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Isodityrosine Cross-Linking Mediates Insolubilization of Cell Walls in Chlamydomonas

Sabine Waffenschmidt,[a] Jeffrey P. Woessner,[b,1] Karin Beer,[a] and Ursula W. Goodenough[b]

[a] Institut für Biochemie, Universität zu Köln, Köln, Germany

[b] Department of Biology, Washington University, St. Louis, Missouri 63130-4899

Enzymatic removal of the cell wall induces vegetative *Chlamydomonas reinhardtii* cells to transcribe wall genes and synthesize new hydroxyproline-rich glycoproteins (HRGPs) related to the extensins found in higher plant cell walls. A cDNA expression library made from such induced cells was screened with antibodies to an oligopeptide containing the $(SP)_x$ repetitive domains found in Chlamydomonas wall proteins. One of the selected cDNAs encodes an $(SP)_x$-rich polypeptide that also displays a repeated YGG motif. Ascorbate, a peroxidase inhibitor, and tyrosine derivatives were shown to inhibit insolubilization of both the vegetative and zygotic cell walls of Chlamydomonas, suggesting that oxidative cross-linking of tyrosines is occurring. Moreover, insolubilization of both walls was concomitant with a burst in H_2O_2 production and in extracellular peroxidase activity. Finally, both isodityrosine and dityrosine were found in hydrolysates of the insolubilized vegetative wall layer. We propose that the formation of tyrosine cross-links is essential to Chlamydomonas HRGP insolubilization.

INTRODUCTION

Chlamydomonas has for many decades served as a model system for photosynthesis research (reviewed in Rochaix, 1992). More recently, it has become apparent that studies of the Chlamydomonas cell wall might provide important insights for understanding the role of higher plant cell walls in growth and development. Hydroxyproline-rich glycoproteins (HRGPs; including extensins, solanaceous lectins, and arabinogalactan proteins), glycine-rich proteins, and proline-rich proteins are the major types of cell wall structural proteins in both higher plants and Chlamydomonas (Goodenough et al., 1986; Woessner and Goodenough, 1989, 1992; Showalter, 1993; this study). Tissue print studies of higher plants have established a high degree of cell specificity in the expression of wall protein genes (Ye et al., 1991), and it is widely assumed that these patterns reflect the establishment of such important parameters as cell shape and extensibility. However, it is difficult to study these events in a plant tissue where diverse cell types are expressing diverse genetic programs. Chlamydomonas cells, in contrast, are clonal, and the switch between expression of their two cell wall programs is readily manipulated. Moreover, the presence of an apparently common epitope, $(SP)_x$, on the Chlamydomonas HRGPs has allowed us to clone their corresponding cDNAS and genomic coding sequences (Woessner and Goodenough, 1989, 1992; this study). Finally, cell wall–less mutants are viable and readily obtained (Davis and Plaskitt, 1971; Hyams and Davies, 1972), permitting a genetic dissection of the system.

[1] To whom correspondence should be addressed.

That the Chlamydomonas HRGPS are evolutionary antecedents of higher plant HRGPs is argued by their repeating motifs of Hyp and Ser, their ara-gal glycosylation (Miller et al., 1972), and their fibrous morphology (Goodenough et al., 1986; Woessner and Goodenough, 1992). In addition, both Chlamydomonas and higher plant HRGPs become insolubilized during wall deposition (Roberts et al., 1972; Imam et al., 1985; Showalter, 1993). Our study provides evidence that in Chlamydomonas, insolubilization involves the formation of isodityrosine (IdT) cross-bridges, a mechanism long postulated to drive the insolubilization of higher plant HRGPs (Fry, 1982; Cooper and Varner, 1983). Hence, Chlamydomonas should provide plant researchers with an excellent system to study how such cross-bridges are catalyzed and regulated.

The major structural proteins of primary plant cell walls are commonly insolubilized in muro. Early work showed that extensins can be extracted from mature walls with acidified chlorite, which under the proper conditions cleaves phenolic cross-links (O'Neill and Selvendran, 1980; Biggs and Fry, 1990), and the amount of soluble extensin in mature walls can be increased if free-radical scavengers and/or peroxidase inhibitors (e.g., *n*-propylgallate, potassium cyanide, 3,4,5-trichlorophenol, and L-ascorbic acid) are present during wall formation (Cooper and Varner, 1981, 1983). Fry (1982) proposed a candidate insolubilization mechanism, oxidative cross-linking of tyrosines, after demonstrating the existence of a novel form of tyrosine cross-link, IdT, in extensin hydrolysates. Support for this mechanism includes the observation that when secreted extensin monomers are insolubilized in muro, the process is

accompanied by IdT formation and is inhibited by antioxidants such as ascorbic acid (Cooper and Varner, 1983). Moreover, the amino acid sequences of almost every cell wall structural protein contain repeating motifs of tyrosine residues (reviewed in Showalter and Rumeau, 1990). Enthusiasm for the IdT-insolubilization hypothesis was tempered when Epstein and Lamport (1984) reported that they could find only intramolecular IdT in extensin peptides, whereas matrix insolubilization would require intermolecular IdT. However, this result may simply indicate that extensins carrying intermolecular cross-links are extremely difficult to extract from the wall.

A recent study (Bradley et al., 1992) has implicated tyrosine cross-linking in the local response to wounding or fungal infection in soybean: proline-rich protein insolubilization in the perturbed tissues was shown to be stimulated by H_2O_2 and inhibited by ascorbate. Although the response of plant tissues to local wounding is of major interest, the mechanisms employed may or may not be similar to those utilized to insolubilize wall proteins during plant growth and development. The goal of elucidating the latter mechanisms has been confounded by the complex composition of the plant wall and the diversity of cell types in plant tissues.

Chlamydomonas elaborates two distinct types of cell wall during its life cycle, the vegetative/gametic (V) wall and the zygotic (Z) wall, and each undergoes insolubilization after it is assembled. Both types of walls lack the matrix polysaccharides found in higher plant walls and are instead composed solely of HRGPs (Miller et al., 1974). Moreover, homogeneous cell populations will undergo wall formation in a highly synchronous fashion, permitting direct experimental analysis of the insolubilization mechanism.

The V wall is particularly suited to experimental manipulation. It is composed of two distinct layers—the salt-soluble outer layer called W6 and the SDS/β-mercaptoethanol–insoluble inner layer called W2 (Roberts et al., 1972; Goodenough and Heuser, 1985)—and it can be completely removed from the cell by exposure to gametic lytic enzyme (GLE; Claes, 1971; Schlösser, 1976; Kinoshita et al., 1992), a species-specific enzyme secreted by mating gametes to lyse their walls in preparation for cell fusion. GLE treatment of vegetative cells has two important consequences. First, GLE cleaves the W2 layer into subunits that can be recovered by centrifugation and subjected to biochemical analysis; with transmission electron microscopy, such subunits display the morphology of "fishbones" carrying a central "spine" and radiating "ribs" of 100 and 200 nm (Goodenough and Heuser, 1985). Second, GLE-treated vegetative protoplasts proceed to synthesize and assemble new cell walls within 2 hr (Robinson and Schlösser, 1978). As first shown by Adair and Apt (1990) and Su et al. (1990), this response initiates with the upregulation of wall-specific mRNA synthesis, facilitating the molecular identification of wall-specific genes.

In previous studies, we identified and sequenced two cDNAs encoding Z wall HRGPs that displayed repetitive SP domains ($(SP)_x$), raised an antiserum against a $(SP)_{10}$ oligopeptide (α-$(SP)_{10}$), and showed that this would immunoprecipitate a discrete set of in vitro translation products of mRNA derived from GLE-treated vegetative cells and from young zygotes (Woessner and Goodenough, 1989, 1992). Henceforth, we refer to these polypeptides and their corresponding genes as VSP (vegetative, SP rich) and ZSP (zygotic, SP rich).

In this paper, we present data indicating that oxidative cross-linking is responsible for cell wall insolubilization in Chlamydomonas. We first demonstrate that a cloned VSP, believed to be a component of the insoluble W2 layer, contains a domain of repetitive tyrosine-containing motifs with a consensus YGG unit. We further show that at the time of both V wall and Z wall insolubilization, there is a concomitant burst of H_2O_2 production and peroxidase activity, and that the addition of ascorbate or tyrosine derivatives delays the onset of the insolubilization process. Finally, we document that hydrolysates of W2 fishbones contain both IdT and dityrosine (dT). Thus, the Chlamydomonas system appears amenable to a detailed analysis of an HRGP-insolubilization mechanism that may be utilized throughout the plant kingdom.

RESULTS

VSP cDNA Clones

A cDNA expression library was constructed in λ ZAPII using poly(A)$^+$ mRNA isolated from vegetative cells that had been exposed to GLE for 1 hr, washed, and incubated for an additional hour without GLE. Plaques were screened with α-$(SP)_{10}$, and 16 immunopositive clones were assigned to five groups that did not cross-hybridize, as shown in Table 1. A representative cDNA from each group was used as a probe on RNA gel blots to determine mRNA size and to confirm that the transcript abundance increases in response to GLE treatment (data not shown). This study focuses on one of these groups, which is designated VSP-1.

DNA sequence analysis of a full-length VSP-1 cDNA revealed an open reading frame of 900 bp that could encode a protein of 300 amino acids with a molecular mass of 31 kD, as shown in Figure 1. The guidelines of von Heijne (1985) were used to identify an apparent signal sequence of 28 amino acids that would yield a mature protein with a molecular mass of 28 kD.

Table 1. VSP cDNA Clones

Group	Number of Members	Longest cDNA Insert (kb)	mRNA Length (kb)	mRNA Enhanced after GLE
VSP-1	5	2.1	2.0	Yes
VSP-2	1	1.1	1.9	Yes
VSP-3	6	2.0	2.0	Yes
VSP-4	1	1.9	3.9, 3.2, 2.5	Yes
VSP-5	3	3.1	3.1, 2.8	Yes

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                    cttagaccagccagcttgcctgacccgttggcaagt   36
  37 ggttaccccgcgcgtctcccccctcgcgttggacgtctttggcaatctgcgctaaaaca   95
  96 gcctgcgacaagtatcgtagtggtcagcccgaggagcatactgttacgataaggtacat  154
 155 aaaagaggagagaaattcgccgcggcgcgaggtcataggcgtgtgaacatacggccgtt  213
 214 ctggagctctcaacggtgccataatcagccttggaatatccgtttccgtttggctgaag  272

 273 atg aaa agc tct cat cgg gcc ctg tgg gcc tct gcc gtg ttg gcc  317
      M   K   S   S   H   R   A   L   W   A   S   A   V   L   A
      1                                                      15
 318 atg gcg gtg ctc gcg tgc gtt gtg cca aca gcg gat gct gcg aaa  362
      M   A   V   L   A   C   V   V   P   T   A   D   A   A   K
     16                                                ▲     30
 363 aag tcg ccc cca cct gcg gat gcg cca ccg ccg gac agc ggc cag  407
      K   S   P   P   P   A   D   A   P   P   P   D   S   G   Q
     31                                                      45
 408 tac ccc ccg ccc act gac tgg gtg act tgg ccg ccg ccg gtg gag  452
      Y   P   P   P   T   D   W   V   T   W   P   P   P   V   E
     46                                                      60
 453 gag cac gga cat cac tcg ccc tcg ccc tcg ccg gag gat tcg ccg  497
      E   H   G   H   H   S   P   S   P   S   P   E   D   S   P
     61                                                      75
 498 tcc tca acg ccc atc ccg acg cca gac atc ttg gaa aac cgc gac  542
      S   S   T   P   I   P   T   P   D   I   L   E   N   R   D
     76                                                      90
 543 ctg ccc tct ccc tcg cct gag gcg gaa gac tcg ccg tct cct tcg  587
      L   P   S   P   S   P   E   A   E   D   S   P   S   P   S
     91                                                     105
 588 cct gac ctt gag gac tcg cct tcc cat tct cct gag cct gag gtg  632
      P   D   L   E   D   S   P   S   H   S   P   E   P   E   V
    106                                                     120
 633 gag tcc ccg tcc ccg gag act gag act gag tcc ccg tct ccc tcg  677
      E   S   P   S   P   E   T   E   T   E   S   P   S   P   S
    121                                                     135
 678 ccc gag cct gag gtt gag gac tcc ccc tct ccc tcg cct gag cct  722
      P   E   P   E   V   E   D   S   P   S   P   S   P   E   P
    136                                                     150
 723 gag gtt gag gac tcc ccc tct ccc tcg cct gag ccc atg gac gaa  767
      E   V   E   D   S   P   S   P   S   P   E   P   M   D   E
    151                                                     165
 768 tct cct gcg cca gag ccg tcc ccc gag ccc gtt ccc agc ccc gag  812
      S   P   A   P   E   P   S   P   E   P   V   P   S   P   E
    166                                                     180
 813 acc ccc tcg ccc gct ccg tac tac ggc gcc tcc ccc tcc ccc tcc  857
      T   P   S   P   A   P   Y   Y   G   A   S   P   S   P   S
                              ___________
    181                                                     195
 858 ccc tat tac ggc ggc tac ggc aac tac ggc ccc agc gtg cca acg  902
      P   Y   Y   G   G   Y   G   N   Y   G   P   S   V   P   T
          _______________________________________
    196                                                     210
 903 tac ggc agc agc ccc tct ccc gcg cct tcg ccg tac ggc ggc tac  947
      Y   G   S   S   P   S   P   A   P   S   P   Y   G   G   Y
     _______                                     _______________
    211                                                     225
 948 ggt ggc tac ggc ggt tac ggc ggt tac ggc ggt tac ggg tcg tat  992
      G   G   Y   G   G   Y   G   G   Y   G   G   Y   G   S   Y
     ___________________________________________________________
    226                                                     240
 993 ggc gat gat gat gag gag gag gag gac ccc atg tct gcc gcg cgc 1037
      G   D   D   D   E   E   E   E   D   P   M   S   A   A   R
     ___
    241                                                     255
1038 cgc ctg atg ctg gat gtg cct aac cag ggt gcg ggg cgg cag ctg 1082
      R   L   M   L   D   V   P   N   Q   G   A   G   R   Q   L
    256                                                     270
1083 tct ggt gat gcc gtt gac ttg ttg gcg gga ggg gag gag ccg gag 1127
      S   G   D   A   V   D   L   L   A   G   G   E   E   P   E
    271                                                     285
1128 ctg gag gag cgt tcc act cgc gtc agc cgc cgc ctg ctg cgc atc 1172
      L   E   E   R   S   T   R   V   S   R   R   L   L   R   I
    286                                                     300
1173 tag   tagacccgtgcgccacatgggtagagcagctctccggcgccgtcaaatgcgtt 1228
      *
1229 tgggcgctggaatgcgcactcagacgcgctgcggctcggagctggcggcggcggcgtgc 1287
1288 gtgttgcgtgcgcgccgccggtgggctgtgaggaatgtgtgacagcatacggacaccgt 1346
1347 caggaacagcaaaagccccggcccagggtctgggaaaaggaaaccctctaaaccaggtc 1405
1406 ctgttgtgcggttgtgagacaaggatgaccaacgcaaggatggacacggctgtatggct 1464
1465 agacaggaccggagcgagcaggagtgcttagtgcttaggcaggtgtgtgactcagtctt 1523
1524 ttgtgacgttgcttaactgtagttagtgacgccatgtatggtattacgttcatggacga 1582
1583 ttgattcaaacccgtaaccgtgtgacgttgactaagttcgtgtgcgtgcgcgtgtgcgt 1641
1642 cgcggctgtgactgctattatgatttactgcaaggggccaacagcaggggaaatgggcg 1700
1701 cgcagggcggctttgcggcttttggctagctactatatggcaggattgcgcttgcttga 1759
1760 taacgcgttgattgcacgcgtgaggttggcggctggctagctgtgtgaagattgcgcac 1818
1819 acttgggcggcaatttacagtgctacaagaagaagcaggtagtgaccagggttgtgcag 1877
1878 gtgacttttgggagctgtgctgtcagtgctgttcagggcagcagcgcggctacccgtca 1936
1937 gggcgtggattccctaagtaggacagaaagcaggcaggcctggaataactgcgccaaag 1995
1996 gacagaggcaacctgctccaggtagtgcacgtgaacacacatgtccaaaatgcaattgc 2054
2055 atcaagcctgagctcgcgacccaaagctgtgtaacacatgccgagcaaaaaaaaaaaaa 2113
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Figure 1. Nucleotide and Derived Amino Acid Sequence of a VSP-1 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 within the open reading frame. The cleavage site for the putative signal sequence is marked by an arrowhead, and a putative polyadenylation signal sequence is underlined. The YGG domain in the amino acid sequence is bold underlined. The GenBank accession number for this sequence is L16461.

The sizes of the 5′ (213-bp) and 3′ (923-bp) untranslated regions are typical for nuclear genes in Chlamydomonas. There is a putative polyadenylation signal (TGTAA) 17 bp upstream of the poly(A) tail. The length of the sequenced cDNA (2113 bp) corresponds closely to the size of the VSP-1 mRNA determined by RNA gel blot analysis, as shown in Figure 2.

Total RNA was isolated from vegetative cells at various times after GLE treatment, electrophoresed on an RNA gel, transferred to nitrocellulose, and probed with ^{32}P-labeled VSP-1 cDNA. VSP-1 mRNA levels were extremely low in vegetative cells, increased during the first 2 hr following GLE treatment of the cells, and then declined once wall regeneration was completed, as shown in Figure 2. This pattern of expression, in response to GLE treatment, is a diagnostic feature of a V wall structural protein–encoding sequence (Adair and Apt, 1990; Su et al., 1990).

Further support for the identification of VSP-1 as a V wall gene was found in the derived amino acid sequence that contains the $X(P)_3$ and $(SP)_x$ motifs typically found in Chlamydomonas HRGPs (Woessner and Goodenough, 1989, 1992). The sequence is DE rich (19%) and lacks N, C, F, and Q, distinguishing this acidic (charge = −40) molecule from any of the proteins found in the salt-extractable W6 layer of the V wall (Goodenough et al., 1986). We propose, therefore, that VSP-1 is a component of the insoluble W2 layer.

A particularly distinctive feature of the VSP-1 polypeptide is its tyrosine content (5.5%). The tyrosines are confined to a central domain (bold underlined in Figure 1), which is interrupted by short SP-rich regions, is glycine rich and highly repetitive, and displays the consensus motif YGG. The central domain, hereafter designated the YGG domain, is itself uncharged and is flanked by an acidic (23% D + E content) SP-rich N-terminal region and by an acidic (28% D + E content) SP-free C-terminal region.

H_2O_2 and Peroxidase Elevation during Wall Insolubilization

The presence of the YGG domain in VSP-1, a putative W2 component, suggested that tyrosine cross-linking might contribute to W2 insolubilization. We therefore monitored levels

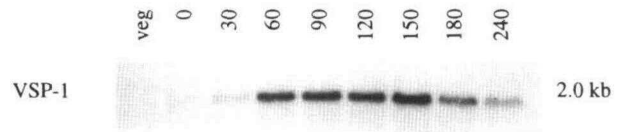

Figure 2. Time Course Analysis of VSP-1 mRNA Abundance.

Vegetative cells (veg) were treated with GLE to remove their walls and then resuspended in fresh media lacking GLE (time 0) to allow V wall regeneration. Ten-microgram samples of total RNA, isolated from aliquots of cells removed every 30 min, were electrophoresed, blotted, and probed with radioactively labeled VSP-1 cDNA. The approximate length of the VSP-1 mRNA is indicated at right in kilobases.

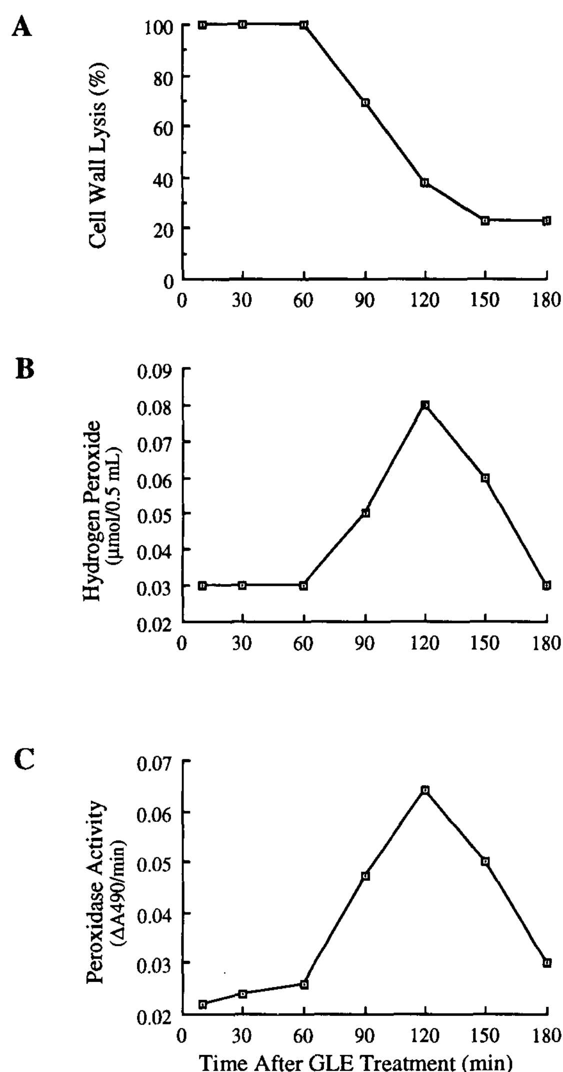

Figure 3. Analysis of V Wall Regeneration.

Vegetative cells were treated with GLE for 1 hr, and the resultant protoplasts were pelleted, washed, and resuspended in fresh media lacking GLE (time 0). Aliquots were taken at 30-min intervals and analyzed as follows.

(A) Detergent sensitivity. Each aliquot was mixed with an equal volume of 0.2% Nonidet P-40 and pelleted in a microcentrifuge. The OD_{440} of the supernatant was read to quantitate chlorophyll release from lysed cells. The highest absorbance reading was designated as 100% cell wall lysis, and the other readings were converted to percentages accordingly. The detergent-insoluble mature wall is re-formed at ~120 min after treatment.

(B) Hydrogen peroxide accumulation. The cells were pelleted from each aliquot, and the supernatant was mixed with an equal volume of 1 M KI. The oxidized I_2 was determined from an OD_{390} reading taken 15 min after mixing at room temperature. Concentrations were determined from a standard calibration curve using known concentrations of hydrogen peroxide.

(C) Peroxidase activity. The cells were pelleted from each aliquot, and the supernatant was mixed with sodium acetate buffer, exogenous H_2O_2, and vanillin (final concentrations were 5 mM sodium acetate, pH 5.5, 2 mM $MnCl_2$, 40 μM H_2O_2, and 900 μM vanillin). The change in the OD_{490}, read immediately after mixing and 5 min later, was used to determine peroxidase activity.

of H_2O_2 and peroxidase activity during the course of V wall regeneration.

Figure 3A shows the normal kinetics of V wall insolubilization in regenerating cells. Cells were fully sensitive to Nonidet P-40 lysis for the first hour, but as wall insolubilization proceeded over the next hour, they lost their sensitivity and became detergent resistant. There are a number of cells that lyse in Nonidet P-40 irrespective of GLE treatment, explaining why the percentage of cell wall lysis does not return to zero.

At each time point when Nonidet P-40 sensitivity was tested, parallel aliquots of the growth medium were monitored for H_2O_2 levels (Figure 3B) and for peroxidase activity (measured in the presence of added H_2O_2) (Figure 3C). Coincident with the onset of detergent resistance, maximal levels of H_2O_2 and peroxidase activity were detected, and both returned to background levels when full detergent resistance was acquired.

In Figure 4, similar analyses are shown for Z wall assembly. In this case, 0 hr represents the time when gametes of opposite mating type are mixed. At the initial 15-min time point (Figure 4A), all of the cells were wall-less due to the mating-induced release of GLE, and by phase microscopy it was ascertained that most had fused to form zygotes. During the next 2.5 hr, Z wall proteins were synthesized and secreted (Minami and Goodenough, 1978) but were not insolubilized, and the zygotes remained detergent sensitive. Insolubilization then initiated, and the cells became detergent resistant. Figures 4B and 4C document that once again, H_2O_2 production and peroxidase activation coincided with insolubilization.

Preliminary studies on substrate specificity of crude preparations of the Z wall and V wall peroxidases have revealed that both enzymes show highest activity when tyrosine is used. In contrast, the Z wall peroxidase is much less active than the V wall peroxidase when vanillin is the substrate (S. Waffenschmidt, K. Beer, J.P. Woessner, and U.W. Goodenough, unpublished data).

Effects of Ascorbate and Free Tyrosine

If, as Figures 3 and 4 suggest, H_2O_2 production and peroxidase activation participate in V wall and Z wall insolubilization, then inhibitors of peroxidase should affect the acquisition of detergent resistance. Figure 5A shows that increasing concentrations of ascorbate, a free-radical scavenger and, hence, a peroxidase inhibitor, progressively postponed the onset of V wall insolubilization and decreased the final percentage of detergent-resistant cells. In Figure 5B, a second aliquot of 10 mM ascorbate was added at 150 min, when the inhibitory influence of the first aliquot was about to abate (as determined

by ascorbate titration curves, data not shown), and this is seen to delay the onset of insolubilization for an additional 1.5 hr. Figures 6A and 6B show that comparable results were obtained when ascorbate was presented to zygotic cells.

If wall insolubilization is caused by the cross-bridging of tyrosine residues, then free tyrosine would also be expected to inhibit the reaction. Figures 7A and 7B document that this is indeed the case: both V wall and Z wall insolubilization were delayed by the addition of tyrosine or a tyrosine derivative, whereas phenylalanine, at similar concentrations, had no inhibitory effect (data not shown).

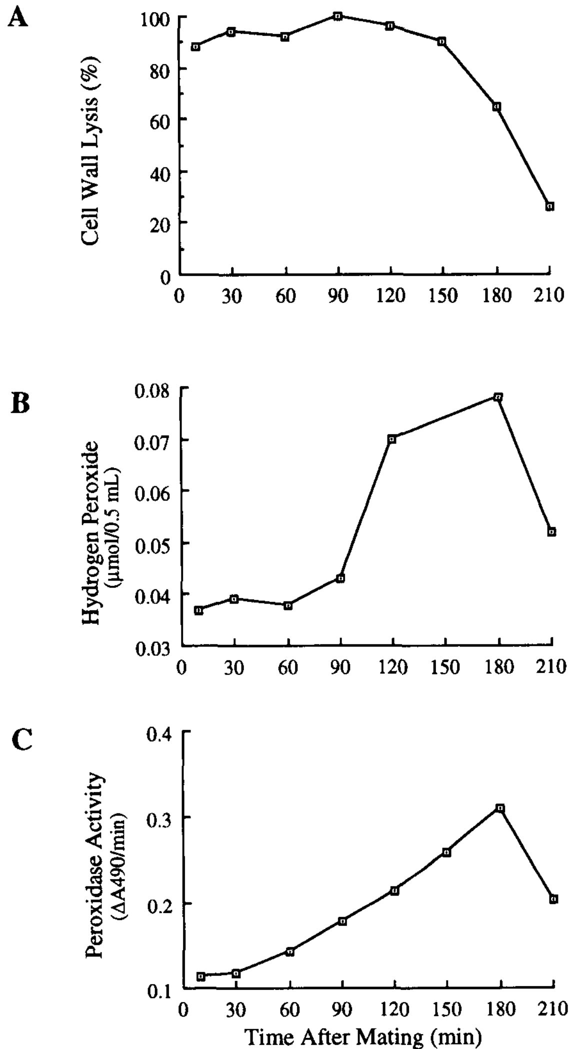

Figure 4. Analysis of Z Wall Formation.

Equal numbers of gametes of both mating types were mixed (time 0), and at 30-min intervals aliquots were removed and analyzed as follows.

(A) Detergent sensitivity (see Figure 3A). The detergent-insoluble mature wall is formed at ~180 min.

(B) Hydrogen peroxide accumulation (see Figure 3B).

(C) Peroxidase activity. Activity was determined as described in Figure 3C except that 900 μM tyrosine was used as a substrate (in place of vanillin), and the absorbance was read at OD_{315}.

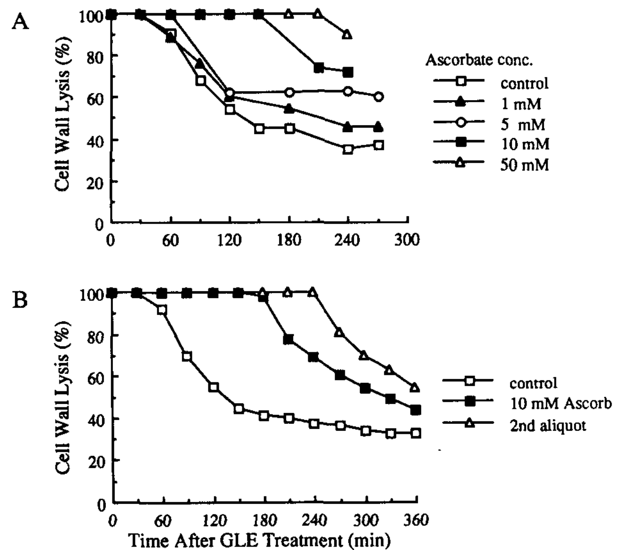

Figure 5. V Wall Regeneration in the Presence of Ascorbate.

(A) Vegetative cells were treated with GLE, as described in Figure 3, and resuspended in media containing various concentrations (conc.) of ascorbate. Detergent resistance (see Figure 3A) was tested at 30-min intervals.

(B) Analysis of detergent sensitivity was done as described above except that, at 150 min after GLE treatment, the cells in 10 mM ascorbate (Ascorb) were divided into two tubes, and one tube received a second aliquot of 10 mM ascorbate.

IdT and dT in Fishbones

To ascertain whether cross-linked tyrosine derivatives are components of the insoluble W2 layer of the V wall, large numbers of cells were treated with GLE to disassemble their walls. The cells were then pelleted, and the supernatants were subjected to ultracentrifugation, which pellets the W2-derived fishbones (Goodenough and Heuser, 1985) but leaves the salt-soluble W6 components in the supernatant. The fishbone material was hydrolyzed in 6 N HCl and subjected to phosphocellulose chromatography, as shown in Figure 8A. Three UV-absorbant peaks were obtained. Peaks 1 and 3 contained many components when examined by thin-layer chromatography (TLC); however, none of these spots corresponded to tyrosine, dT, or IdT (data not shown). The second peak was characterized further because it eluted in similar fractions to a dT standard (Figure 8B). The thin-layer chromatograph of peak 2 revealed a mixture of two components: a major nonfluorescent spot (retardation factor [R_f] 0.18) and a minor blue fluorescent spot (R_f 0.15). When compared with standards given in Table 2, these data indicate that peak 2 is comprised mainly of IdT with some dT.

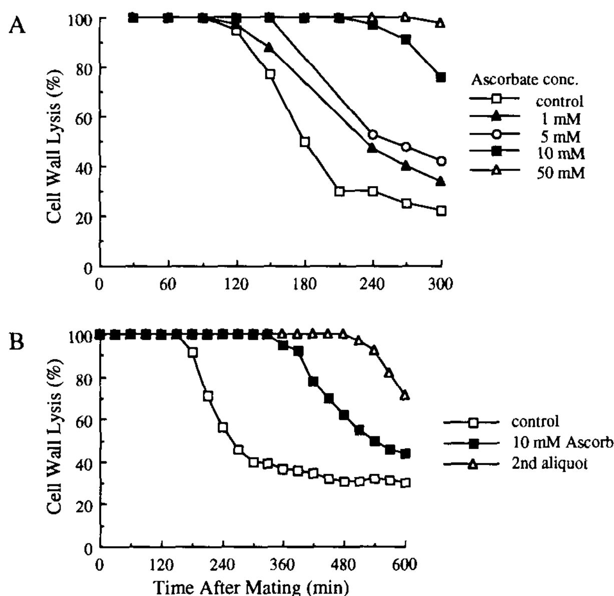

Figure 6. Z Wall Formation in the Presence of Ascorbate.

(A) Equal numbers of gametes of both mating types were mixed (time 0) and allowed to mate for 15 min. The cells were pelleted, washed, and resuspended in media containing various concentrations (conc.) of ascorbate. At 30-min intervals, Nonidet P-40 sensitivity was determined, as described in Figure 3A.

(B) Analysis of detergent sensitivity was done as described above except that, at 200 min after mating, the cells in 10 mM ascorbate (Ascorb) were divided into two tubes and one tube received a second aliquot of 10 mM ascorbate.

Peak 2 fractions were further purified using reverse phase HPLC. A small peak containing tyrosine alone was followed by a large second peak with two small shoulders constituting 10% of the total peak area. The material between the shoulders was collected and subjected to UV absorption analyses, as shown in Figure 9. As summarized in Table 3, the UV spectra indicate that IdT is the major component of peak 2. There is absorbance above 320 nm in our spectra (Figure 9A) that is not seen in spectra of IdT standards (Epstein and Lamport, 1984). Although we cannot rule out the possibility that this absorbance is due to a contaminant, we believe this is a tailing effect resulting from analysis of a smaller concentration of IdT in our sample than in the standards. In addition, we did not detect any new spots on the TLC plate of our sample that might have indicated the presence of a contaminant.

DISCUSSION

VSP-1

In previous papers, we described an SP-rich zygote-specific cDNA that, using our present nomenclature, we designate as ZSP-1 (Woessner and Goodenough, 1989), and we showed that α-$(SP)_{10}$ would immunoprecipitate specific in vitro translation products driven by either vegetative or zygotic mRNA (Woessner and Goodenough, 1992). Here, we present the sequence of a full-length V wall cDNA, designated VSP-1. Both ZSP-1 and VSP-1 share a similar overall organization: a series of XP_3 repeats at the N terminus is followed by an SP-rich domain, whereas the C-terminal domain displays little serine or proline and no obvious repeats. Two cloned wall sequences from Volvox, ISG and SSG-185, also display distinct SP-rich and SP-poor domains (Ertl et al., 1989, 1992), although neither Volvox protein employs alternating SP dyads. With transmission electron microscopy, most of the wall proteins from the Volvocales display discrete fibrous and globular domains (Goodenough and Heuser, 1985, 1988a, 1988b; Goodenough et al., 1986; Ertl et al., 1992), and it seems likely that these domains correspond, respectively, to the SP-rich and SP-poor sequences.

Our focus of interest in this report is a domain that occurs at the SP-rich/SP-poor boundary of VSP-1, an imperfect YGG repeat within a 50-amino acid region that also contains short SP-rich blocks. The abundance of tyrosine (5.5%) distinguishes

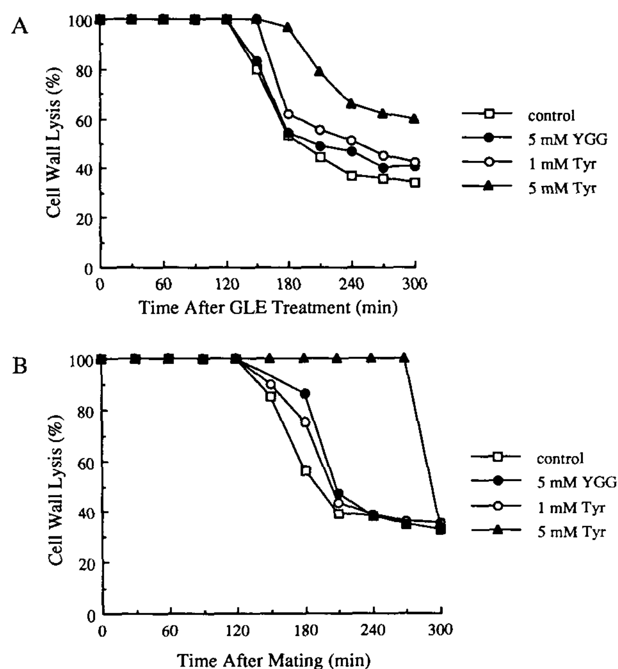

Figure 7. Tyrosine Inhibition of Wall Insolubilization.

(A) Vegetative cells were treated with GLE as described in Figure 3 and resuspended in media containing the tripeptide YGG or various concentrations of tyrosine. Detergent sensitivity was tested every 30 min (see Figure 3A).

(B) Gametes were mated as described in Figure 6A, and the resultant zygotes were resuspended in media containing the YGG peptide or various concentrations of tyrosine. Detergent resistance was tested every 30 min.

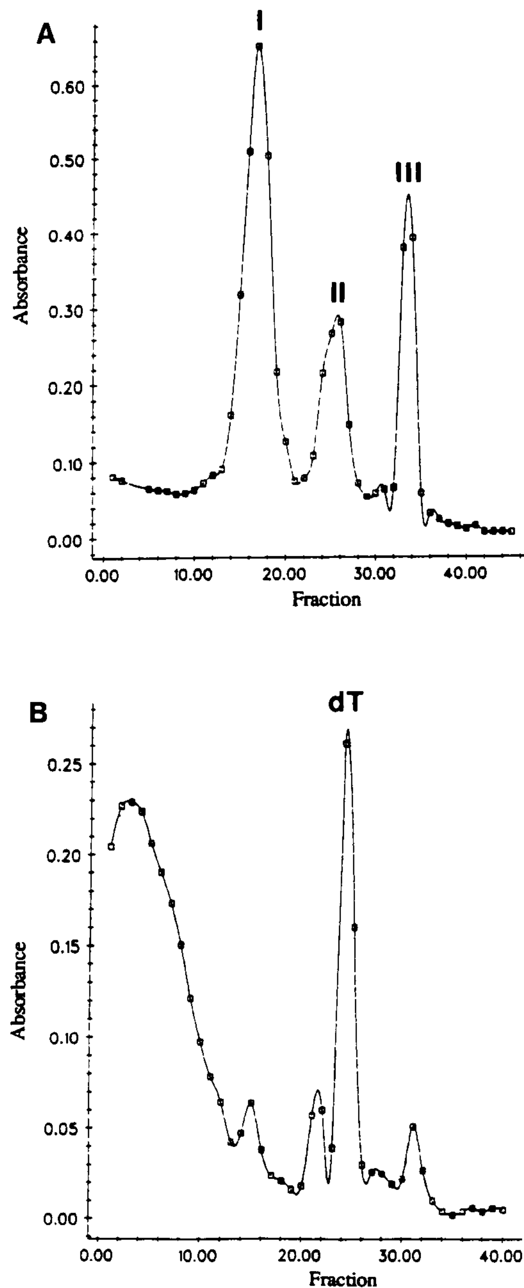

Figure 8. Phosphocellulose Elution Diagrams.

(A) Elution pattern of the W2 fish-bone hydrolysate. Vegetative cells were treated with GLE, the cells were pelleted, and the supernatant was subjected to ultracentrifugation, which pellets the fish bones. The fish-bone material was hydrolyzed in 6N HCl and loaded on a phosphocellulose column. Elution was continuously monitored at 280 nm. The three peaks are labeled in order of elution.

(B) Elution pattern of a dT standard. dT was synthesized in vitro and run on the same phosphocellulose column.

VSP-1 from any of the salt-soluble proteins of the V wall (Goodenough et al., 1986), indicating that it localizes to the salt-insoluble W2 layer, but we have as yet no direct evidence that the YGG domain of VSP-1 participates in W2 insolubilization. On the other hand, our data strongly indicate that peroxidase-catalyzed tyrosine cross-linking is integral to the insolubilization process. Tyrosine cross-linking has been proposed to occur in two steps: peroxidase/H_2O_2 first generates tyrosine free radicals, and these then dimerize in an enzyme-independent reaction (Fry, 1987). The probability of dimerization would presumably be enhanced by displaying the tyrosines in a clustered repetitive fashion because this would obviate the need for a precise alignment between participating polypeptides. Taken together, the data suggest, but do not yet prove, that the YGG domain of VSP-1 becomes cross-linked in muro.

Repeating units of tyrosines are not unique to Chlamydomonas wall proteins. A review of repeated tyrosine motifs in higher plant extracellular matrix (ECM) proteins reveals the interesting and unexplained fact that the tyrosines are usually found in three contexts: at the N-terminal side of a lysine (YK), or a serine/threonine (YS/YT), or a glycine (YG). In the extensins and proline-rich proteins, the YK dyad (included in a proline or hydroxyproline block) is found either as KYK or coupled with an uncharged amino acid (VYK, IYK, and YYK) (Chen and Varner, 1985; Datta et al., 1989; Datta and Marcus, 1990; Hong et al., 1990; Showalter and Rumeau, 1990). A repeated YS motif is found encoded in the matrix genes of dicots (YYS and TYS) (Keller and Lamb, 1989), whereas YT is encoded in monocot wall genes (TYT and PYT) (Stiefel et al., 1988, 1990; Raz et al., 1991; Caelles et al., 1992). Of particular relevance to the present study is the distribution of YG motifs. These are found in the glycine-rich proteins of higher plant matrices, where long strings of G residues are interrupted only by Y residues (Lei and Wu, 1991; Didierjean et al., 1992). They are also found in short repeats at the N termini of various monocot wall proteins (DAGYGYGGGY in maize and sorghum, Stiefel et al., 1990, Raz et al., 1991; DAGGYGGGY in rice, Caelles et al., 1992). Possibly these various tyrosine contexts represent recognition motifs for specific peroxidase enzymes (e.g., the extensin peroxidase characterized by Everdeen et al. [1988]). Alternatively, they may influence how polypeptides align with one another and thereby influence which sets of molecules undergo cross-linking.

Table 2. Thin-Layer Chromatography

	Tyr[a]	dT[a]	IdT[a]	Peak 2
R_f values	0.45	0.16	0.19	0.15 0.18
Fluorescence	No	Yes	No	Yes No

[a] Data from Fry (1982).

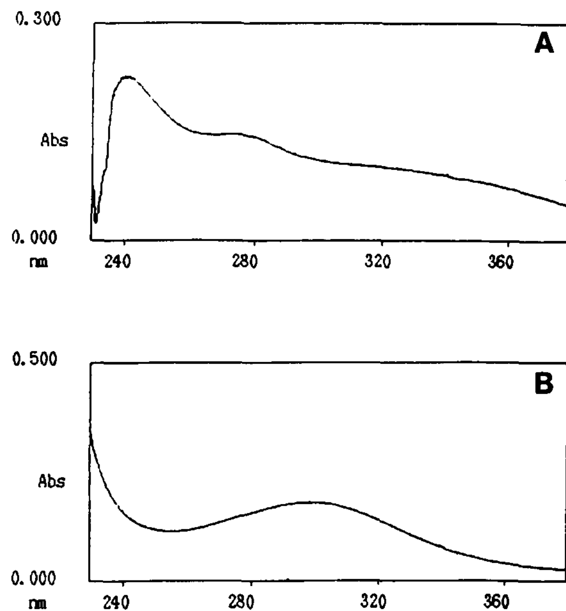

Figure 9. UV Spectra of HPLC-Purified Peak 2 Material.

HPLC was used to further purify the mixed components of peak 2 (see Table 2) followed by UV absorbance analyses at pH 2 and pH 13. The UV maxima at each pH are compared to standards in Table 3.
(A) Analysis at pH 2.
(B) Analysis at pH 13.

Peroxidation Reactions

Two recent papers have documented that the insolubilization of ECM components is accomplished by a burst of H_2O_2 production and the (presumed) cross-linking of tyrosine residues. In the first paper (Shapiro, 1991), a carefully orchestrated sequence of events following the fertilization of the sea urchin egg entails the secretion of tyrosine-rich substrates and peroxidase enzymes into the extracellular space and then the occurrence of a respiratory burst and H_2O_2 production, which drives the formation of dT cross-links, thereby hardening the vitelline layer. In the second paper (Bradley et al., 1992), soluble forms of two proline-rich proteins in the soybean wall are insolubilized in response to wounding or fungal elicitors, both of which stimulate an oxidative burst and H_2O_2 production. Each system is highly sensitive to ascorbate, indicating that peroxidase activity is essential to insolubilization.

We show here that in Chlamydomonas, both the V wall and the Z wall insolubilization events are also accompanied by a burst of H_2O_2 production. The burst occurs after the synthesis and secretion of wall proteins have been underway for 1 to 3 hr; the stimuli for its onset and its cessation remain to be identified. Electron microscopy of V wall regeneration has shown that the salt-soluble W6 layer assembles first, W2 material then accumulates beneath it, and finally insolubilization occurs (Goodenough and Heuser, 1985). An attractive notion, paralleled by the model proposed for the hardening of the sea urchin vitelline layer (Shapiro, 1991), is that the crystalline array of W6 components organizes the W2 components, perhaps placing tyrosine-rich domains into the appropriate orientation for the ensuing peroxidase-catalyzed cross-linking. Electron microscopy of Z wall formation, on the other hand, reveals no obvious template: a thicket of fibers first assembles and then becomes cross-linked (Minami and Goodenough, 1978; J.P. Woessner, J.E. Heuser, and U.W. Goodenough, unpublished data). There may, of course, be a Z wall template as well, one that is not sufficiently close packed to be recognized as crystalline.

In any case, the observation that an H_2O_2 burst accompanies both V wall and Z wall insolubilization, despite the fact that two different sets of HRGPs are assembling into two architecturally distinct walls, suggests strongly that each H_2O_2 burst is involved in insolubilization. We also detect a parallel rise and fall in the activity of the peroxidases released into the growth medium (as assayed in the presence of added H_2O_2). If the peroxidases have short half-lives, then one explanation for this pattern might be that secretion of the enzymes initiates at the time of wall insolubilization, that the enzymes find their way into the medium until they become trapped behind insolubilized walls, and that the activity released into the medium subsequently decays. Because many peroxidases are known to be stable enzymes, however, a second possibility is that Chlamydomonas may engage in the direct activation/deactivation of the secreted peroxidase enzymes. In either case, this would contrast with the soybean and sea urchin systems that are thought to be driven solely by H_2O_2 availability (Shapiro, 1991; Bradley et al., 1992).

Inhibitor data fully support the thesis that peroxidase/H_2O_2 drives wall insolubilization in Chlamydomonas and that tyrosine cross-linking is involved. As with the sea urchin, soybean, and carrot (Cooper and Varner, 1984) systems, the addition of ascorbate delays wall insolubilization in a dose-dependent fashion. As would be expected if the ascorbate were being oxidized and thereby inactivated, the Chlamydomonas cells recover from ascorbate inhibition (again in a dose-dependent fashion), and the insolubilization process resumes. This resumption can be prevented by adding a second aliquot of ascorbate at the time the cells start to recover from the first (a similar phenomenon was also observed in the carrot system; Cooper and Varner, 1984). The addition of tyrosine and tyrosine derivatives also delays insolubilization, whereas phenylalanine has no effect. The limited solubility of tyrosine precludes dose-response experiments, but again the cells eventually "recover" and resume insolubilization, presumably

Table 3. UV Absorbance Maxima

	Tyr[a]	dT[a]	IdT[a]	Peak 2 (HPLC)
pH 2	275	283	273	275
pH 13	293	317	298	296

[a] Data from Fry (1982).

because native cross-linking events are rendered less probable, but nonetheless continue to occur, in the presence of free tyrosine. Some of the exogenous tyrosine may also become directly cross-linked to wall monomers, compromising their ability to interact with one another.

IdT and dT

When IdT was first detected in plant cell wall material, it was proposed that such cross-links might account for the insolubilization of extensin monomers in muro (Fry, 1982; Cooper and Varner, 1983). To date, however, only intramolecular cross-links have been detected (Epstein and Lamport, 1984). Intermolecular cross-links would be expected to generate, at a minimum, dimeric polypeptides with two N termini, two C termini, and IdT cross-link(s), and these are difficult to extract from the wall and to identify (Fry, 1986). Indeed, although the intramolecular IdTs may be of significance, it is also possible that they represent abortive attempts at intermolecular cross-links in polypeptides that fail to become insolubilized.

An indirect but interesting argument that tyrosines are involved in cross-linking, noted by Goldman et al. (1992), is suggested by the sequences of extensin-like proteins expressed only in the pistil of tobacco. The genes for these proteins encode the $S(P)_4$ repeats of classic extensins but are either devoid of tyrosine (Chen et al., 1992) or carry no repetitive tyrosine-containing motifs (Goldman et al., 1992). Whereas the expression of extensin genes in vegetative tissues is induced by wounding (Showalter and Rumeau, 1990) and the extensin proteins are subsequently insolubilized in muro, none of the pistil extensin-like genes is induced by wounding. In fact, one of these pistil genes is actually turned off following wounding (Chen et al., 1992). Whether the pistil proteins remain soluble in muro is an important question that has not yet been addressed.

Two earlier papers demonstrated that acidified chlorite treatment of the insolubilized inner layer (W2) of the V wall releases components that are the primary substrates for proteolysis by GLE (Jaenicke et al., 1987; Waffenschmidt et al., 1988). The solubilization of these wall molecules with acidified chlorite suggests that they are held in the ECM by phenolic cross-links such as IdT. The identification of tyrosine cross-links in fishbone (W2) preparations (obtained from GLE-treated cells) is consistent with the thesis that the cross-links participate in W2 insolubilization, particularly in light of the peroxidase/H_2O_2 and inhibitor data. As with higher plants, however, the thesis will be proven only by identifying the dimeric/multimeric polypeptides. We hope that future work on the molecules that form the fishbones will yield clear examples of intermolecular cross-links.

Just as insolubilization of higher plant walls most likely involves more than just IdT cross-links between extensins (Showalter, 1993), we have evidence that disulfide linking and glutamyl–lysine side chain bridges also play a role in insolubilization of the Chlamydomonas walls (J.P. Woessner, S. Waffenschmidt, K. Beer, and U.W. Goodenough, manuscript in preparation). Nevertheless, our data on the tyrosine cross-links have important implications.

First, we have shown by several criteria that most (90%) of the cross-links in fishbones that involve tyrosine take the form of IdT. To date, this phenolic configuration has been identified only in higher plants (Fry, 1982, 1986), placing Chlamydomonas squarely in the higher plant lineage, which is an evolutionary position supported as well by its ribosomal DNA sequences (Sogin, 1991), by its photosynthetic apparatus (Harris, 1989), and by its endowment of HRGPs (Miller et al., 1974). In contrast, dT cross-links have not been detected in vivo in higher plant HRGPs. However, when soluble carrot extensins are exposed to native peroxidases and H_2O_2 in vitro, they form both IdT and dT linkages (Cooper and Varner, 1984). In addition, purified peroxidases from peanut suspension cultures can oxidize tyrosine to either dT or IdT, depending on the pH (Zheng and van Huystee, 1991). Therefore, particular orientations of tyrosine residues may be established in the wall, and lost in vitro, which assure the exclusive formation of the IdT linkages; alternatively, or in addition, the wall may provide cofactors or optimal conditions (pH or hydrophobicity) for IdT formation that are imperfectly mimicked in vitro. In any case, the appropriate in muro conditions for IdT formation appear to exist in Chlamydomonas during V wall insolubilization.

Second, we show that a minor proportion (~10%) of the cross-links in fishbones take the form of dT, which has to date been identified in vivo only in the metazoa (Foerder and Shapiro, 1977). Because, as noted above, dT will form in vitro in the carrot and peanut system (Cooper and Varner, 1984; Zheng and van Huystee, 1991), one possibility is that the dT in the Chlamydomonas fishbones results from aberrant events in muro; these "aberrations" are not detected in higher plants. The alternate possibility is that Chlamydomonas employs both modes of tyrosine cross-bridging during the insolubilization of its matrices. If evidence for specific dT cross-bridges between particular peptides and/or motifs is obtained, then the "aberration model" will yield to the notion that Chlamydomonas uses both types of linkages during wall assembly. If so, then this is consistent with the suggestion (Gunderson et al., 1987) that a flagellated progenitor of Chlamydomonas figured in the lineage of both the metazoa and the higher plants.

METHODS

Preparation and Screening of a λ ZAPII cDNA Library

Vegetative cells (CC-621) were grown in 500 mL of TAP media (Harris, 1989) to a density of 5×10^6 cells per mL. Cells were pelleted in sterile GSA bottles by bringing the rotor up to 4000 rpm and then immediately back down and then were resuspended in 200 mL of gametic lytic enzyme (GLE; prepared from a mating of 5×10^9 gametes of each mating type, Jaenicke et al., 1987) for 1 hr. The cells were again pelleted as described above and resuspended in 200 mL of TAP. The cells were allowed to regenerate their walls for 1 hr, and then total RNA

was isolated according to the method of Kirk and Kirk (1985). Approximately 20 μg of poly(A)$^+$ mRNA was prepared from the total RNA using the PolyATtract system (Promega). Five micrograms of poly(A)$^+$ mRNA was used to create a λ ZAPII cDNA expression library of 7 × 10^6 plaque-forming units (pfu)/μg arms in the Uni-ZAP XR vector using a ZAP-cDNA synthesis kit (Stratagene).

(Ser/Pro)$_x$ antibodies were affinity purified, as detailed in Woessner and Goodenough (1992), and used to screen 350,000 recombinant plaques at a density of 5 × 10^4 pfu per 15-cm diameter plate. *Escherichia coli* PLK-F′ was infected with λ ZAP and allowed to form a lawn at 37°C. Nitrocellulose filters soaked in 10 mM isopropyl-β-D-thiogalactopyranoside were laid down on each plate and incubation continued for an additional 6 hr at 37°C. The filters were removed and screened with affinity-purified (Ser/Pro)$_x$ antibodies, as described in Woessner and Goodenough (1992), resulting in 16 selected phage. After plaque purification, the pBluescript SK− phagemid containing the cDNA insert was excised using the Stratagene protocol. Each DNA was labeled, using a random primed DNA labeling kit (Boehringer Mannheim), and used to probe a filter lifted from a plate where all 16 selected phage had been spotted onto a lawn of C600 cells. In this manner, all selected phage were placed into five groups that did not cross-hybridize: vegetative Ser/Pro-rich (VSP)-1 through VSP-5.

DNA Sequencing and Analysis

Nested deletions of both strands of the VSP-1 cDNA were generated using the Erase-a-Base kit (Promega), and single-stranded DNA was generated from selected clones for dideoxy sequencing with the Sequenase kit (U.S. Biochemicals). DNA sequences were assembled and analyzed with the Genetics Computer Group Sequence Analysis Software Package (University of Wisconsin, Madison) for VAX/VMS computers.

RNA Gel Blot Analysis

Vegetative cells (CC-621) were grown in a 500-mL culture of TAP media treated with GLE for 1 hr and resuspended in 250 mL of TAP as described above. A 30-mL aliquot was removed every 30 min, and total RNA was isolated. Total RNA (10 μg) from each time point was electrophoresed on a 1.1% formaldehyde gel, transferred onto nitrocellulose, and baked as described by Maniatis et al. (1982). The blots were hybridized and washed according to the Church and Gilbert (1984) conditions.

Analysis of Insolubilization of *Chlamydomonas reinhardtii* Walls

Regenerating Vegetative Wall

Vegetative cells (CC-620) were grown in TAP to a density of 1 to 3 × 10^6 cells per mL. The cells were pelleted in a Sorvall HB4 rotor by bringing the rotor up to 4000 rpm and then immediately back down, and the pellet was resuspended in 15 mL of GLE for 1 hr at room temperature. GLE was removed by two successive washings in TAP. After the second wash, the cells were resuspended in TAP (or TAP and various inhibitors, with the pH adjusted to 7.4) to a density of 5 × 10^6 cells per mL and assayed as described below.

Formation of Zygote Wall

Equal numbers of gametes of each mating type were mixed at a density of 5 × 10^7 cells per mL and analyzed as described below. For inhibitor studies, gametes were mated for 15 min and pelleted; pellets were washed as described above and resuspended in HSM-N media (Harris, 1989) or HSM-N and various inhibitors, with the pH adjusted to 7.4.

Detergent Sensitivity

Insolubility of the wall (either vegetative [V] or zygotic [Z]) was monitored by measuring chlorophyll released by Nonidet P-40 lysis of the cells. Aliquots (0.5 mL) were removed at 30-min intervals, mixed with an equal volume of 0.2% Nonidet P-40, spun for 5 min at 13,000 rpm in a microcentrifuge, and the optical density of the supernatant read at 440 nm. The highest absorbance reading was set at 100% cell wall lysis and the other readings were calibrated accordingly.

H_2O_2 Levels

At each time point, a 0.5-mL aliquot was pelleted at 13,000 rpm in a microcentrifuge, and the supernatant was mixed with an equal volume of 1 M KI. After 15 min at room temperature, the mixture was assayed for iodine formation by reading the OD at 390 nm. The absorbance at 390 nm was stable for at least 3 hr. Concentrations were determined from a standard calibration curve using known concentrations of H_2O_2.

Peroxidase Activity

Enzyme activity was measured as described by Vilter et al. (1983). At each time point, a 50-μL aliquot of the supernatant after a 13,000-rpm pelleting was mixed with 50 μL of acetate buffer (0.1 M sodium acetate, pH 5.5, with 40 mM $MnCl_2$) containing 0.8 mM H_2O_2 and 900 μL of 1 mM substrate (vanillin for V wall or tyrosine for Z wall). The OD (490 nm for vanillin or 315 nm for tyrosine) was read immediately and again after 5 min. Enzyme activity was determined from the change in absorbance.

Analysis of Tyrosine Cross-links in the Vegetative Wall

Preparation of Fishbone Hydrolysates

Gametes from 100 plates of each mating type were mated for 15 min. Cells were pelleted at 6000*g* for 10 min. Fishbones were isolated from the supernatant by ultracentrifugation at 40,000*g* for 20 min. The pellet was hydrolyzed in 6 N HCl under N_2 at 100°C overnight.

Preparation of Dityrosine and Isodityrosine

A dityrosine (dT) standard was prepared, as described by Fry (1982), using 25 mM tyrosine, 52 μL H_2O_2 (30%), and horseradish peroxidase (0.1 mg/mL) and incubated at 30°C for 20 hr. Isodityrosine (IdT) was synthesized according to the protocol of Fry (1988).

Separation on Phosphocellulose

Phosphocellulose chromatography was done with a slight modification of the procedure published by Foerder and Shapiro (1977). Samples were dissolved in 8% acetic acid and loaded on a 50-mL column

equilibrated with 8% acetic acid. One hundred and fifty milliliters of 8% acetic acid was used to wash off nonbound material; this was followed by 200 mL of a NaCl gradient (0 to 1 M) in 8% acetic acid for elution of tyrosine cross-links. Elution was constantly monitored at 280 nm, and 5-mL fractions were collected. Figures 8A and 8B present absorbances for the first 45 and 40 fractions, respectively. The remaining fractions in both instances did not have absorbances above 0. Fractions containing the major peaks were pooled, freeze-dried, and used for thin-layer chromatography (TLC) analysis.

TLC Analysis

Analytical silica TLC plates (Merck, Darmstadt, Germany) were chromatographed in propan-1-ol/25% NH_3 (7:3). dT was detected under UV light after exposure to NH_3 vapor (25%). Nonfluorescent spots were visualized after spraying with ninhydrin reagent and developing at 110°C for 10 to 15 min. NaCl was not removed from the freeze-dried samples prior to application to the TLC plates. IdT and dT standards (synthesized in vitro by us and also a gift of Dr. Stephen Fry, University of Edinburgh) were eluted from the column and treated similarly before being applied to the TLC plates.

HPLC Separation

Samples were separated on a 300-mm reverse phase column (C 18, 5 μm, Macherey & Nagel, Dueren, Germany) using MeOH/25% NH_3 (3:2) as solvent. Elution was monitored at 280 nm, and peaks were quantified with a Hitachi integrator.

UV Absorption

Spectra of the components were obtained by scanning the absorbance from 230 to 380 nm at both pH 2 and pH 13. Spectra of dT and IdT standards were also determined to verify that our techniques yielded UV maxima consistent with the published values (Fry, 1982).

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