O-linked glycosylation.

The VSP-3 and WP6 genes from *C. reinhardtii* and *C. eugametos* both have some X(Pro)$_{2-4}$ units that are a variation of the (X)HypHyp(Hyp)$_n$ motif. But we show here that a prominent *Chlamydomonas* repeat unit is (SerPro)$_x$. A SerPro unit is present in the Ser(Pro)$_4$SerPro-Ser(Pro)$_4$ block in some tomato, petunia and bean extensins [46]. There are also a number of XPro doublets found in the flower cell wall proteins of tobacco [59]. Since serine and alanine codons differ only at the first nucleotide position, the (SerPro)$_x$ repeats in *Chlamydomonas* might be evolutionary precursors of the (AlaHyp)$_x$ repeats that are found in the higher plant arabinogalactan proteins [13, 14] and that have recently been found in a cell wall extensin from maize [24] and Douglas fir [11]. There is no potential isodityrosine motif in either WP6 or VSP-3; in fact, no tyrosines at all are found within the repetitive domains. The abundance of serine, threonine and (potential) hydroxyproline residues suggests that the repetitive domains are highly glycosylated in both molecules.

An invariant feature of all of the volvocalean cell wall proteins that have been sequenced to date is their subdivision into distinct domains: domains that are repetitive and Pro-rich, and domains that lack repeating motifs, have few prolines, and frequently are Cys-rich [7, 8, 53, 56, present study]. EM analysis of vegetative wall HRGPs from *C. reinhardtii* [16, 37], *C. eugametos* [18], and *V. carteri* [7, 8, 17] reveals these molecules to be rods of various lengths with knobs along the shaft and/or at one terminus. The higher plant extensins lack these knobs and appear in the EM as simple rods [47, 50]. Correspondingly, the genes for higher plant extensins code for only repetitive proline-rich domains. Parallels to the multidomain structure of volvocalean wall proteins have, however, been reported in several interesting cases.

1. The potato tuber lectin [26] consists of two protein domains: a glycine- and cysteine-rich, chitin-binding domain that is homologous to hevein lectins, and a serine- and hydroxyproline-rich domain that is homologous to the extensins. Although the N-terminal domain of WP6 is cysteine-rich, neither it nor the N-terminal domain of VSP-3 is detectably homologous to any of the known lectins.

2. A family of cysteine-rich extensin-like proteins (CELPs) has been isolated from tobacco flower cell walls [59]. These CELPs are found almost exclusively in floral organs and have two distinct structural domains: an extensin-like domain with numerous $(XPro)_x$ and $X(Pro)_{3-7}$ repeats, and a cysteine-rich domain that does not appear to be lectin-like.

3. A class of pollen-specific, extensin-like proteins has been isolated from maize which also displays two domains: a C-terminal putative rod domain with $Ser(Pro)_4$ repeats, and a N-terminal putative globular domain that is leucine-rich and proline-poor (P. Bedinger, Colorado State University, pers. comm.). Perhaps the structural plan of rod and knob found in *Chlamydomonas* HRGPs was conserved for specific multifunctional roles in the sexual tissue proteins of the vascular plants.

Although we do not have direct proof of a correspondence between aa sequence domains and protein secondary structure domains for WP6 and VSP-3 since we have not yet isolated the proteins, there is evidence from another *C. reinhardtii* vegetative cell wall protein, GP1, that $(SerPro)_x$ repeats can yield a rod-like protein. GP1 is an HRGP component of the outer wall layer that has been solubilized, purified and shown by EM to be a long cane with a globular knob on one end [16]. DNA sequencing of the GP1 gene reveals that the open reading frame codes for a long N-terminal domain of $(SerPro)_x$ repeats with a C-terminal domain lacking any repeated motifs or abundant prolines [1, Woessner and Goodenough, unpubl.]. Furthermore, a recent review on proline-rich regions in proteins from many different taxa, in addition to plants, states that $(XPro)_x$ sequences adopt an extended helical structure and provide binding sites for other proteins leading to the creation of large networks [54]. The *V. carteri* ISG that has been purified and subjected to EM and molecular analysis [8] also has two regions: a terminal knob, which corresponds to an N-terminal domain lacking numerous prolines or repeated units, and a rod coded for by a C-terminal domain dominated by hydroxyamino acids and repeating motifs of $Ser(Pro)_{3-7}$.

Neither ISG nor SSG, another cell wall protein characterized in *V. carteri* [7], have any $(SerPro)_x$ repeats, but our data make it seem likely that $(SerPro)_x$ -containing proteins will be found when *Volvox* cDNA expression libraries are screened with α-$(SerPro)_{10}$. Thus, it appears that several groups of cell wall HRGPs have arisen within the volvocalean lineage.

Figure 7 diagrams the comparisons among the three volvocalean cell wall proteins: WP6, VSP-3 and ISG. While the C-terminal rod domain of ISG shows no homology to the putative rod domains of WP6 and VSP-3, the N-terminal globular domain of ISG is homologous to the N-terminal domain of VSP-3. Therefore, we propose that the N-terminal portion of VSP-3 will also be globular. Even though the N-terminal domain of WP6 is not homologous to these other N-terminal domains, it has an abundance of cysteines and potential N-linked glycosylation sites,

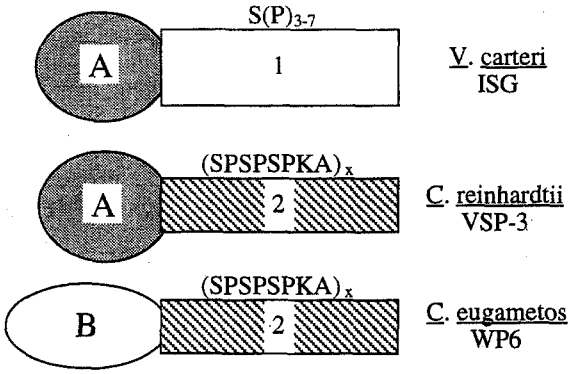

Fig. 7. Domain conservation in volvocalean cell wall proteins. Each protein is represented in cartoon fashion with a globular N-terminal domain and a rod-like C-terminal domain. Homologous N- and C-terminal domains are shaded and the characteristic amino acid repeat units are presented for each protein.

and a paucity of prolines and repeating amino acid units, leading us to propose that it also codes for a globular structure. It appears that VSP-3 and WP6 have a rod coded for by homologous C-terminal domains, while VSP-3 and ISG have a knob coded for by homologous N-terminal domains.

If it clear from morphological, biochemical and cladistic analyses that the genus *Chlamydomonas* is not monophyletic [reviewed in 58]. Several phylogenetic trees [3, 4, 28, 35] show that *C. reinhardtii* is more closely allied with the colonial and multicellular Volvocales (e.g. *V. carteri*) than with *C. eugametos* (a separation of 350 million years). Comparing *C. reinhardtii* to *C. eugametos* is equivalent (in terms of evolutionary distance) to comparing species of *Equisetum* and *Nicotiana* among the vascular plants [23]. Thus, our results suggest domain conservation over 350 million years of cell wall protein evolution in the Volvocales.

Homologous recombination and unequal crossing over have been proposed and examined as possible mechanisms for the generation of the repeated sequences that code for the conserved rod domain in higher plant extensins [15, 36, 45, 59]. In addition, both the CELPs and the volvocalean wall proteins discussed here provide evidence for exon shuffling, whereby functional domains of various proteins are recombined to produce proteins with novel properties. Such non-homologous rearrangements between genes have been postulated to occur within introns flanking conserved protein domains [12]. The strongest evidence for exon shuffling has come from studies of extracellular and cell surface proteins, specifically in the vertebrate immunoglobulin superfamily [34]; in contrast, the data for exon shuffling in intracellular proteins is not convincing [38].

In a recent review, Schmitt *et al.* [44] found many examples of intron additions when comparing various volvocalean genes encoding the same intracellular component, and they concluded that there was no obvious proof for exon shuffling in any intracellular protein. Here we can compare three different extracellular proteins from the Volvocales. The gene for ISG is interrupted by two introns: one lies between aa 4 and 5 of the mature polypeptide and the other falls between amino acids 207 and 208 [8]. This results in 3 exons: one includes the signal sequence and 4 additional amino acids, the second contains all but 16–17 amino acids of the globular domain, and the third carries the rod domain. DNA sequencing of a VSP-3 genomic clone revealed three introns: the first falls within amino acid 4 of the mature polypeptide; the second lies between amino acids 29 and 30; and the third is positioned within amino acid 69 (data not shown). Therefore, as in ISG, the first exon of VSP-3 contains the signal sequence and 3 additional amino acids, and in both genes the intron occurs within or next to a conserved tyrosine. However, neither of the other two introns in VSP-3 falls in a similar position to the second intron in ISG, suggesting that these represent inserted introns or that the second intron in ISG was lost in VSP-3. The number of introns in WP6 has not been determined, but the regions of the gene corresponding to the beginning of the mature polypeptide and to the start of the rod domain have been sequenced in a WP6 genomic clone (data not shown), and no intron was found at either of these locations.

It is not easy to recognize ancestral versus re-

cently inserted introns [5], and while the exact mechanisms are not readily deduced, it does appear that some type of exon shuffling has taken place during the evolution of the volvocalean cell wall HRGPs, leading to novel rod/knob pairings, and presumably generating new molecules with different functional roles. With the characterization of more volvocalean cell wall proteins, it may become possible to identify ancestral rod and knob domains and perhaps provide insight into the mechanism by which these domains were brought together to form novel proteins.

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