The *Chlamydomonas* Cell Wall: Characterization of the Wall Framework

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ABSTRACT The cell wall of the biflagellated alga *Chlamydomonas reinhardtii* is a multilayered, extracellular matrix composed of carbohydrates and 20–25 polypeptides. To learn more about the forces responsible for the integrity of this cellulose-deficient cell wall, we have begun studies to identify and characterize the framework of the wall and to determine the effects of the cell wall–degrading enzyme, lysin, on framework structure and protein composition. In these studies we used walls released into the medium by mating gametes. When isolated shed walls are degraded by exogenously added lysin, no changes are detected in the charge or molecular weight of the 20–25 wall proteins and glycoproteins when analyzed on one- and two-dimensional polyacrylamide gels, which suggests that degradation of these shed walls is due either to cleavage of peptide bonds very near the ends of polypeptides or that degradation occurs via a mechanism other than proteolysis. Incubation of walls with Sarkosyl-urea solutions removes most of the proteins and yields thin structures that appear to be the frameworks of the walls. Analysis by polyacrylamide gel electrophoresis shows that the frameworks are highly enriched in a polypeptide of M_r 100,000. Treatment of frameworks with lysin leads to their degradation, which indicates that this part of the wall is a substrate for the enzyme. Although lysin converts the M_r 100,000 polypeptide from an insoluble to a soluble form, there is no detectable change in M_r of the framework protein. Solubilization in the absence of lysin requires treatment with SDS and dithiothreitol at 100°C.

These results suggest that the *Chlamydomonas* cell wall is composed of two separate domains: one containing ~20 proteins held together by noncovalent interactions and a second domain, containing only a few proteins, which constitutes the framework of the wall. The result that shed walls can be solubilized by boiling in SDS-dithiothreitol indicates that disulfide linkages are critical for wall integrity. Using an alternative method for isolating walls from mechanically disrupted gametes, we have also shown that a wall-shaped portion of these non-shed walls is insoluble under the same conditions in which shed walls are soluble. One interpretation of these results is that wall release during mating and the wall degradation that follows may involve distinct biochemical events.

The cell wall of the biflagellated alga *Chlamydomonas reinhardtii* provides structural support for the cell and may regulate movement of molecules into and out of the cell. This complex extracellular matrix is composed of carbohydrates and several hydroxyproline-rich proteins arranged into at least seven distinct layers visible in the electron microscope with the outer layers forming a crystalline lattice (3, 4, 15, 17, 18). It is not clear whether all of the wall carbohydrate is in glycoproteins, but, unlike the cell walls of higher plants, the *Chlamydomonas* wall contains no cellulose (8, 17). This observation raises the question as to what provides the underlying structural support or framework for the wall. Since walls contain a substantial amount of carbohydrate, high molecular weight carbohydrate polymers other than cellulose might be essential for wall structure. On the other hand, these walls also contain substantial amounts of protein; therefore, it may be that proteins alone or in a complex with polysaccharides form the integral part of the wall.

Studies by Roberts (17) and Hills et al. (4) have shown that walls isolated from the medium of vegetatively growing cells can be separated into soluble and insoluble fractions by chaotropic salts such as lithium chloride or sodium perchlor-

ate. The insoluble portion retains the overall saclike morphology of the wall, although it appears to be much thinner than the intact wall. Electron microscopic examination showed that this part of the wall is amorphous. These results suggest that the salt-insoluble part of the wall may be the actual framework for the wall and that the soluble portion includes the crystalline lattice. In support of this idea, Hills et al. (4) have reported that removal (by dialysis) of the solubilizing agent leads to reassembly of the walls, including the reappearance of the crystalline lattice. If the insoluble portion of the wall is removed before dialysis, fragments of the crystalline lattice reassemble but not into a wall-shaped structure.

Other than these results there is little information on the nature of the forces that maintain the integrity of the wall. Lefebvre et al. (11) reported that incubation of intact vegetative or gametic cells at 100°C in SDS and β-mercapoethanol solubilized most cellular proteins but left cell ghosts that had the same size and morphology of intact cell walls, which suggested that at least part of the wall with the overall shape of the wall is held together by nondisulfide, nonionic bonds. These results are consistent with work by Roberts et al. (18), who have reported that the chaotropic salt–insoluble portion of vegetative walls shed during cell division is also insoluble after boiling in SDS and reducing agents. And Voigt (22) has reported that part of the cell wall is not solubilized after incubation for 16 h at room temperature in buffer containing 2% SDS, 200 mM β-mercaptoethanol, and 8 M urea.

We recently developed a procedure for rapid isolation of cell walls shed by gametes during the mating reaction (20) and have begun biochemical studies on these walls with the goal of learning more about the intermolecular forces responsible for wall integrity. Gametic shed walls are particularly amenable to these studies because they are completely soluble in SDS and diothiothreitol (DTT).[1] Our results indicate that shed walls contain a framework composed of from one to four proteins which represent ~10% of the total protein of the wall. The data suggest that this framework, which is a critical site of action of the cell wall–degrading enzyme lysin (1, 6, 13, 19, 20, 21), contains disulfide bonds essential for the integrity of the shed walls. Using an alternative method for isolating walls from mechanically disrupted gametes, we have also shown that a wall-shaped portion of these mechanically isolated walls is insoluble under the same conditions in which the gametic, shed walls are soluble. One interpretation of these results is that wall release during mating and the wall degradation that follows might involve distinct biochemical events. Part of this work has appeared in abstract form (5).

MATERIALS AND METHODS

Cultures: Methods for maintaining stock cultures, cell growth in suspension, cell harvesting, and induction of gametes were essentially the same as reported previously (20).

Isolation of Shed Walls: 50 ml each of mt^+ and mt^- gametes (1×10^7 cells/ml) were centrifuged at 2,600 *g* (3,000 rpm, 4°C, Sorvall RC-3B refrigerated centrifuge, rotor H-6000A, DuPont Instruments–Sorvall Biomedical Div., Wilmington, DE) for 3 min. The sedimented cells were resuspended in nitrogen-free medium (M-N), recentrifuged, and the cells in each tube were resuspended in 5 ml M-N. After the temperature of each cell suspension was brought to 12°C the gametes of opposite mating types were mixed and incubated with gentle agitation at 12°C. After 3 min, 10 ml ice-cold TE containing 10 mM Tris, 10 mM EDTA, pH 7.4, was added, and the suspension was centrifuged at 2,500 rpm for 2 min at 4°C as above. The supernatant containing cell walls was collected, and the centrifugation step was repeated once or twice until the remaining cell bodies had been removed. The walls were harvested from the supernatant by centrifugation at 27,000 *g* (16,000 rpm, Sorvall RC-5b centrifuge, rotor SA600), for 30 min at 4°C. The sedimented cell walls were resuspended in TE and stored at −20°C.

Isolation of Walls from Mechanically Disrupted Gametes: Mt^+ gametic cells (32 L in M-N) were harvested by centrifugation, resuspended in Tris-sucrose buffer (4% sucrose, 10 mM Tris, pH 7.8), and deflagellated by the pH-shock method of Witman et al. (23). The suspension of cells and flagella was underlaid with 20 ml 25% Tris-sucrose buffer, pH 7.8, and centrifuged at 2,000 *g* for 10 min at 4°C to sediment the cell bodies. The deflagellated cells were resuspended in 5 vol of 95% ethanol, stirred with a magnetic stirrer for 5 min, and centrifuged at 4,600 *g* for 5 min at 4°C. The sedimented cells were extracted two more times with 95% ethanol to yield an orange-brown pellet of cell bodies which was resuspended in 1 vol 10 mM HEPES, pH 7.2. This suspension was shaken by hand for 5 min with 1 vol 0.5-mm glass beads (VWR Scientific Inc., San Francisco, CA). The beads were allowed to settle and the liquid phase that contained the partially disrupted cells, cell walls, and undisrupted cell bodies was decanted, pooled with two additional washings of the beads, and centrifuged at 4,600 *g* for 5 min at 4°C. The cell walls remained both in the supernatant and in a loose, semigelatinous layer above the firm pellet of cell bodies. The tube was gently swirled to resuspend the loose layer of cell walls into the supernatant, which was then decanted from the cell bodies. The intact cells were treated two more times with beads as above and the cell walls were harvested from the 4,600-*g* supernatants by centrifugation at 20,000 *g* for 20 min at 4°C. The walls were gently resuspended away from a denser layer consisting of contaminating cell bodies and purified by sequential centrifugations at 4,600 and 20,000 *g*, taking the supernatants and pellets, respectively, as described above. Walls were stored as 20,000 *g* pellets in azide.

Fractionation of Intact Shed Walls: To 1 vol (200–400 μl) concentrated ice-cold shed cell walls (~200–400 μg protein) was added 1.5 vol of concentrated solubilization buffer to give a final concentration of 0.7% Sarkosyl in 10 mM Tris, pH 7.2, 10 mM EDTA, 0.1 M KCl, 6 M urea, and 1 mM DTT. The suspension was incubated in a microfuge tube at room temperature. After 60 min, when microscopic examination of stained samples indicated that the walls appeared much thinner, 1 ml TE was added to the suspension and this mixture was centrifuged at 26,000 *g* (12,500 rpm, Sorvall RC-5b centrifuge, rotor HB4) at 4°C for 25 min. (For the experiment shown in Fig. 5, the sample was centrifuged without the addition of TE.) This supernatant (SI) was collected and the pellet was resuspended in 1.5 ml TE (1.0 ml for the experiment in Fig. 5) and recentrifuged. This supernatant (SII) was saved and the pellet was stored in TE at −20°C for subsequent experiments. SI and SII were dialyzed overnight against 4 L TE at 4°C and stored at −20°C. Determinations of protein and carbohydrate were made on dialyzed samples using the methods of Lowry et al. (12) and Kochert (9), respectively.

Preparation of Crude Lysin: Crude lysin was prepared as previously described (20). In brief, mt^+ and mt^- gametes were mixed together in M-N at a final cell concentration of 1×10^8 cells/ml at 26°C. After 4 min, the suspension was centrifuged at 2,600 *g* at 4°C for 3 min. The supernatant was clarified by centrifugation at 20,000 *g* at 4°C for 15 min. For some experiments the lysin was partially purified using diethyl aminoethyl ion-exchange chromatography before being stored at −20°C in 10 mM HEPES, pH 7.2.

SDS PAGE: Linear 4–16% acrylamide slab gels with a gradient of 1–8 M urea were prepared according to the Jarvik and Rosenbaum (7) modification of the Laemmli procedure (10). Samples were mixed with 4× sample buffer (to give final concentrations of 2% SDS, 1 mM EDTA, 400 mM DTT, 10 mM Tris, pH 8.0), heated in a boiling water bath for 3 min, loaded onto the gels, and electrophoresed overnight at 5–7 mA. Gels were fixed and stained according to Merrill et al. (14).

Two-Dimensional Gel Electrophoresis of Lysin-degraded Shed Walls: Walls isolated as above were washed twice with ice-cold M-N and resuspended in 300 μl of freshly isolated lysin at room temperature. Cell wall degradation was monitored microscopically. At 6 and 90 min portions were withdrawn and mixed with 100 μl of lysis buffer containing 9.5 M urea, 2% Nonidet P-40, 5% β-mercaptoethanol, and 2% ampholines, pH 3.5–10. The samples were frozen quickly using dry ice and acetone and stored at −20°C. Samples were thawed simultaneously and analyzed by two-dimensional gel electrophoresis using nonequilibrium pH gradient electrophoresis as described by O'Farrell et al. (16). In the second dimension 4–16% polyacrylamide gradient gels were used as described above.

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; M-N, nitrogen-free medium; TE, Tris-EDTA buffer.

RESULTS

Isolation of Shed Cell Walls and Degradation of the Walls by Lysin

Aggregation between mt^- and mt^+ gametes of *Chlamydomonas reinhardtii* is accompanied by the release of cell walls into the medium (Fig. 1 *A*). At room temperature the released walls are almost immediately degraded and, thus, 10–15 min after the gametes are mixed together few, if any, walls are visible in the medium (not shown). If the cells are mixed at 12°C, however, and EDTA is added 3 min after mixing, wall release occurs but, as reported previously (20), further wall degradation is prevented. Differential centrifugation of the suspension yields isolated walls (Fig. 1 *B*). These preparations of walls, which appear very similar to walls prepared by other workers (17), have been used to learn about the molecular interactions responsible for wall integrity.

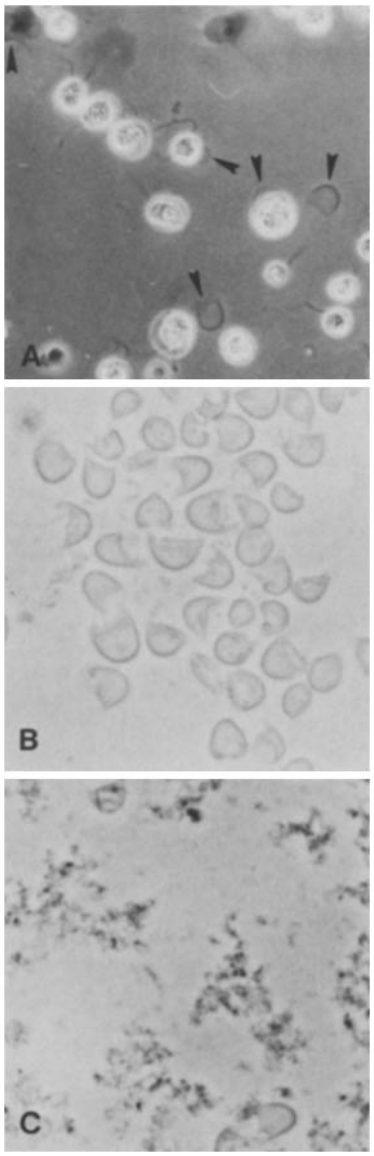

FIGURE 1 Phase-contrast micrograph of *Chlamydomonas* cell walls. (*A*) Walls released into the culture media by mating gametes are indicated by the arrows. × ~1,000. (*B*) Purified preparation of isolated, intact walls, × ~1,500, and (*C*) the same preparation treated with exogenously added cell wall degrading enzyme, lysin, × ~1,000.

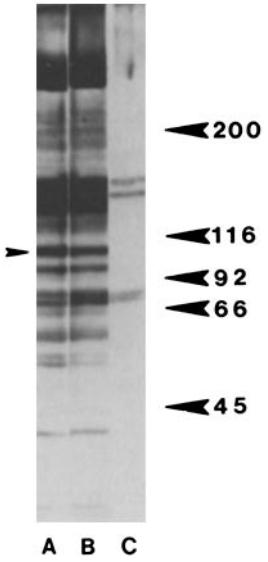

FIGURE 2 SDS PAGE analysis of intact and lysin degraded walls. Intact walls (20 μl) were washed out of TE and resuspended in either 60 μl TE (control, lane *A*) or in 20 μl 10 mM Tris buffer, pH 7.2, and 40 μl lysin in M-N (lane *B*). The lysin sample alone (30 μl) was diluted with 20 μl M-N (lane *C*). The samples were incubated at 26°C for 60 min and prepared for electrophoresis. 60% of each sample was loaded on the gel.

If associations among one or a few proteins were responsible for the basic structure of the cell wall, these proteins might have been identifiable by changes in their molecular weights or charges after degradation of the walls by lysin. To attempt to identify degradation-associated changes in wall proteins, walls isolated as above were incubated with lysin to bring about wall degradation (Fig. 1 *C*), and the proteins of the degraded walls were analyzed by one- and two-dimensional gel electrophoresis. Confirming our previous results (20), although now with much more sensitive silver staining methods for visualizing proteins, one-dimensional SDS PAGE revealed no differences in the protein composition of control (Fig. 2, lane *A*) and lysin-treated walls (Fig. 2, lane *B*). Fig. 2, lane *C* is a gel of the lysin preparation used in this experiment. Except for the proteins contributed by the added lysin, both preparations contained the same amounts of the ~20 proteins detectable on these gels, and no new bands appeared in the degraded sample.

Similar results were obtained by use of two-dimensional gel electrophoresis with the nonequilibrium pH gradient electrophoresis system of O'Farrell (16) (Figs. 3, *A* and *B*). Although there were occasionally small differences in intensity of some of the protein spots on the two-dimensional gels of nondegraded (Fig. 3 *A*) and degraded walls (Fig. 3 *B*), no consistent differences were observed. These results suggested either that wall degradation was not due to proteolytic modification or changes in charge of the 20–25 polypeptides detected on the one- and two-dimensional gels or that proteolytic cleavages clipped just a few amino acids from the ends of polypeptides.

Purification of Shed Wall Frameworks

Since we could not use the above approach as even a first step in identifying proteins responsible for shed wall integrity, it became necessary to identify and characterize the framework of the wall more precisely. To do this, shed walls isolated as described above were incubated with solubilization buffer for 60 min at room temperature. Microscopic examination of the sample revealed remnants or ghosts of the walls (not shown). These wall frameworks, which probably corresponded to the chaotropic salt insoluble portion of the walls described earlier by Hills et al. (4), were barely visible under

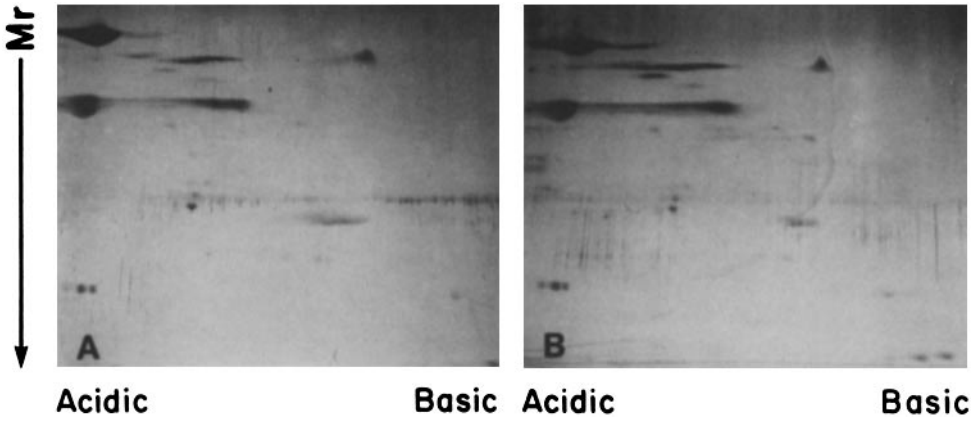

FIGURE 3 Two-dimensional gel electrophoresis of lysin-treated cell wall proteins. Intact walls were treated with lysin for 6 min (*A*) and 90 min (*B*) as described in the text.

the phase-contrast microscope and required staining with Giemsa for better contrast. We should emphasize that suboptimal alignment and adjustment of the phase-contrast microscope prevented detection of unstained frameworks.

To allow further study, the wall frameworks were harvested by centrifugation at 26,000 *g* and washed once as described in Materials and Methods, yielding SI and SII supernatants and a final pellet of wall frameworks. Fig. 4, *A* and *B* are micrographs of stained (*A*) and unstained (*B*) washed frameworks. It quite often was observed that the washed and harvested frameworks were smaller and more fragmented than frameworks examined before the centrifugation and washing steps. Apparently the centrifugal forces and subsequent resuspension with Pasteur pipettes caused some breakage.

Table I, which presents results on the protein and carbohydrate content of the fractions obtained from the Sarkosyl-urea extraction procedure, shows that the frameworks represented 8% of the protein and 16% of the carbohydrate recovered from the fractionated walls. This indicated that the frameworks composed a small but significant portion of the entire wall and that most of the wall proteins and carbohydrates were associated with shed walls through noncovalent interactions.

SDS PAGE analysis was used to study the protein components of the fractions. Because other workers had reported that the salt-insoluble portion of the walls was insoluble in SDS and reducing agents (18), the samples prepared for electrophoresis were first inspected by phase-contrast microscopy. By this criterion the walls appeared to be completely solubilized by boiling in the SDS PAGE sample buffer because there were no visible walls or wall fragments in stained or unstained samples. In addition, centrifugation of such samples did not yield visible pellets. The SDS PAGE analysis of the fractions (Fig. 5) revealed that the Sarkosyl-urea soluble and insoluble portions contained different polypeptides. Many of the polypeptides of the walls were recovered in the extract, including the three major wall polypeptides of M_r 300,000, 150,000 and 140,000. Absent from the extract was a quite heavily stained polypeptide of M_r 100,000 that was quantitatively recovered in the Sarkosyl-urea-insoluble fraction. A polypeptide of this molecular weight can be seen in the control and lysin-degraded samples shown in Fig. 2 (small arrowhead). Two other polypeptides of 225,000 and 185,000 M_r also were present in the framework fraction and absent from the extract (Fig. 5, asterisks). The M_r 185,000 polypeptide was consistently present (albeit in small amounts relative to the M_r 100,000 polypeptide) in most of our framework preparations, whereas the M_r 225,000 protein varied in amount from preparation to preparation. The molecules migrating at M_r 300,000, 150,000, and 140,000 probably were contaminants from the soluble fraction, since these three polypeptides represented the major polypeptides found in intact walls.

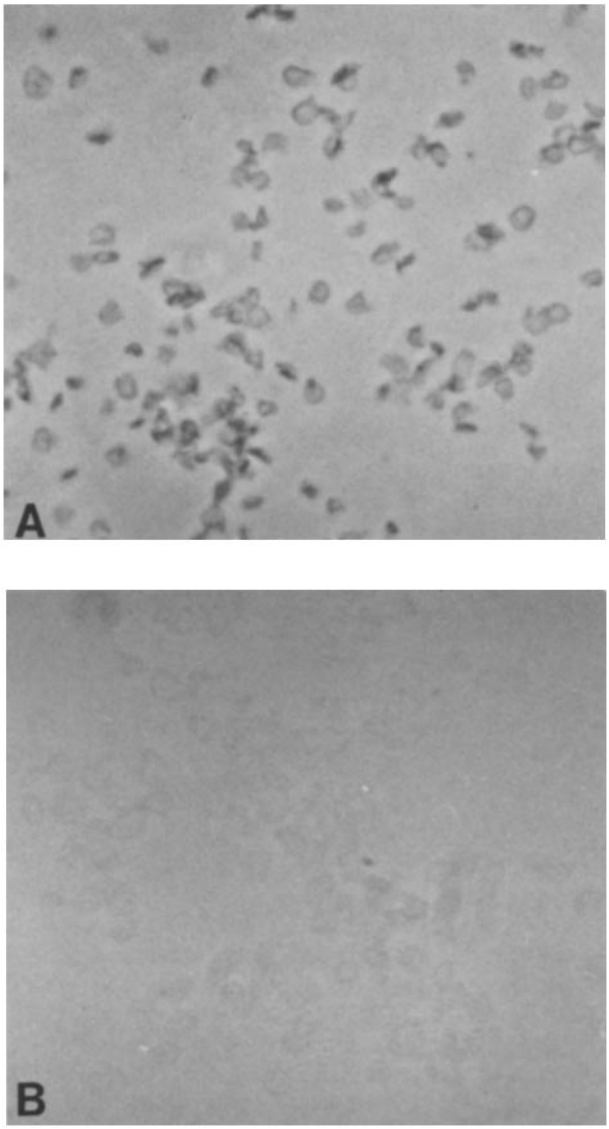

FIGURE 4 Phase-contrast micrographs of wall frameworks prepared as described in the text and (*A*) stained with Giemsa or (*B*) unstained. × ~800.

TABLE I. *Fractionation of Cell Walls**

	Protein		Carbohydrate	
	mg	*%‡*	*mg*	*%‡*
Walls	1.2	—	3.0	—
SI	0.86	67	2.0	69
SII	0.32	25	0.45	15
Pellet (frameworks)	0.11	8	0.47	16

* Walls were fractionated as described in Materials and Methods.
‡ These are percentages of recovered material that represented 107 and 97% of the starting protein and carbohydrate, respectively.

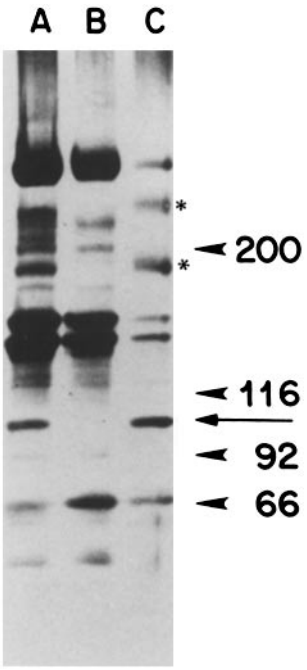

FIGURE 5 SDS PAGE analysis of the cell wall fractions after solubilization in Sarkosyl-urea solution as described in the text. Lane *A*, intact walls; lane *B*, combined supernatants SI and SII; lane *C*, frameworks. The amounts of protein loaded in each lane were equivalent to 3.75% (*A*), 1.76% (*B*), and 3.75% (*C*) of the starting material. The arrow indicates the M_r 100,000 framework protein. Proteins of M_r 225,000 and 185,000 that also are present in the framework fraction are shown by asterisks.

Frameworks Are Degraded by Lysin and by SDS and DTT

To determine if frameworks isolated as described above could be degraded by lysin, they were incubated with the wall-degrading enzyme in the presence of divalent cations, stained with Giemsa, and viewed under the light microscope. The results shown in Fig. 6 indicated that whereas untreated frameworks showed the typical framework morphology (Fig. 6*A*), the lysin-treated samples were degraded (Fig. 6*B*).

To study the behavior of the framework polypeptides during lysin treatment, we incubated frameworks at room temperature for 2, 8, and 16 h with lysin. The long incubation was required to get complete degradation with this particular preparation of lysin. Examination by light microscopy revealed that, with time, the frameworks were degraded. Frameworks incubated at room temperature for 16 h without lysin were not degraded. At the end of the incubations, the samples were centrifuged and the supernatants and pellets were analyzed by SDS PAGE. The results shown in Fig. 7 indicated that initially all of the framework polypeptides were insoluble, but with time in the presence of lysin, much of the M_r 100,000 polypeptide became nonsedimentable. An important aspect of these results was that lysin degradation of the frameworks was not accompanied by the appearance, disappearance, or detectable change in molecular weight of any framework proteins. They simply went from an insoluble to a soluble form, which suggested either that lysin was not acting as a protease or that there was proteolytic removal of only a few amino acids from the polypeptides.

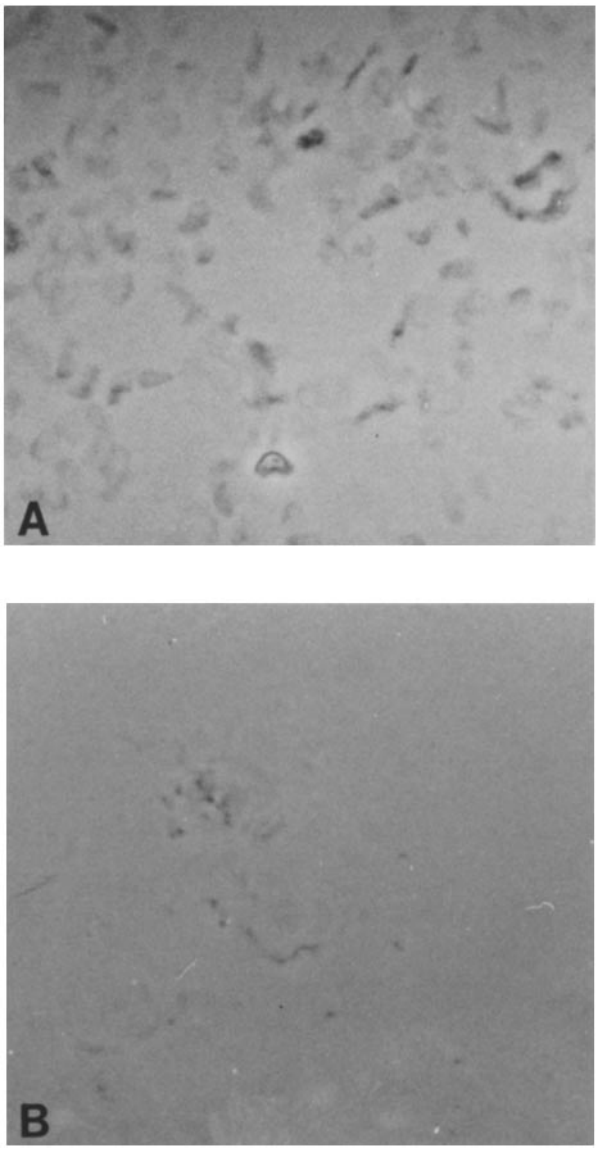

FIGURE 6 Phase-contrast micrographs of wall frameworks incubated with exogenously added lysin for 0 min (*A*) and 90 min (*B*). × ~880.

Because microscopic examination revealed that walls were also completely solubilized by boiling in SDS PAGE sample buffer, we wanted to learn more about the requirements for solubilization. To do this, intact walls were boiled in SDS-containing sample buffer with and without DTT. Microscopic examination revealed that whereas the walls were completely solubilized in the samples with DTT, structures with the shape of walls were still present in the samples without DTT (Table II). The samples were then centrifuged and the supernatants were transferred to a fresh tube. Sample buffer with DTT (equal to the original sample volume) was then added to the first set of tubes and all of the samples were boiled for 3 min and loaded on gels. In addition, a sample of walls was incubated in sample buffer with DTT at 37°C for 15 min, centrifuged, and then prepared for electrophoresis as above. Microscopic examination showed that this latter treatment did not solubilize the wall-shaped structures (Table II).

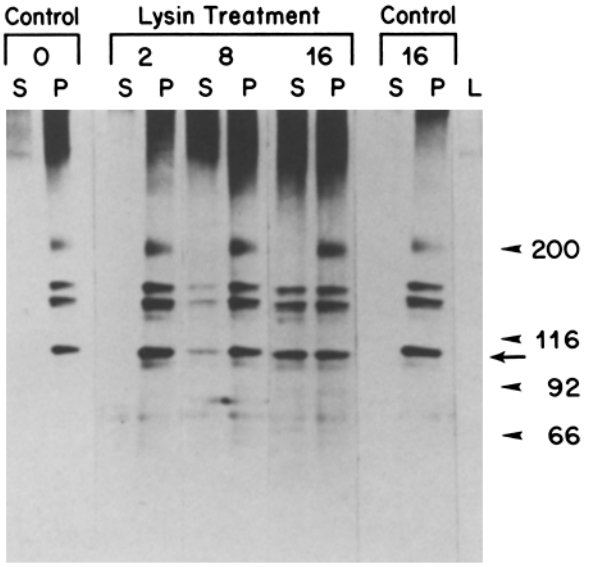

FIGURE 7 SDS PAGE analysis of frameworks incubated with lysin for various times. Aliquots (20 μl) of wall frameworks in 10 mM HEPES, pH 7.2, containing 10 mM $CaCl_2$, 0.2% azide were mixed with 20 μl lysin. The samples were incubated at 26°C for 2, 8, or 16 h. Controls were incubated for 0 or 16 h with TE instead of lysin. At the end of the incubation the samples were centrifuged (26,000 g, 4°C) for 25 min. The supernatants (*S*) and pellets (*P*) were separately mixed with Laemmli sample buffer containing 2% SDS and 400 mM DTT, incubated at 100°C for 3 min, and analyzed by SDS PAGE. The purified lysin (*L*) preparation (20 μl) used in the experiments is also shown.

TABLE II. *Conditions for Solubilization of Shed Walls*

Treatment	SDS	DTT	State of walls*
37°C (15 min)	+	+	Intact
100°C (3 min)	+	+	Solubilized
100°C (3 min)	+	−	Intact
100°C (3 min)	−	+	Intact

* Walls were treated as described in the text and then examined by phase-contrast microscopy; the walls that remained intact were much thinner than the starting walls.

The results shown in Fig. 8 indicated that, in the presence of SDS, both DTT and boiling were required for complete solubilization of the M_r 100,000 major framework polypeptide. The pellet of the samples boiled in sample buffer with both SDS and DTT (Fig. 8, lane *C*) contained only residual amounts of the M_r 300,000, 150,000, and 140,000 molecules and no M_r 100,000 polypeptide. All of the wall polypeptides were in the supernatant fraction. This was not true for the sample boiled without DTT; although there was some of the M_r 100,000 polypeptide in the supernatant (Fig. 8, lane *E*), most of it was in the pellet fraction (lane *F*). In addition, little if any was solubilized at 37°C in the sample buffer with DTT (lanes *A* and *B*). In other experiments we found that boiling samples in sample buffer with DTT but without SDS did not solubilize frameworks (Table II) and the M_r 100,000 framework protein did not become soluble (not shown).

Mechanically Isolated Walls Are Not Soluble in SDS and DTT

Because we were puzzled about the apparent discrepancies between our results on the solubility of gametic shed walls in SDS and DTT and the results of Lefebvre et al. (11) and Roberts et al. (18), who found that a wall-shaped portion of walls was insoluble under similar conditions, we did experiments with intact cells and with walls isolated from mechanically disrupted cells. Attempts to solubilize intact gametes by boiling in SDS and 400 mM DTT confirmed the results of Lefebvre et al. (11) that such treatment fails to solubilize a portion of the cells that resembles the cell wall (Fig. 9*A*). On the other hand, if the gametes were first treated with lysin, no wall-shaped structures were visible after boiling in SDS and DTT (not shown).

Because these results suggested that wall release during mating might cause biochemical changes in walls, we developed a method for purification of walls from mechanically disrupted gametes. By use of this method, it was possible to obtain walls (shown in Fig. 9*B*) that had not been exposed to lysin. Boiling the mechanically isolated walls in SDS and DTT caused them to become much thinner but left a portion of the wall that still had the shape of the wall (Fig. 9*C*). If, however, the mechanically isolated walls were briefly (1–5

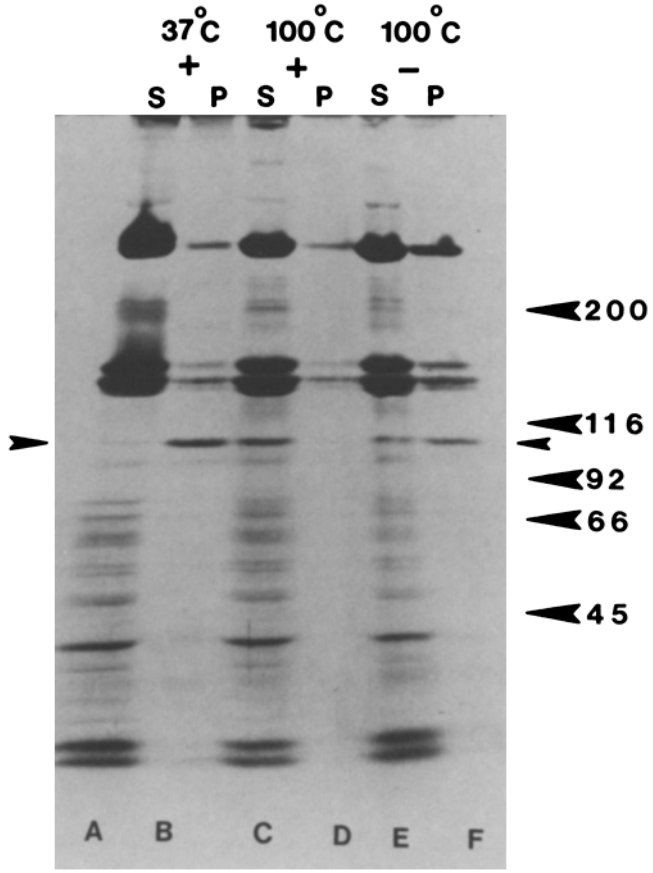

FIGURE 8 SDS PAGE analysis of the effects of SDS, DTT, and temperature on the solubility of wall fractions. Intact walls (60-μl aliquots) were incubated under the following conditions: 15 min at 37°C in Laemmli sample buffer containing 2% SDS and 400 mM DTT (lanes *A* and *B*); 3 min at 100°C in Laemmli sample buffer containing 2% SDS and 400 mM DTT (lanes *C* and *D*); 3 min at 100°C in Laemmli sample buffer containing 2% SDS but no DTT. At the end of the incubations the samples were centrifuged at 26,000 *g* for 30 min at 4°C. The pellets were resuspended in 60 μl Laemmli sample buffer containing 2% SDS and 400 mM DTT, all of the samples were incubated at 100°C for 3 min, and 50 μl of each supernatant (*S*) and pellet (*P*) fraction was loaded into the gels.

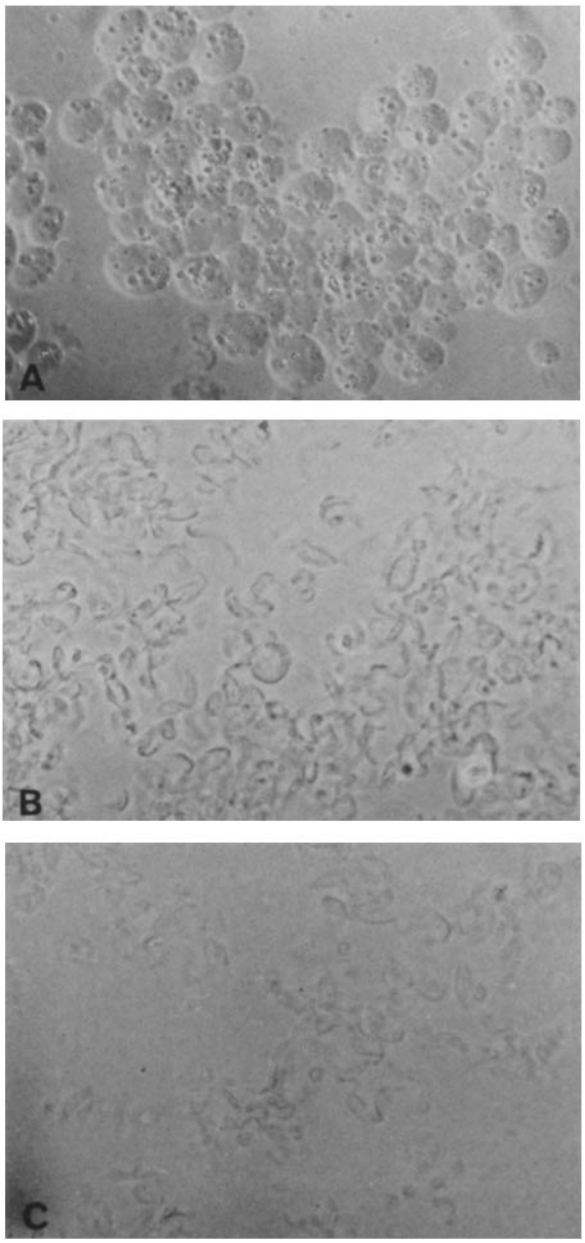

FIGURE 9 Solubility in SDS PAGE sample buffer of intact gametes and walls isolated from mechanically disrupted gametes. (*A*) Phase-contrast micrograph of intact mt^+ gametes that were boiled for 3 min in Laemmli sample buffer containing 2% SDS and 400 mM DTT. × ~1,200. (*B*) Untreated walls isolated from mechanically disrupted gametes as described in the text. × ~950. (*C*) Mechanically isolated walls boiled for 3 min in Laemmli sample buffer as above and stained with Giemsa. × ~950.

min) treated with a crude preparation of lysin, the walls were rendered soluble by boiling in SDS and DTT (not shown).

DISCUSSION

The studies reported here have shown that *Chlamydomonas* cell walls shed during mating retain many of the properties of mechanically isolated walls in that they still have the morphological appearance of walls and are degraded upon addition of the cell wall degrading enzyme, lysin. On the other hand, although a wall-shaped portion of mechanically isolated walls is not soluble in SDS and DTT at 100°C, shed walls are soluble under these conditions. We have taken advantage of these properties of shed walls to study the molecular interactions responsible for wall integrity. The Sarkosyl-urea insoluble portion of the shed wall, which we have designated the wall framework, can be acted upon by lysin. Examination by phase-contrast microscopy of frameworks incubated with lysin revealed that the frameworks were degraded (Fig. 6*B*). This result, along with the evidence that the proteins and glycoproteins that make up the peripheral portion of shed walls are associated with each other and with the framework through noncovalent interactions, suggests that the framework may be a critical site of action of lysin. That is, walls are degraded as a consequence of the action of lysin on the frameworks. This interpretation is consistent with the result that none of the ~20 Sarkosyl-urea-soluble peripheral shed wall polypeptides showed detectable alterations in charge or molecular weight as a consequence of wall degradation by lysin, whereas the framework proteins changed from an insoluble to a soluble form. This view is also consistent with recent ultrastructural studies by Goodenough and Heuser (2). Using the quick-freeze, deep-etch method to analyze the *Chlamydomonas* cell wall, these workers showed that the salt-insoluble (framework) portion of the wall is the primary target of lysin. In their studies, as the salt-insoluble layer was degraded by lysin, fibrous units shaped like fishbones appeared in the medium.

The implication of these results is that the Sarkosyl-urea-insoluble portion of the wall is a framework or infrastructure for the wall and thus can serve as a template for the assembly of the peripheral portion of the wall. Some information already available about the assembly properties of the peripheral wall proteins indicates that the in vitro assembly of a wall-shaped structure requires a template. Hills et al. (4), using perchlorate or lithium chloride to fractionate the walls, reported that the peripheral wall components reassembled to form intact walls if the insoluble portion of the wall was present. The walls reassembled in vitro had the same crystalline lattice appearance as freshly isolated, intact walls. (This group presented electron microscopic evidence that the salt-insoluble portion corresponded to the innermost layer of the wall. Because we have not yet done ultrastructural studies, we do not know the location of the Sarkosyl-urea-insoluble portion of the wall and, therefore, have used the term framework to indicate its functional role.) In the absence of the frameworks there was no reassembly of wall-shaped structures, although there was some aggregation of the proteins to form sheets.

Our biochemical studies have indicated that the framework is a unique portion of the shed wall. SDS PAGE analysis showed that frameworks contained from one to three polypeptides that were found only in this part of the wall. A polypeptide of M_r 100,000 was consistently a primary constituent of the frameworks and molecules of M_r 185,000 and 225,000 also were present, albeit in amounts varying from experiment to experiment. These polypeptides were quantitatively recovered in the framework fraction and were absent from the Sarkosyl-urea soluble fraction. We do not yet understand this variability, and the relationship between the M_r 100,000 polypeptide and these other two polypeptides is not clear. Monk et al. (15) have reported that walls contain a group of proteins of M_r ~100,000 that can be labeled by surface iodination methods. Further work is needed to determine whether the M_r 100,000 polypeptide may be one of

these proteins. Three other molecules of M_r 300,000, 150,000, and 140,000 were present both in the framework preparations and as the major constituents of the Sarkosyl-urea-soluble portion of shed walls. Since the amounts of these molecules relative to the M_r 100,000 polypeptide also varied from experiment to experiment, these three were probably not completely removed by the extraction.

Studies on solubilization of shed walls showed that incubation at 37°C with SDS and DTT did not solubilize the M_r 100,000 polypeptide or disrupt the frameworks; in addition, boiling in either SDS or DTT alone did not solubilize the frameworks, and the 100,000 M_r polypeptide was not solubilized. The solubility of the frameworks in SDS PAGE sample buffer (Table II) was somewhat unexpected because Roberts et al. (18) reported that the salt-insoluble portion of vegetative walls was not solubilized after incubation at 37°C for 2 h in buffers containing 1% SDS or 1% SDS and 1% β-mercaptoethanol. In addition, Roberts has indicated (personal communication) that the inner portion of the vegetative wall is insoluble even after boiling in buffers containing SDS and DTT. Our evidence that the frameworks were solubilized by an SDS PAGE sample buffer that contained 2% SDS and 400 mM DTT was that, after boiling for 3 min, the frameworks were no longer visible by phase-contrast microscopy. In addition centrifugation of samples boiled in SDS and DTT yielded no visible pellets.

Initially we felt that the insolubility of the walls used by Roberts' group might be unique to the strain of cells they used. This group has taken advantage of a particular wild-type strain of *C. reinhardtii* in which the walls released during vegetative growth are not degraded but accumulate in the medium (4). Hills et al. indicate (4) that their strain may be unusual in this regard because wild-type cells from other sources, including 11/32a, c, and 11/45 of the Culture Centre of Algae and Protozoa, Cambridge, produce far less wall material in the medium; and, in our experience, intact walls do not accumulate in the medium during growth of the 21gr and 6145c strains used in the experiments reported here.

For these reasons we did experiments with walls purified from mechanically disrupted gametes and discovered that wall-shaped structures, probably the wall frameworks, were resistant to solubilization in SDS and DTT at 100°C. On the other hand, a brief pretreatment with lysin rendered the walls soluble under these conditions. These results are important because they resolve the apparent discrepancy and lead to a new way of viewing wall structure and wall degradation during mating. Except for being opened so that the cells can slip out of them, the shed walls used in our experiments appear intact. And, by both light-microscopic and biochemical criteria, these shed walls are solubilized by boiling in SDS and DTT. Thus, unless this treatment is cleaving bonds other than disulfide bonds, this type of linkage is sufficient to maintain the integrity of shed walls. On the other hand, the results with the mechanically isolated walls suggest that frameworks of non-lysin-treated walls contain additional, nondisulfide bonds that contribute to the form of the wall. These bonds are not necessary for maintenance of the sac-like shape of the wall, however, since wall release during mating leads to cleavage of these bonds without a significant effect on the morphology of the walls.

Although we cannot yet completely rule out that the biochemical events that cause wall degradation are a continuation of those that cause wall release, these results suggest that wall release and wall degradation during the mating reaction may be biochemically distinct events. According to this idea, during wall release one class of nonionic, nondisulfide bonds would be broken. These might be glycosidic bonds, since the frameworks contain a substantial amount of carbohydrate. During wall degradation or disintegration, a second group of bonds would be broken. Since treatment at 100°C with ionic detergents and reducing agents can mimic this step, it may be that inter- or intramolecular protein disulfide bonds are reduced during this second stage. Alternatively, in vivo degradation of walls might occur by cleavage of peptide bonds close enough to the ends of the framework polypeptides to be undetectable on our SDS PAGE system. In support of this idea, Matsuda et al. have shown that protease inhibitors reduce lysin activity (13). Thus, even though reducing agents can mimic the in vivo events, disulfide reduction may not be the natural mechanism for wall degradation. These hypotheses are testable and experiments are in progress both to identify biochemical differences between mechanically isolated and shed gametic walls and to determine if two distinct wall-specific enzymes are activated during the mating reaction.

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Note Added in Proof: Matsuda et al., (1985, *J. Biol. Chem.*, 260: 6373–6377) have reported that lysin treatment of the salt-insoluble portion of the non-shed walls leads to the disappearance of some polypeptides and the appearance of new polypeptides on SDS-gels.

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