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A Transglutaminase Immunologically Related to Tissue Transglutaminase Catalyzes Cross-Linking of Cell Wall Proteins in *Chlamydomonas reinhardtii*[1]

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The addition of primary amines to the growth medium of the unicellular green alga *Chlamydomonas reinhardtii* disrupts cell wall assembly in both vegetative and zygotic cells. Primary amines are competitive inhibitors of the protein-cross-linking activity of transglutaminases. Two independent assays for transglutaminase confirmed a burst of extracellular activity during the early stages of cell wall formation in both vegetative cells and zygotes. When non-inhibiting levels of a radioactive primary amine (^{14}C-putrescine) were added to the growth medium, both cell types were labeled in a reaction catalyzed by extracellular transglutaminase. The radioactive label was found specifically in the cell wall proteins of both cell types, and acid hydrolysis of the labeled material released unmodified ^{14}C-putrescine. Western blots of the proteins secreted at the times of maximal transglutaminase activity in both cell types revealed a single highly cross-reactive 72-kD band when screened with antibodies to guinea pig tissue transglutaminase. Furthermore, the proteins immunoprecipitated by this antiserum in vivo exhibited transglutaminase activity. We propose that this transglutaminase is responsible for an early cell wall protein cross-linking event that temporally precedes the oxidative cross-linking mediated by extracellular peroxidases.

Cell wall formation in the unicellular green alga *Chlamydomonas reinhardtii* has been intensely scrutinized for over two decades (for review, see Woessner and Goodenough, 1994). During its life cycle, *C. reinhardtii* elaborates two structurally and biochemically distinct cell walls. The vegetative wall (V-wall) surrounding both vegetative and gametic cells has salt-soluble glycoproteins comprising the outer wall layers, and salt- and detergent-insoluble components found only in the inner wall layers. During mating, gametic lytic enzyme (GLE) cleaves off the V-wall, and the early zygotes assemble a desiccation-resistant, insoluble zygote wall (Z-wall). Both the V- and Z-walls lack the abundant complex polysaccharides typical of higher plant extracellular matrices (cellulose, hemicelluloses, and pectins), facilitating analyses of structural wall protein assembly in *C. reinhardtii*.

GLE-treated vegetative cells and early zygotes secrete developmentally specific Hyp-rich glycoproteins (HRGPs), evolutionary homologs of higher plant HRGPs that self-assemble in a predictable, ordered manner. This self-assembly has been well documented in vitro for the salt-extractable outer V-wall HRGPs: when the salt is dialyzed away from these proteins, they form a crystalline structure similar to that found in muro (Hills et al., 1975; Goodenough et al., 1986). In addition, salt-extracted vegetative cells added to the dialysis bag can nucleate the assembly of the soluble HRGPs to reform a crystalline layer (Adair et al., 1987).

One of the terminal steps in the formation of both the V-wall and Z-wall is insolubilization of many of the assembled HRGPs. In previous work (Waffenschmidt et al., 1993), we determined the time of insolubilization for each wall and demonstrated that insolubilization is due, at least in part, to the formation of isodityrosine cross-links, a mechanism also proposed to be responsible for insolubilization of higher plant HRGPs (Fry, 1982; Cooper and Varner, 1983). Here we present evidence that another cross-linking enzyme, transglutaminase, is also involved in the assembly and insolubilization of both walls.

Transglutaminases (TGases; E.C. 2.3.2.13) catalyze the covalent linkage of protein-bound glutaminyl residues with primary amines (e.g. polyamines) or with protein-bound lysines to create intermolecular $\epsilon(\gamma$-glutamyl) lysyl cross-links. These cross-links are stable, resistant to all known proteases and to boiling in 1% (w/v) SDS. The family of TGases is widespread, with examples found in bacteria, fungi, plants, and animals. Known biological functions involving TGase include formation of the fibrin clot (Lorand and Conrad, 1984), coagulation of seminal plasma to form the copulation plug in rodents (Williams-Ashman, 1984), keratinization of epidermis and hair (Rice et al., 1992), assembly of the fertilization envelope in sea urchin (Battaglia and Shapiro, 1988), formation of the teleost fish eggshell (Oppen-Bernsten et al., 1990), and construction of a desiccation-resistant fungal spherule (Klein et al., 1992). In each instance, TGase catalyzes the cross-linking of structural proteins into a large insoluble network.

Recently, it was proposed that TGase is involved in cytoskeletal rearrangement during pollen germination and pollen tube formation in apple trees (Del Duca et al., 1997), and intracellular and chloroplast membrane-bound TGase activity have been demonstrated in several instances. Both the large subunit of Rubisco (Margosiak et al., 1990) and

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chlorophyll *a/b* binding proteins (Del Duca et al., 1994) have been identified as major TGase substrates. A potential role for TGase in cross-linking plant extracellular proteins has been suggested in two studies (Falcone et al., 1993; Serafini-Fracassini et al., 1995). The present study demonstrates that *C. reinhardtii* secretes an extracellular TGase immunologically related to guinea pig tissue TGase whose substrates include cell wall HRGPs, and that inhibition of this activity disrupts normal wall assembly.

MATERIALS AND METHODS

Wall Regenerating Vegetative Cells (RVC)

Vegetative cells (CC-621), grown in 600 mL of Tris-acetate-phosphate (TAP; Harris, 1989) medium to a density of 3×10^6 cells mL^{-1}, were pelleted at 4,000*g* for 10 min in a rotor (model HB4, Sorvall, Newtown, CT). The cells were resuspended in 30 mL of TAP medium and 30 mL of gametic lytic enzyme (GLE; prepared as described by Jaenicke et al. [1987]), and incubated for 1 h at room temperature. GLE was removed by two successive washes in TAP medium (pelleting in the rotor by bringing it up to 4,000*g* and immediately back down), the cells were resuspended in 200 mL of TAP medium (or TAP medium and inhibitor with the pH adjusted to 7.4) and allowed to regenerate a new cell wall.

Developing Zygotes (DZ)

Zygotes were formed by mixing 100 mL (5×10^7 cells mL^{-1}) of gametes of each mating type (CC-620 and CC-621). After a 30-min period of mating, the cells were pelleted, washed, and resuspended in 200 mL of high-salt medium lacking nitrogen (Harris, 1989) or the same medium plus inhibitor with the pH adjusted to 7.4.

Spectrophotometric Assays

Insolubilization of the wall (in both vegetative and zygotic cells) was monitored by measuring chlorophyll release following Nonidet P-40 treatment (Waffenschmidt et al., 1993). H_2O_2 concentration and peroxidase activity were determined using the methods described in Waffenschmidt et al. (1993).

In Vitro TGase Assays

Putrescine Method

At each time point, a 500-μL aliquot of cells was pelleted (2 min at 12,000 rpm in a microcentrifuge) and 40 μL of the supernatant was mixed with 200 μL of the test solution (150 mM Tris-HCl, pH 7.4, 4 mM $CaCl_2$, 30 mM glutathione, 1 mg mL^{-1} *N,N*-dimethyl casein, and 0.01 mCi mL^{-1} [1,4-^{14}C]putrescine). The specific activity of the labeled putrescine was 0.05 mCi mL^{-1}, 117 mCi $mmol^{-1}$. The samples were incubated for 30 min at 25°C and the reaction was stopped by addition of 1 mL of ice-cold 10% (w/v) TCA. The precipitate was washed three times with ice-cold acetone, resuspended in 100 μL of 0.1 N NaOH, added to 1 mL of scintillation fluid, and counted in a BG Betaszint 5000 liquid scintillation counter. Two control samples were also analyzed in the same manner. One control was a 40-μL aliquot of supernatant that had been heated for 15 min at 60°C to kill the enzyme. The second control was just test solution with no supernatant added. Both control samples yielded background levels of 60 to 80 cpm and all incorporation values were then corrected for these levels.

The incorporation time courses were done with a 1-mL sample of supernatant collected from RVC at the time of maximal TGase activity as determined above. This sample was mixed with 5 mL of the test solution, incubated at 25°C and at various time points aliquots were removed, and processed to measure incorporation. Similarly, a second incorporation time course was generated by taking aliquots from a mixture of 0.5 mL of supernatant and 5.5 mL of test solution.

Hydroxamate Formation Method

Twenty-five microliters of cell-free supernatant exhibiting maximal TGase activity as determined by method A were added to a 350-μL reaction cocktail containing 200 mM Tris-acetate-buffer, pH 6.0, 30 mM carbobenzoxy-L-glutamylglycine, 5 mM $CaCl_2$, 10 mM glutathione, and 100 mM hydroxylamine, as described previously (Folk and Cole, 1966). This was mixed well and incubated for 5 min at 37°C, then 375 μL of 1 M $FeCl_3$ in 5% (w/v) TCA were added. The suspension was mixed, the precipitate was removed by centrifugation (12,000 rpm, 2 min), and the supernatant was assayed for iron-hydroxamate-complex formation at 525 nm in a spectrophotometer.

Inhibition of TGase in Vitro

The supernatant isolated from RVC or DZ at the time of maximum enzyme activity (as determined by the putrescine method detailed above) was used as a crude preparation of TGase. Enzyme activity was determined by the hydroxamate formation assay and this value was set to 100%. Then, EGTA, Zn^{2+}(buffered with Tris, pH 7.6), *N*-ethylmaleimide, or *p*-chloromercuribenzoate was added to aliquots of the supernatant, enzyme activity was remeasured, and the values were converted to percentages of control activity. $CaCl_2$ was omitted from the TGase reaction cocktail for the EGTA experiment. These assays were performed in triplicate and the average percentages are presented.

In Vivo Incorporation of ^{14}C-Putrescine

One-hundred milliliters of RVC or DZ was prepared as described above except that the final resuspension was in medium containing 2.5 μCi of ^{14}C-putrescine. One milliliter of each suspension was removed, immediately pelleted (2 min at 12,000 rpm), and 100 μL of the supernatant added to the scintillation cocktail. The counts per minute of input label was determined in a scintillation counter and set to 100%. At 20-min intervals, 250 μL was removed to test for

detergent sensitivity (as above). At the same time, the cells were pelleted out of a 1-mL aliquot (2 min at 12,000 rpm), washed eight to 10 times to eliminate non-incorporated radioactivity, and finally resuspended in 1 mL of medium. One-hundred microliters of this cell suspension was added to the scintillation cocktail and counted. The resultant counts per minute were converted to percentages of the total input.

After the last time point was collected (200 min for zygotes, 180 min for vegetative cells) all of the remaining cells were pelleted, extensively washed, and resuspended in 30 mL of medium. A 1-mL aliquot was taken and the total incorporation was determined as described above. The remaining cells were pelleted.

The vegetative cell pellet was first resuspended in 30 mL of 2 M $NaClO_4$ and incubated for 20 min at room temperature. The sodium perchlorate solubilizes the outer crystalline wall layers and kills the cells, but does not lyse them (Goodenough et al., 1986). One-hundred microliters of the supernatant was taken after the cells were pelleted, and added to scintillation cocktail for counting. The cell pellet was washed three times to remove the $NaClO_4$, and then resuspended in 30 mL of GLE for 1 h. After pelleting the protoplasts, a 100-μL aliquot of the supernatant was removed and counted.

The pelleted zygotes were extracted with 30 mL of 0.3% (w/v) $NaClO_2$ in 0.12% (w/v) acetic acid at 70°C for 2 h while bubbling with nitrogen (Jaenicke et al., 1987). This treatment solubilizes the isodityrosine cross-links within the wall (Waffenschmidt et al., 1993). The suspension was pelleted and 100 μL of the supernatant was analyzed in the scintillation counter.

Identification of ^{14}C-Putrescine-Labeled Wall Proteins

One-hundred milliliters of GLE-treated and washed vegetative cells was resuspended in TAP medium and 5 μCi of ^{14}C-putrescine (this concentration, 0.43 μM, is far below the inhibiting levels of putrescine). After 3 h of regeneration, the cells were pelleted and extracted with 2 M $NaClO_4$ as described above. The supernatant was lyophilized and resuspended in 2 mL of water. Both the perchlorate-solubilized proteins and the lyophilized medium were dialyzed against water, and two aliquots (400 and 800 μL) were removed, precipitated with 10% (w/v) TCA in 50% (w/v) acetone, resuspended in loading buffer, and subjected to SDS-PAGE. After fixing for 30 min in 25% (v/v) isopropyl alcohol and 10% (w/v) acetic acid and shaking for 30 min in Enhance (Amersham, Uppsala), the gel was dried and exposed to Kodak XAR-5 film.

Analysis of Self-Assembly Competency

Sixty milliliters of GLE-treated and washed vegetative cells (1×10^6 cells mL^{-1}) was divided into two equal volumes and pelleted; one half was resuspended in TAP medium and the other half in TAP medium plus 50 mM putrescine. After 3 h, each sample was pelleted and extracted with 3 mL of 2 M $NaClO_4$. The supernatant from each sample was saved and lyophilized as well. These lyophilates were resuspended in 3 mL of 2 M $NaClO_4$ and, along with the sodium-perchlorate-solubilized components, dialyzed overnight against water. This dialysis leads to wall crystal formation when the protein concentration is greater than or equal to 1 mg mL^{-1} (Goodenough et al., 1986). Wall crystals were visible in the dialysis tubing, pelleted out of solution, and subjected to SDS-PAGE. The gel was stained using the periodic acid-Schiff reagent as described in Monk et al. (1983).

In Vitro Nucleation Assay

A 100-mL culture of vegetative cells was divided into three samples (A, B, and C). The cells in samples B and C were GLE treated and washed as usual. Sample B was resuspended in TAP medium and C was resuspended in TAP medium plus 50 mM putrescine. After 3 h all samples were fixed in paraformaldehyde (1% [w/v] in 15 mM HEPES, pH 7.0) for 1 h, incubated for 2 h in 25 mM Gly, pH 7.0, washed, and resuspended in 2 M sodium perchlorate. The extracted cells were pelleted, washed, and mixed with 50,000 cpm of labeled outer wall glycoproteins solubilized in 2 M sodium perchlorate; the labeled proteins were obtained by perchlorate-extracting vegetative cells regenerating in the presence of ^{14}C-putrescine. Additional unlabeled outer wall glycoproteins (0.2 mg mL^{-1}) were added to increase the protein concentration. The three samples were dialyzed overnight and then transferred to a test tube for five successive washes in water. Each sample was then perchlorate extracted and 100 μL of the soluble fraction was added to 1 mL of scintillation cocktail and assayed in the scintillation counter.

Electron Microscopy

Quick-freeze, deep-etch electron microscopy of vegetative cells regenerated for 3 h in the presence or absence of 50 mM putrescine was performed as described in Heuser (1980).

Examination of Cross-Linking on the Plasma Membrane

To label RVC, the same protocol as described in "In Vivo Incorporation of ^{14}C-Putrescine" was followed.

One-milliliter aliquots of cells were removed at 5-min intervals. The cells were pelleted and extracted with sodium perchlorate to remove soluble wall glycoproteins. The extracted cells were pelleted again and lysed with 5 mL of 0.006 digitonin in 5 mM KPO_4 buffer, pH 7.2, and 1 M KCl. Cell debris and the chloroplast were removed by centrifugation for 15 min at 8,000*g*. Microsomal preparations of the supernatant were isolated by ultracentrifugation for 1.5 h at 100,000*g* in a rotor (model 50.3Ti, Beckman Instruments, Fullerton, CA). The pellets were resuspended in 50 μL of loading buffer and subjected to SDS-PAGE on

a 7.5% (w/v) running gel with a 3.5% (w/v) stacker. The gel was fixed, dried, and exposed to x-ray film as described above.

Recovery of Putrescine from Labeled Protein Conjugates

To 1 mL of a crude cell-free TGase preparation (described above), 20 μCi of ^{14}C-putrescine and 1% (w/v) dimethylcasein were added. The same amount of label was also added to 100 mL of RVC (prepared as outlined above). Both samples were incubated for 30 min at room temperature, and the cells were removed from the second sample by centrifugation for 10 min at 5,000*g* in a rotor. The proteins from both samples were precipitated with ice-cold 10% (w/v) TCA, 50% (w/v) acetone, and 10 mM unlabeled putrescine, and then hydrolyzed in 6 N HCl at 110°C under nitrogen for 48 h. The HCl was removed under vacuum and the remaining sample washed twice with methanol and finally dissolved in 1 mL of phosphate buffer (30 mM, pH 7.6). The respective solutions were run on a phosphocellulose column (1 × 6 cm) equilibrated with the phosphate buffer. The elution was according to Signori et al. (1991) by first washing with 20 mL of phosphate buffer, then with 20 mL of 100 mM borate buffer (pH 8.1) containing 25 mM NaCl, and finally a 100-mL gradient to a limit buffer of 200 mM borate and 600 mM NaCl, pH 8.6. Two-milliliter fractions were collected and assayed for radioactivity in a scintillation counter. The column was calibrated by following the elution of ^{14}C-putrescine.

Immunoprecipitation with Anti-TGase Serum

Forty milliliters of RVC or DZ was harvested at the time of maximal TGase activity. The cell-free supernatants from these were used in two experiments. First, a 1-mL aliquot from each supernatant was mixed with 5 μL of serum against guinea pig tissue TGase (Aeschlimann et al., 1993), incubated 1 h at 4°C, and the resulting IgG complex was adsorbed onto 50 μL of protein A-agarose (Sigma Chemicals, St. Louis) during a 1-h room temperature incubation. After pelleting the agarose (10 min, 12,000 rpm), 40 μL of supernatant and 40 μL of the pellet (resuspended in 1 mL of TGase assay buffer) were tested for TGase activity using the putrescine method. Second, the remainder of each cell-free supernatant was lyophilized and resuspended in 5 mL of distilled water. Twenty-five-microliter samples of this resuspension were mixed with an equal volume of Laemmli dissociation buffer (0.715 M β-mercaptoethanol, 8 M urea, 0.36 M Suc, 4% [w/v] SDS, 0.04% [w/v] pyronin y, and 100 mM Na_2CO_3) and subjected to SDS-PAGE. The gel was either silver-stained or electroblotted onto nitrocellulose. The immunoblot was developed using anti-TGase serum as the primary antibody and anti-rabbit IgG linked to alkaline phosphatase (Sigma Chemicals) as the secondary antibody. Color was detected using nitroblue tetrazolium chloride and 5 bromo-4-chloro-3-indolyl-phosphate toluidine salt. For the competition experiments 2.5 or 10 μg of purified TGase from guinea pig (a gift of M. Paulsson, University of Cologne) was incubated with the primary antibody for 30 min prior to adding it to the blot.

RESULTS

Inhibition of Cell Wall Insolubilization with Primary Amines

The effect of exogenously added compounds on cell wall insolubilization can be measured by a simple spectrophotometric assay for detergent sensitivity (Waffenschmidt et al., 1993). Mature V-walls and Z-walls rendered cells detergent insensitive, and a plot of percent cell lysis versus time reflects the progress of wall insolubilization. During the course of our work on peroxidase-mediated cross-linking, we discovered that the addition of Gly ethyl ester delayed insolubilization of both V-walls and Z-walls. Since transglutaminases, which have been shown to generate covalent intermolecular cross-links in animal systems, are competitively inhibited by primary amines such as Gly ethyl ester (Lorand et al., 1979), we tested several other primary amines (cadaverine, putrescine, spermine, and spermidine) in cultures of RVC or DZ. Cadaverine, spermine, and spermidine all inhibited or delayed insolubilization of both wall types, but all three ultimately led to cell death and thus were not utilized further (data not shown). Putrescine, like Gly ethyl ester, did not cause any obvious cell lethality as determined by visual inspection under the light microscope.

Figure 1 shows detergent-sensitivity plots for RVC and DZ in medium containing various concentrations of putrescine. Prior to time 0 in Figure 1A, the vegetative cells were incubated for 1 h in GLE to remove the V-wall. At the end of this incubation, the cells were washed free of GLE (time 0) and began to synchronously assemble a new V-wall. As indicated by the control curve in this panel, a detergent-resistant V-wall is normally completed within 2 h (Robinson and Schlösser, 1978; Waffenschmidt et al., 1993). When vegetative protoplasts were resuspended in media with increasing concentrations of putrescine, a proportional decrease in the number of cells completing the insolubilization program was observed. Washing the putrescine away from the treated cells did not reinitiate the wall-insolubilization program (data not shown).

In Figure 1B, gametes of the opposite mating type were mixed together at time 0, and within 30 min the culture contained synchronous DZ. The early zygotes were wall-less and assembled a new detergent-resistant Z-wall over the next 3 h (control curve). The DZ were pelleted at 30 min and resuspended in increasing concentrations of putrescine. As seen in the RVC, greater concentrations of putrescine resulted in proportionally fewer cells becoming insolubilized. The concentrations of putrescine found to be most effective at blocking the insolubilization of both walls in *C. reinhardtii* were directly comparable to the levels of putrescine required to inhibit the TGase activity in assembly of the sea urchin fertilization envelope (Battaglia and Shapiro, 1988).

In Vitro TGase Assays

With suggestive evidence that TGase activity might be involved in the wall insolubilization program, our next

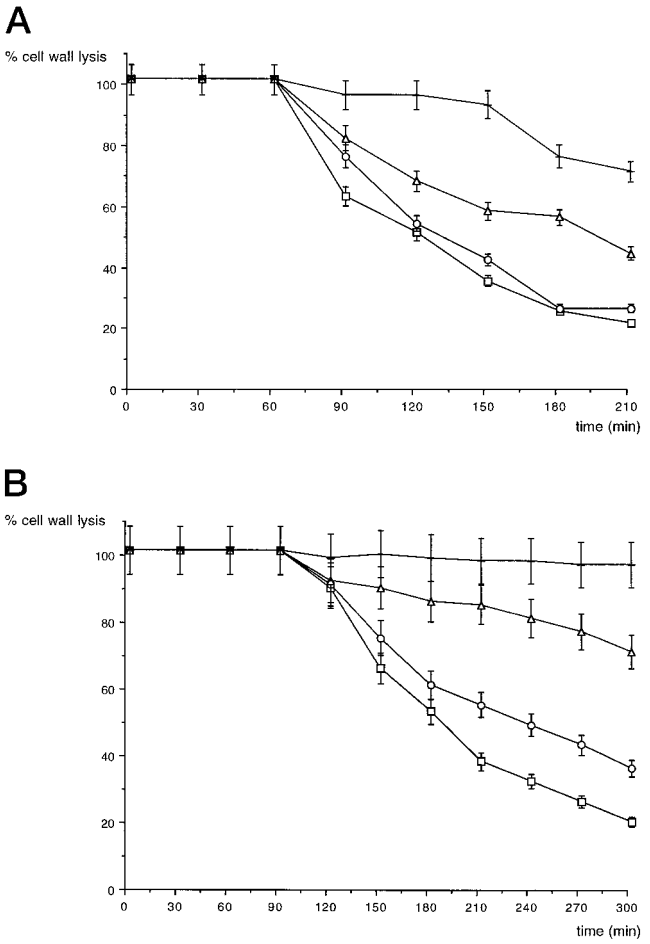

Figure 1. Effect of putrescine on cell wall insolubilization. A, Vegetative cells were treated with GLE for 1 h and the resultant protoplasts were pelleted, washed, and resuspended in fresh medium (time 0) without (control) or with various concentrations of putrescine. Aliquots were taken at 30-min intervals and analyzed for detergent sensitivity by mixing with an equal volume of 0.2% (w/v) Nonidet P-40 and pelleting the cells and cellular debris in a microfuge. The A_{440} of the supernatant was read to quantitate chlorophyll release from lysed cells, and the highest absorbance reading was set to 100% cell lysis. The other values were converted to percentages accordingly. B, Equal numbers of gametes of both mating types were mixed (time 0), allowed to mate for 30 min, then pelleted, washed, and resuspended in fresh medium lacking (control) or containing various concentrations of putrescine. At 30-min intervals, aliquots were removed and analyzed for detergent sensitivity as above. Error bars at each time point indicate the results from three independent assays. □, Control; ○, 5 mM putrescine ; △, 10 mM putrescine; +, 50 mM putrescine.

step was to perform in vitro TGase assays. To determine whether the culture medium of RVC or DZ contains TGase activity, small aliquots were taken over time from a culture of RVC or DZ, the cells were pelleted, and the supernatant assayed for TGase activity in vitro by measuring ^{14}C-putrescine incorporation into dimethyl casein (Battaglia and Shapiro, 1988). At each time point, aliquots were also removed to test for detergent sensitivity, H_2O_2 concentration, and peroxidase activity. The results for both RVC and DZ are shown in Figure 2. Figure 2A documents a sharp peak of TGase activity around 60 min after GLE removal from vegetative cells. The peak of TGase activity, unlike the peak of H_2O_2 secretion and peroxidase activity (indicated in the figure by the arrow), preceded the time of V-wall insolubilization. Likewise, Figure 2B shows a peak of TGase activity in DZ that also precedes the time of Z-wall insolubilization and the peak of H_2O_2 and peroxidase activity (arrow). Figure 2C shows the incorporation time courses for a crude cell-free preparation of TGase isolated from RVC at the time of maximum enzyme activity, as determined from the data presented in Figure 2A. Curve A represents incorporation values from a crude preparation that was twice as concentrated as the sample used to generate curve B. The two curves clearly demonstrate enzymic behavior. The activity for the V-wall TGase was 78.4 pmol incorporation h^{-1} mg^{-1} and for the Z-wall enzyme, 90 pmol h^{-1} mg^{-1}. The K_m values were 1.8 mM (RVC) and 2.0 mM (DZ). V_{max} for the RVC enzyme was 180 pmol h^{-1} mg^{-1} $casein^{-1}$ and for the DZ enzyme 185 pmol h^{-1} mg^{-1} $casein^{-1}$. These values for K_m and V_{max} are somewhat misleading because the crude enzyme preparation we assayed contains natural substrates for the TGase (wall proteins secreted into the media) that compete with the substrate (casein) we were using to measure activity. Thus, truly meaningful characterization of the *C. reinhardtii* TGase enzyme activities will have to be done with more purified enzyme preparations.

Response of *C. reinhardtii* TGase to Various Inhibitors

Using the supernatants from aliquots taken at the times of maximum TGase activity in RVC and DZ (as determined by ^{14}C-putrescine incorporation), we tested this enyzme activity for Ca^{2+} dependence and sensitivity to thiol inhibitors and Zn^{2+} with the hydroxamate-formation assay of Folk and Cole (1966). The hydroxamate assay offers direct evidence, independent of the ^{14}C-putrescine/dimethyl casein assay, of a *C. reinhardtii* TGase activity capable of cross-linking hydroxylamine (amine donor) to a carbobenzoxy-L-glutaminylglycine (substrate), forming an iron-hydroxamate complex. The rate of hydroxamate formation in RVC was 30.8 pmol h^{-1} mg^{-1} and in DZ was 45.1 pmol h^{-1} mg^{-1}.

While the vast majority of animal TGases show a strong Ca^{2+} dependence, the plant TGases examined to date do not (Falcone et al., 1993; Serafini-Fracassini et al., 1995). Animal TGases are also inhibited by low levels (<50 μM) of Zn^{2+}; the effect of Zn^{2+} on plant TGases has not been studied. As shown in Table I, *C. reinhardtii* TGases, like those of higher plants, do not appear to have a strict requirement for Ca^{2+} and are only slightly inhibited by 1 mM Zn^{2+}. The lack of inhibition by Zn^{2+} is not surprising since the growth medium for both vegetative cells and zygotes normally contains 76 μM Zn^{2+} (Harris, 1989). The thiol inhibitors *N*-ethylmaleimide and *p*-chloromercuribenzoate completely blocked both *C. reinhardtii* enzymes, which is typical for TGases.

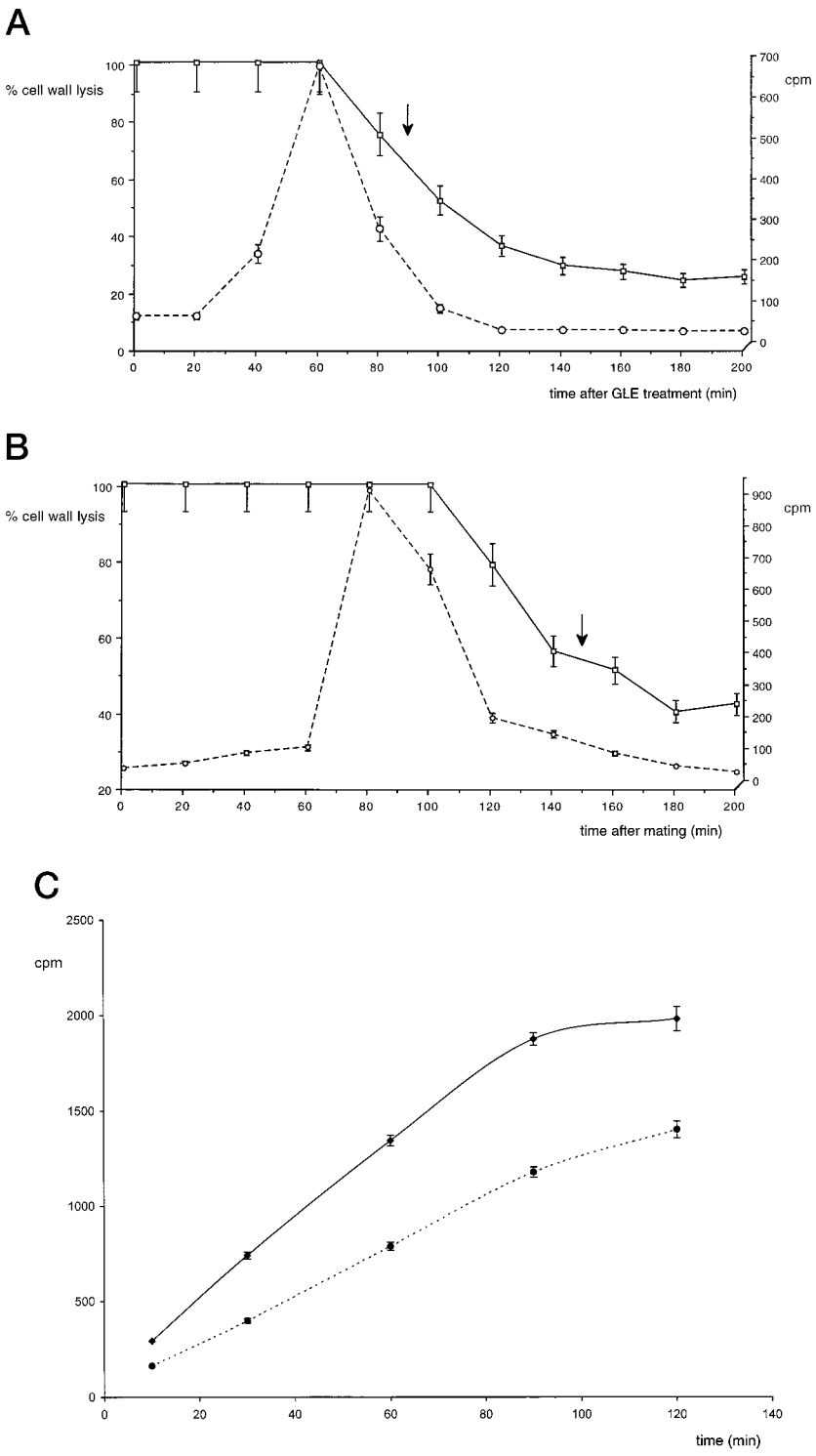

Figure 2. TGase, peroxidase, and H_2O_2 levels during cell wall formation. A, Vegetative cells were treated with GLE for 1 h, washed, and resuspended in fresh medium (time 0). Aliquots were removed at 20-min intervals and analyzed for detergent sensitivity (%), TGase (cpm), peroxidase activity, and H_2O_2 production. TGase activity was assayed by measuring ^{14}C-putrescine incorporation into dimethyl casein. Peroxidase activity and H_2O_2 accumulation were determined spectrophotometrically using vanillin and KI, respectively, as substrates. Only the time point (arrow) of maximum peroxidase activity and highest H_2O_2 level is indicated in this figure. □, % Cell wall lysis; ○, counts per minute. B, Equal numbers of gametes of each mating type were mixed (time 0) and aliquots were taken at 20-min intervals to assay for detergent sensitivity (%), TGase (cpm), peroxidase activity, and H_2O_2 accumulation. Again, only the time point of peak peroxidase activity and H_2O_2 accumulation is indicated (arrow). □, % Cell wall lysis; ○, counts per minute. C, Incorporation time courses were derived from aliquots of supernatants collected from RVC at the time of maximum TGase activity. Curve A (◆) presents the incorporation for an undiluted aliquot of supernatant, while curve B (●) shows the incorporation for a sample that is diluted 1:1 with buffer. Error bars represent the results from three independent assays.

In Vivo TGase Assays

Given the presence of TGase activity in culture supernatants, we next looked for TGase activity associated with wall-regenerating cells. For these experiments, RVC or DZ were resuspended in medium containing ^{14}C-putrescine. The concentration of putrescine in these cultures (0.22 μM) was far below the inhibiting levels shown in Figure 1 because in this case we were testing whether the labeled putrescine could be cross-linked by TGase onto cell surface substrates (e.g. wall proteins). At each time point, an aliquot was removed and the cells were pelleted, washed, and assayed for ^{14}C-putrescine incorporation. Another aliquot was taken to determine detergent sensitivity. Figure 3 shows the two curves (incorporation and detergent sensi-

Table I. *Inhibitors of C. reinhardtii TGase activity*

Substance	TGase Activity	
	RVC	DZ
	% of max. activity	
EGTA		
0.1 mM	79	80
1.0 mM	75	58
Zn^{2+}		
0.1 mM	92	92
1.0 mM	73	67
N-Ethylmaleimide 1.0 mM	0	0
p-Chloromercuribenzoate 1.0 mM	0	0

tivity) for RVC (Fig. 3A) and DZ (Fig. 3B). The incorporation values are given as percentages of the counts per minute originally added to the medium. Both cell types were labeled with ^{14}C-putrescine, and the maximum rates of incorporation occurred within the time of highest TGase activity, as determined in vitro with the culture supernatants (see Fig. 2).

Identification of Potential TGase Substrates

The in vitro assays indicated that the TGases are extracellular, suggesting that the in vivo labeling shown in Figure 3 may have occurred on cell wall substrates. We took the remaining vegetative cells from the in vivo labeling experiment described above and treated them with sodium perchlorate. This salt extraction solubilizes glycoproteins of the outer layers of the V-wall, leaving behind the glycoproteins of the insoluble inner layer (Hills et al., 1975). As seen in Table II (which presents data averaged over six separate extractions), when this salt-soluble fraction was assayed for radioactivity, 53% of the total counts per minute incorporated into the RVC were released. Next, the salt-extracted cells were incubated with GLE, which cleaves off the insoluble inner V-wall layer (Goodenough and Heuser, 1985; Imam and Snell, 1988; Waffenschmidt et al., 1988). This treatment released another 39% of the total counts per minute. This leaves 8% of the counts still on the cell surface, possibly attached to the radiating wall fibers left behind after GLE cleavage (Goodenough and Heuser, 1985). Since the Z-wall is not salt-soluble or susceptible to GLE, we used acidified chlorite to cleave the phenolic cross-links within the Z-wall (Jaenicke et al., 1987; Biggs and Fry, 1990; Waffenschmidt et al., 1993). This released 86% of the counts incorporated into DZ (Table II).

Since 53% of the counts incorporated into RVC are found within the well-studied outer layers of the V-wall (see Woessner and Goodenough, 1994), we wished to determine which salt-soluble components are substrates for TGase. Vegetative cells that had regenerated for 3 h in the presence of non-inhibiting concentrations of ^{14}C-putrescine were pelleted and extracted with sodium perchlorate, and the supernatant was lyophilized. Samples of both the salt extract and the supernatant were analyzed by SDS-PAGE and autoradiography. As shown in Figure 4, most of the previously identified salt-soluble glycoproteins (Goodenough et al., 1986) were labeled and present in both the extract (lane A) and the supernatant (lane B). There were, however, other polypeptides in the supernatant that were not labeled (lane C), which is consistent with the finding that TGases recognize only certain protein-bound Gln as acceptor substrates (Folk and Cole, 1966; Dutton and Singer, 1975).

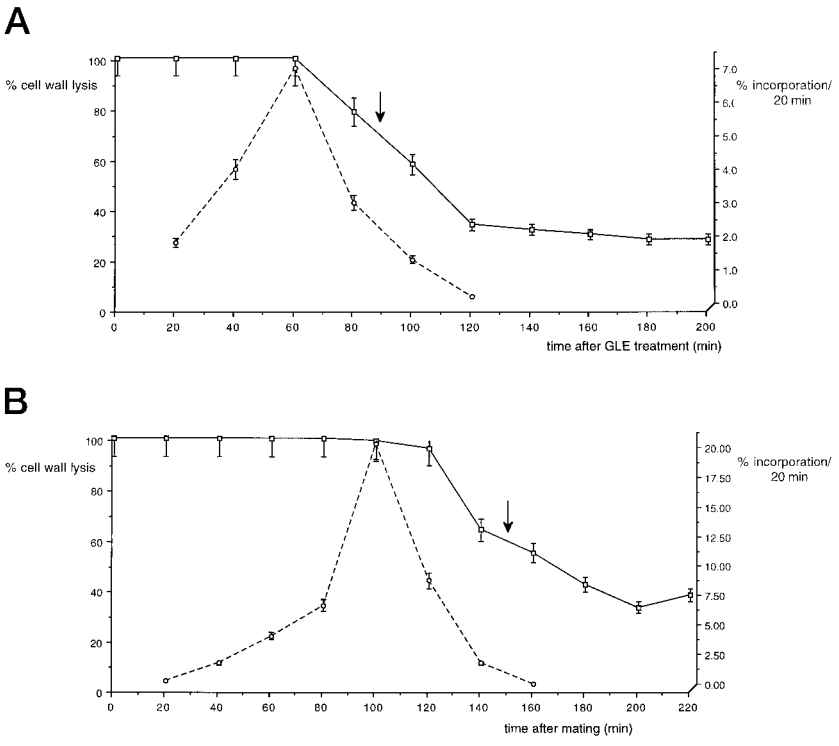

Figure 3. ^{14}C-Putrescine incorporation during cell wall formation. A, Vegetative cells were treated with GLE for 1 h, washed, and resuspended in fresh medium containing a non-TGase-inhibiting concentration of ^{14}C-putrescine (time 0). Every 20 min aliquots were taken and the cells were either tested for detergent sensitivity, as described in Figure 1, or pelleted, washed, and assayed for radioactivity. Incorporation is presented as a percentage of the original quantity of ^{14}C-putrescine added to the medium. B, Gametes of both mating types were mixed in equal numbers. After 30 min of mating, a non-inhibiting quantity of ^{14}C-putrescine was added to the medium. Samples were taken every 20 min and the cells were tested for detergent sensitivity and incorporation of radioactivity as above. Error bars represent the results from three independent assays. □, % Cell wall lysis; ○, % incorporation over 20 min.

Table II. *Analysis of ^{14}C-putrescine-labeled cells*

	RVC	DZ
	cpm	
Total incorporation	11,480	20,160
$NaClO_4$-soluble	6,140	ND[a]
GLE extractable	4,530	ND
Chlorite extractable	ND	17,260

[a] ND, Not determined.

Involvement of TGase in V-Wall Assembly

Having identified outer wall glycoproteins as potential TGase substrates, our next goal was to define the role of TGase in the assembly of these components. One well-documented trait of the salt-soluble glycoproteins is that, upon dialysis of the salt, they can self-assemble into a crystalline array similar to that found in muro (Hills et al., 1975; Goodenough et al., 1986). We first examined whether inhibition of TGase had any effect on self-assembly. RVC were grown in the presence or absence of 50 mM putrescine and after 3 h the cells were pelleted, resuspended, and sodium perchlorate extracted; the supernatants were lyophilized and resuspended in sodium perchlorate. All four samples were then dialyzed to remove the salt and allow self-assembly into wall crystals. Crystals, observed in all four samples, were pelleted and analyzed by SDS-PAGE as shown in Figure 5.

Only wall crystals were loaded on the gel, so everything on the gel was assembly competent and the material derived from equal numbers of cells. The salt-extracted components of RVC in the absence of putrescine displayed all of the glycoproteins in abundance (lane B), whereas in the presence of inhibiting levels of putrescine, relatively few glycoproteins were extracted (lane C). Reciprocally, few outer wall glycoproteins were present in the supernatant of control RVC without putrescine (i.e. the majority had assembled onto the cell surface, lane D), while the supernatant from RVC in putrescine contained abundant outer-wall glycoproteins (lane E). Inhibition of TGase therefore does not block the potential for self-assembly, but it appears to block the ability of outer wall glycoproteins to assemble onto the cell.

The assembly of outer wall glycoproteins onto the cells can be studied in vitro as well (Hills et al., 1975; Adair et al., 1987). When "shells" (vegetative cells that have been perchlorate extracted and concomitantly killed) are dialyzed together with the solubilized glycoproteins, the glycoproteins assemble onto the shells, reforming a normal V-wall (Adair et al., 1987). The experiment documented in Figure

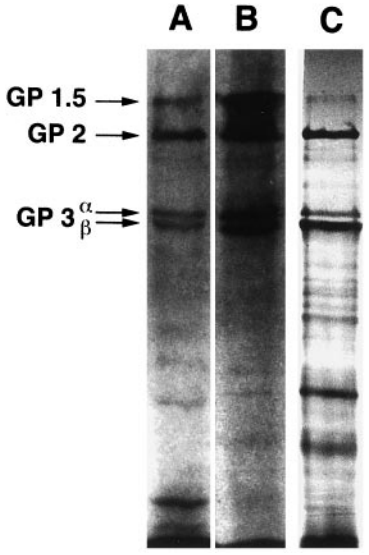

Figure 4. Identification of ^{14}C-putrescine-labeled wall proteins. Vegetative cells were treated with GLE for 1 h, washed, and resuspended in fresh medium with a non-inhibiting quantity of ^{14}C-putrescine. After 3 h, the cells were pelleted and extracted with $NaClO_4$. Both the salt-extracted proteins and the medium from which the cells were pelleted were dialyzed against distilled water, TCA precipitated, and analyzed by SDS-PAGE and autoradiography. Lane A shows the autoradiograph of the $NaClO_4$-extracted proteins, and lane B shows the labeled proteins in the supernatant. Lane C shows an equal loading of the supernatant sample that has been silver-stained to reveal all of the proteins actually present. The outer wall salt-soluble glycoproteins (GP) that have been previously identified and characterized (Goodenough et al., 1986) are indicated adjacent to lane A.

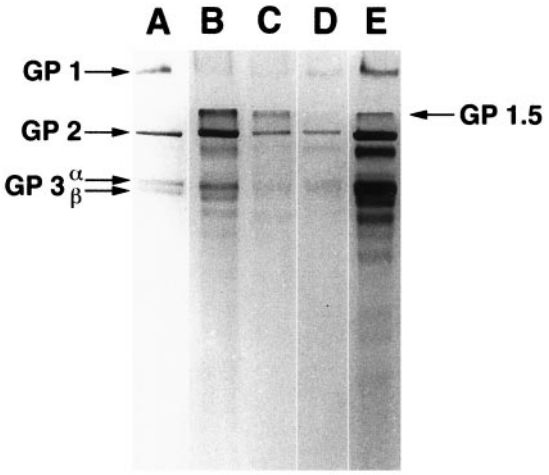

Figure 5. Putrescine's effects on self-assembly of outer wall proteins. GLE-treated vegetative cells were washed and resuspended in medium with or without inhibiting levels of putrescine. After 3 h, the cells in both cultures were pelleted and the two supernatants were lyophilized and resuspended in sodium perchlorate, while the cells were sodium perchlorate extracted. All four samples, the supernatants and the two salt extracts, were dialyzed against distilled water to remove the chaotrope and initiate self-assembly of outer wall proteins into crystals. The crystals of all four samples were analyzed by SDS-PAGE and the gel was stained with periodic acid-Schiff reagent. Lane A is a standard of purified outer wall crystals. The next two lanes are the crystals from the salt extracts of control (lane B) and putrescine-inhibited (lane C) cells. The last two lanes show the crystals from the control (lane D) and putrescine-inhibited (lane E) supernatants. The previously identified outer wall glycoproteins are indicated along the outside of lanes A and E. Periodic acid Schiff-staining of GP1.5 is concentration dependent and there is too little of this glycoprotein (GP) in the standards (lane A) for it to be visibly stained.

6 compared shells from normal vegetative cells and RVC in the absence or presence of inhibiting concentrations of putrescine. Each type of shell was dialyzed with perchlorate-solubilized glycoproteins labeled with ^{14}C-putrescine. After assembly, the shells were washed, re-extracted with sodium perchlorate, and the solubilized fractions counted for radioactivity. Shells from untreated vegetative cells and from RVC unexposed to putrescine nucleated assembly of five to six times the amount of labeled glycoproteins, as did shells from RVC in the presence of putrescine (Fig. 6). This provides quantitative support for the hypothesis that TGase is involved in rendering the cells competent to nucleate the assembly of outer wall glycoproteins.

Direct visual proof that TGase activity is essential to wall assembly in RVC was provided by the electron micrographs shown in Figure 7. These are images of the wall on RVC that have been incubated in medium with or without inhibiting concentrations of putrescine. After regeneration in the absence of putrescine (Fig. 7A), the insoluble V-wall had all of the layers described in detail by Goodenough and Heuser (1985): a recognizable crystalline layer (W6), globular components (W4), and a highly cross-linked inner layer (W2) all interwoven on long W1/W7 fibers radiating out from the plasma membrane. In contrast, the wall found on RVC incubated in putrescine displayed only radiating short fibers (Fig. 7B).

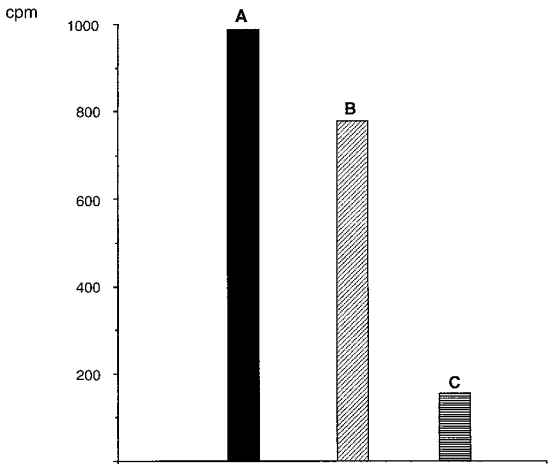

Figure 6. Quantitative determination of putrescine's effects on cell wall assembly. A culture of vegetative cells was divided into three equal volumes (A, B, and C). Volume A was the non-GLE-treated control, while volumes B and C were GLE-treated, washed, and allowed to regenerate for 3 h in medium lacking putrescine (B) or containing inhibiting levels of putrescine (C). After 3 h all samples were sodium-perchlorate-extracted, removing the soluble outer wall proteins and leaving behind extracted cells. These cells were pelleted, washed, and mixed with radioactively labeled assembly-competent outer wall proteins in sodium perchlorate. The three samples were dialyzed overnight against water to initiate assembly onto the cell surface. After extensive washing to remove unassembled proteins, the three samples were re-extracted with sodium perchlorate and the radioactivity of the solubilized components was determined.

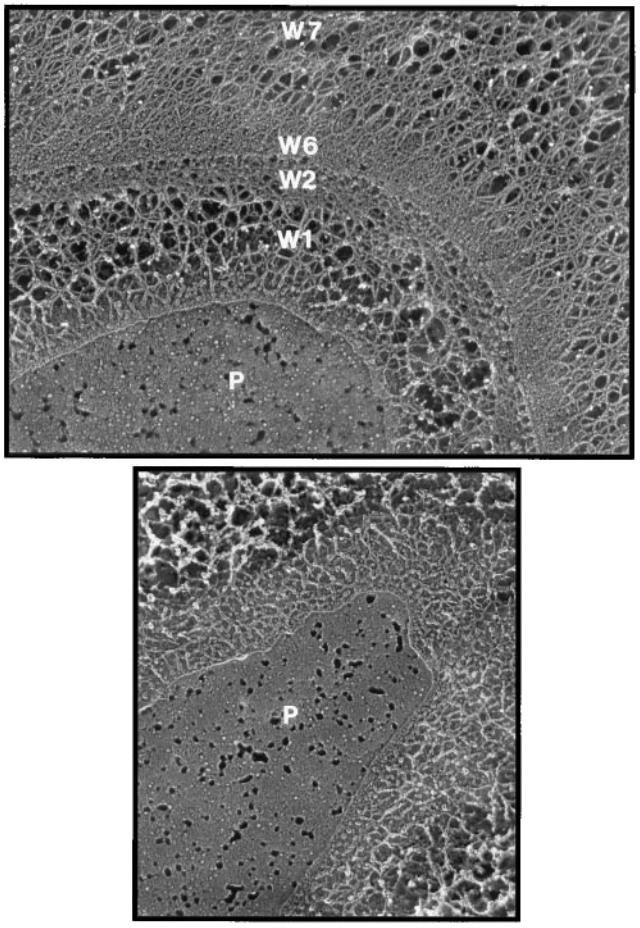

Figure 7. Electron micrographs of cell walls from control (A) and putrescine-treated (B) RVC quick-frozen 3 h after GLE treatment. P, Plasma membrane; W1, W2, W6, and W7, layers of the wall.

Examination of Membrane-Bound Protein Cross-Linking

The electron micrograph images, in accordance with our other studies, suggested that TGase is involved in an early stage of wall assembly. Goodenough and Heuser (1985), after examining many electron micrographs of vegetative wall growth, proposed that the earliest visible event of V-wall formation is the appearance of long fibers (W1/W7) radiating from the plasma membrane. To look for TGase-mediated cross-linking of membrane-bound proteins, we set up an experiment identical to in vivo labeling, resuspending 100 mL of RVC in ^{14}C-putrescine. At 5-min intervals we collected aliquots of cells. Each sample was sodium perchlorate extracted to remove all soluble wall proteins. Then the cells were lysed with digitonin (solubilizing the plasma membrane), subjected to osmotic shock, and a microsomal fraction was prepared by ultracentrifugation. Each microsomal pellet was resuspended in loading buffer and subjected to SDS-PAGE. Figure 8 presents the autoradiograph of three pellets from successive time points. No labeled band is visible by SDS-PAGE until 35 min into the wall formation (lane 1). This molecule is over 200 kD; the largest molecular mass standard on the gel was 193 kD. Within the next 5 min this band disappeared and was

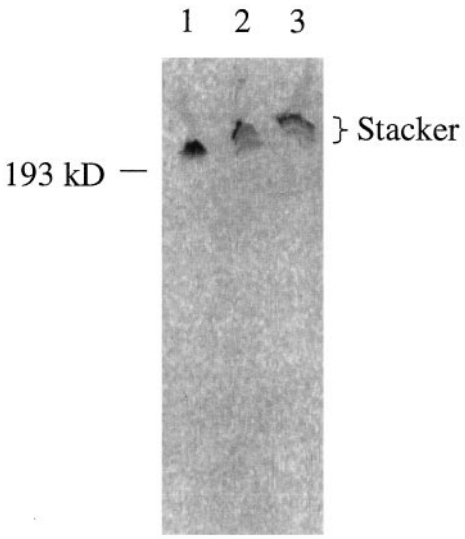

Figure 8. Cross-linking of membrane-bound proteins. GLE-treated vegetative cells were washed and resuspended in media with ^{14}C-putrescine. Samples were collected every 5 min and extracted with sodium perchlorate to remove soluble wall proteins. The plasma membrane of the cells was solubilized in digitonin, subjected to osmotic shock, and a microsomal fraction was isolated by ultracentrifugation. Each microsomal pellet was resuspended in loading buffer and analyzed by SDS-PAGE and autoradiography. Lane 1 shows the sample collected at 35 min, lane 2 is the 40-min sample, and lane 3 is from 45 min. Only the position of the largest molecular mass standard, 193 kD, is indicated to the left of lane 1. The brackets on the right side of lane 3 delineate the boundaries of the stacking gel. Bands located in the stacker are typically diffuse.

replaced by a diffuse band that migrated in the middle of the stacking gel (lane 2). By 45 min, the labeled band had become so large it could not even run through the stacking gel (lane 3). A signal was found at this position in subsequent time points as well (data not shown). Our interpretation of these results is that we were seeing a plasma membrane-bound protein being rapidly cross-linked by TGase.

Characterization of Incorporated ^{14}C-Putrescine

The results of the hydroxamate formation assay can only be ascribed to the presence of an extracellular TGase activity in *C. reinhardtii*. However, it was important to document that the putrescine inhibition and labeling results were not due to amine oxidase activity. Diamine oxidases, enzymes commonly found in plant cells, can oxidize putrescine to mono- and di-aldehydes that indiscriminately inhibit many biological processes, and such aldehydes could also potentially cross-link proteins through non-enyzmatic reactions involving Schiff's base intermediates. If amine oxidases were involved, then the incorporated ^{14}C-putrescine in the in vitro and in vivo labeling experiments would be oxidized to an aldehyde. Therefore, we set up two experiments to characterize the incorporated putrescine molecules. First, 20 μCi of ^{14}C-putrescine was added to our standard in vitro assay using dimethylcasein and cell-free supernatant isolated from RVC at the time of maximal TGase activity. Following a 30-min incubation, the proteins were precipitated out of solution using TCA in the presence of excess cold putrescine. Second, the same amount of label was added to 100 mL of RVC in our standard in vivo assay. After 30 min, the cells were pelleted out of the medium and perchlorate extracted. These salt-soluble glycoproteins and the TCA-precipitated proteins from the in vitro assay were hydrolyzed in 6 N HCl and assayed for radioactivity; 86% of the incorporated radioactivity was recovered for the in vitro experiment and 87% for the in vivo experiment. Both hydrolysates were dried under nitrogen, resuspended in phosphate buffer, loaded onto a phosphocellulose column, and eluted fractions assayed for radioactivity. The column was calibrated using authentic ^{14}C-putrescine. As shown in Figure 9, the hydrolyzed label from both the in vitro and in vivo experiments eluted in the

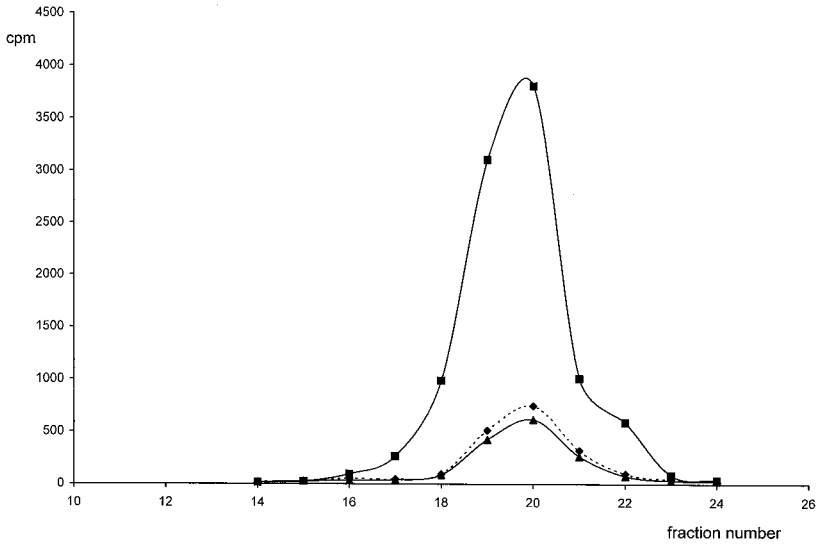

Figure 9. Characterization of incorporated ^{14}C-putrescine. The ^{14}C-putrescine-labeled dimethyl casein substrate from a standard in vitro TGase assay with RVC (▲) and the $NaClO_4$-solubilized outer wall proteins labeled with ^{14}C-putrescine in an in vivo TGase assay (♦) were hydrolyzed in 6 N HCl. The hydrolysates were loaded onto a phosphocellulose column and the eluted fractions were tested for radioactivity. The control curve is the elution of authentic ^{14}C-putrescine. Error bars indicate the results from three independent assays. ■, Control.

same fractions as the control, proving that the labeled putrescine was incorporated without metabolic conversion.

Immunoprecipitation with Antibodies to Mammalian TGase

Confident that we were analyzing a bona fide TGase, we tested for antigenic conservation between this algal enzyme and a mammalian counterpart using polyclonal antibodies to guinea pig tissue TGase (Aeschlimann et al., 1993). The proteins secreted into the medium of RVC and DZ at the times of maximal TGase activity were separated by SDS-PAGE, blotted to nitrocellulose, and probed with the anti-TGase serum. Figure 10, A and B, indicate that the serum cross-reacts with a 72-kD band found in the supernatant of both RVC and DZ. To rule out that this cross-reactivity is due to a nonspecific interaction, we reprobed the western blot using antiserum that had been preincubated with increasing amounts of purified guinea pig tissue TGase. Figure 10C shows that the 72-kD signal was competed away in a concentration-dependent manner. Lastly, we used the anti-TGase serum in immunoprecipitations of the cell-free supernatants of both RVC and DZ isolated at the time points of maximum TGase activity. The resulting antigen/antibody complexes were adsorbed onto protein A-agarose and pelleted out of solution. Following this immunoprecipitation, TGase activity was detected only in the pelleted complexes and not in the treated supernatants (data not shown).

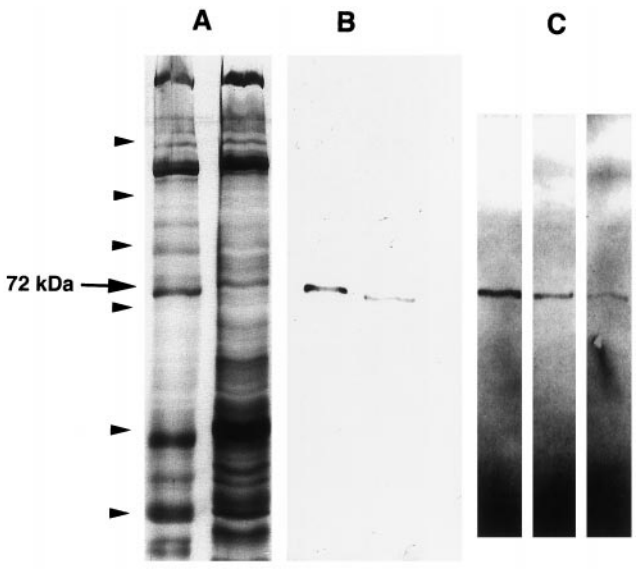

Figure 10. Antigenic conservation between the cell wall TGase of *C. reinhardtii* and the tissue TGase of guinea pig. A, The growth media was collected from RVC (left lane) and DZ (right lane) at the times of maximal TGase activity as determined in Figure 2. The secreted proteins were TCA-precipitated out of the medium, separated by SDS-PAGE, and the silver-stained gel is presented. The positions of the molecular mass markers (193, 112, 86, 70, 57, and 39.5 kD) are indicated by arrowheads. B, Two lanes identical to those in A were blotted onto nitrocellulose and probed with antiserum against guinea pig TGase. C, Three samples of the RVC secreted proteins were isolated, electrophoresed, and blotted as described above. The left blot was probed with only anti-TGase serum as in B. The next two blots were probed with the same antiserum after preincubation with either 2.5 (middle) or 10 μg (right) of purified guinea pig TGase.

DISCUSSION

Delineating the assembly program for a plant extracellular matrix is a challenging task, yet we have made considerable progress toward this goal in our studies of cell wall protein interactions in *C. reinhardtii*. As noted in the introduction, these algal cell walls lack the complex diversity of matrix molecules found in higher plants. Nevertheless, matrix assembly in *C. reinhardtii* is not an elementary process. To date, three distinct assembly events have been identified: the early TGase-catalyzed cross-linking described in this paper, the self-assembly of glycoproteins into a crystalline array (Hills et al., 1975; Goodenough et al., 1986), and an oxidative cross-linking reaction (Waffenschmidt et al., 1993). We propose, as detailed below, that all three events must transpire sequentially to achieve insolubilization of the V-wall.

Although we have demonstrated the existence of an extracellular TGase in *C. reinhardtii* whose activity is essential to cell wall formation, the role of this enzyme in early wall formation remains unclear in both RVC and DZ. Nevertheless, the large body of literature on the V-wall has facilitated a more detailed examination of matrix assembly in RVC than is currently possible in DZ. Following a crude but accepted method, we identified most of the soluble outer wall glycoproteins in RVC as substrates for the TGase when putrescine is added. Since these would no longer be soluble if indeed they were covalently cross-linked to each other, our interpretation is that these glycoproteins carry potential sites for covalent attachment of putrescine by TGase, but these sites are not the preferred substrate and are normally inaccessible, unavailable, and/or unused in muro.

In contrast, the labeling of inner-wall components in vivo is likely to be significant because other data indicate that the TGase activity appears to be responsible, directly or indirectly, for nucleating the assembly of the wall. EM images confirm the lack of both the soluble W6 layer and the insoluble W2 layer on the cell surface of RVC incubated with inhibiting levels of putrescine, and a previous EM study of wild-type RVC indicated that the assembly of W6 precedes that of the W2 layer (Goodenough and Heuser, 1985). The EM image of putrescine-inhibited RVC also shows only small fibers radiating out from the plasma membrane. These short fibers might be precursors of the much longer W1/W7 molecules. These data, together with the results of the SDS-PAGE indicating cross-linking, suggest that the sequence of matrix assembly includes an early TGase cross-linking of yet-to-be-identified membrane-bound wall molecules (possibly comprising the W1/W7 radial fibers). This cross-linking somehow facilitates and/or initiates the self-assembly of the W6 crystalline layer. Assembly of the inner W2 layer components rapidly follows and, finally, a peroxidase-mediated oxidative cross-linking of these proteins leads to full insolubilization of the wall.

When we presented our previous work on peroxidase-mediated cross-linking in *C. reinhardtii* walls (Waffenschmidt et al., 1993), we proposed, based on the model for the hardening of the sea urchin vitelline layer (Shapiro, 1991), that the early assembly of the crystalline layer of salt-extractable W6 and W4 components might serve to organize and position the underlying W2 components prior to the ensuing oxidative cross-linking. Now we can carry the analogy one step further. In the sea urchin, an early TGase-catalyzed event leads to the formation of a non-cross-linked "soft envelope" that, in turn, organizes underlying proteins prior to the oxidative cross-linking responsible for the hardened fertilization membrane (Battaglia and Shapiro, 1988). If this sea urchin TGase is inhibited, then a fertilization envelope is never formed. Likewise, there is an extracellular TGase in *C. reinhardtii* RVC whose inhibition prevents assembly of the "soft envelope" of W4 and W6 proteins and, consequently, insolubilization of the V-wall.

This commonality in matrix insolubilization programs between *C. reinhardtii* and sea urchin could be entirely fortuitous, but TGases participating in matrix hardening have been found in organisms as distant as bacteria and humans (Rice et al., 1993). Indeed, previous studies have even used comparisons of TGase amino acid sequences to develop phylogenetic trees (Tokunaga et al., 1993). One of the most conserved domains of the TGase enzyme at the amino acid level corresponds to the active site, and DNA probes from this region have been used to clone TGases from different organisms (Floyd and Jetten, 1989; Kim et al., 1991). Perhaps the antigenicity of this domain is the reason why antibodies to animal TGases cross-react with homologs in filarial parasites (Mehta et al., 1992), higher plants (Del Duca et al., 1994, 1997), *Dunaliella salina* (Serafini-Fracassini et al., 1995), and *C. reinhardtii*. To date, only intracellular TGases have been reported in higher plants (Serafini-Fracassini et al., 1995; Del Duca et al., 1997; Hou and Lin, 1997). However, high-molecular-mass substrates for TGase in non-green tissue of *Helianthus tuberosus* were identified by Falcone et al. (1993), and these could correspond to cell wall structural proteins. The implication is that there could be extracellular TGases integral to cell wall formation in higher plants as well, as has been speculated (Serafini-Fracassini et al., 1995).

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