Cellulose Synthesis Is Coupled to Cell Cycle Progression at $G_1$ in the Dinoflagellate *Cryptothecodinium cohnii*

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Cellulosic deposition in alveolar vesicles forms the “internal cell wall” in thecate dinoflagellates. The availability of synchronized single cells, the lack of secondary deposition, and the absence of cellulosic cell plates at division facilitate investigation of the possible roles of cellulose synthesis (CS) in the entire cell cycle. Flow cytograms of cellulosic contents revealed a stepwise process of CS in the dinoflagellate cell cycle, with the highest rate occurring at $G_1$. A cell cycle delay in $G_1$, but not $G_2/M$, was observed after inhibition of CS. A cell cycle inhibitor of $G_1/S$, but not $G_2/M$, was able to delay cell cycle progression with a corresponding reduction of CS. The increase of cellulose content in the cell cycle corresponded well to the expected increase of surface area. No differences were observed in the cellulose to surface area ratio between normal and fast-growing $G_1$ cells, implicating the significance of surface area in linking CS to the coupling of cell growth with cell cycle progression. The coupling of CS to $G_1$ implicates a novel link between CS and cell cycle control, and we postulate that the coupling mechanism might integrate cell wall integrity to the cell size checkpoint.

Cell walls are not static. They are dynamic players in cell growth and differentiation and are required for maintenance of cell size and shape and for controlling cell expansion. Therefore, cellulose fibrils, the chief constituent of plant cell walls, play a crucial role in plant growth and differentiation. Cellulose ($\beta-1,4$-linked glucan chains) is the most abundant biopolymer in nature. A billion metric tons of cellulose are produced every year, synthesized by a wide variety of organisms including plants, algae, bacteria, and animals. Cellulose fibrils are synthesized by cellulose synthase, molecules of which are embedded in the plasma membrane, arranged in a rosette configuration (Giddings et al., 1980).

In higher plant and algal systems, the cell wall is external to the plasma membrane. However, dinoflagellates, euglenids, and cryptomonads are three distinct groups that possess an internal cell wall (Morrill and Loeblich, 1983). Dinoflagellates can be classified as thecate or athecate based on the presence or absence, respectively, of cellulosic thecal plates. The cell covering, or amphiesma (Schütt, 1895), of a typical thecate dinoflagellate (Fig. 1) consists of a continuous outermost membrane (plasma membrane), an outer plate membrane, and a single-membrane-bound vesicle beneath the plasma membrane. Inside this vesicle, there are a number of cellulosic thecal plates subtended by a pellicular layer.

Plant cell walls usually consist of the primary and secondary cell walls. Primary cell wall is synthesized immediately after cytokinesis, whereas secondary cell wall is made when growth ceases and differentiation begins. Cellulose synthesis (CS) is required for cell elongation, cell plate formation, and differentiation. Cellulose is deposited in cell plates at late M phase after callose deposition (Samuels et al., 1995). However, it is difficult to study the relationship in higher plants because a cellulose deficiency might be countered or masked by the presence of the old mother cell wall.

Previous reports have examined 2,6-dichlorobenzonitrile (DCB) inhibition of the formation of cellulose microfibrils (Mizuta and Brown, 1992), cell plate formation (Buron and Garcia-Herdugo, 1983; Vaughn et al., 1996), and regeneration of cell walls of protoplasts (Meyer and Herth, 1978; Arad et al., 1994). DCB arrested the cell cycle by blocking a late stage of cell plate formation (Vaughn et al., 1996) and could lead to the formation of multinucleated cells (Meyer and Herth, 1978). In the red microalga *Rhodella reticulata* (Cohen and Arad, 1998), cell division was inhibited in the protoplasts when DCB was added at the end of cell cycle. Cytokinesis gives rise to new cell wall; therefore, inhibition of CS results in a cell cycle arrest in late M phase (Vaughn et al., 1996). Unfortunately, these results do not represent the in vivo situation, especially for cell cycle progression, because a regenerated cell wall is prerequisite for nuclear division (mitosis) in protoplasts. Therefore, we sought a different model system in which to examine relationship between CS inhibition and cell cycle control—the heterotrophic dinoflagellate *Cryptothecodinium cohnii*.

In this report, we examine the effects of CS on cell cycle progression of *C. cohnii* through use of the established CS inhibitor, DCB. In addition, we address the question of whether or not CS and internal wall placement might be involved in cell size control,
and the possible parallel between dinoflagellate CS and protein synthesis in other cell wall-less systems (i.e. mammalian cells; Fingar et al., 2002).

RESULTS

Specificity of Calcofluor White M2R (CFW) Staining

The specificity of CFW staining in *C. cohnii* was confirmed with the acetic-nitric cellulose assay (Fig. 2F). The significant positive correlation between CFW fluorescence intensities and cellulose amount indicates that CFW mainly stained cellulose in *C. cohnii*. The chi square value \( (R^2) \) was higher when the data of **T** = 12 was excluded. At **T** = 12, when many 

G1 cells should be released from the mother cell wall, the cellulose amount (measured by acetic-nitric cellulose assay) reported a higher cellulose content in cells than that of CFW fluorescence intensity (measured by flow cytometry). A plausible explanation is that acetic-nitric cellulose assay only measured the total cellulose amount in the samples, including discarded mother cell walls, whereas flow cytometry only made measurements with intact cells. This would result in an overestimation of cellulose content by acetic-nitric cellulose assay for sample at **T** = 12. Undetectable fluorescence signal was observed for chlorazol black staining (for chitin), whereas a very low level of aniline blue staining (for callose) of *C. cohnii* cells was observed with fluorescence microscopy (data not shown). Flow cytometric analyses of cell cycle variations of aniline blue and chlorazol black fluorescence intensities (data not shown) indicated low and insignificant variations of callose and chitin contents in the *C. cohnii* cell cycle. Therefore, CFW could be used as a specific stain for monitoring CS throughout the *C. cohnii* cell cycle.

CS Is a Stepwise Process in *C. cohnii* Cell Cycle

Relative CFW florescence intensity was plotted against time of cell cycle progression (Fig. 2, A and C). The CFW fluorescence intensity increased throughout the entire G1 phase \( (T = 0 \text{ to } T = 6) \) and peaked at \( T = 6 \). From \( T = 6 \) to \( T = 8 \), when the cells exited S phase and prepared to enter G2 phase, there was a decrease in CFW fluorescence intensity. A similar pattern of cell cycle variation of CFW has been observed in three separate experiments. *C. cohnii* is a thinly thecated species. Despite this, we were able to observe clearly demarcated thecal plates at \( T = 6 \), but not at \( T = 8 \), in photomicrographs of CFW-stained cells in G2 (Fig. 2D). In the late G2 phase \( (T = 8 \text{ to } T = 10) \), the CFW intensity increased again, reaching a maximum at \( T = 10 \). The cells kept synthesizing cellulose before the end of mitosis and the newly synthesized cellulose contents were probably used for the assembly of the new daughter cell walls. At **T** = 12, when most of the cells finished mitosis, CFW intensity dropped again. This drop of CFW intensity corresponded to the dinoflagellates shedding their whole parental amphiesma after cytokinesis (Kubai and Ris, 1969).

Because CFW intensity is a cumulative measurement in the course of the cell cycle and could be affected by cell size, CFW:FSC2 ratio was calculated to examine the cellulose contents per unit surface area at specific time points. In G1, cellulose thecal plates thickened and the CFW:FSC2 ratio increased. A drop of CFW:FSC2 ratio was recorded in G2/M phase \( (T = 7 \text{ to } T = 10) \). This implies that relatively less cellulose was synthesized in the G2/M phase. CS became relatively slower and, therefore, did not keep pace with the increase in cell size. Until \( T = 12 \), when the cells reentered G1 phase, CFW:FSC2 ratio increased again as expected. The substantial increase of CFW:FSC2 ratio recorded at \( T = 12 \) was associated with the increased population of smaller G1 cells. These results showed that CS in *C. cohnii* was a stepwise process, rather than just a continuous increase throughout the cell cycle. To further clarify changes of the mean CFW values and CFW intensity for different subpopulations of cells (G1 and G2/M), dot-plot diagrams were developed (Fig. 2B). During the G1 phase \( (T = 0 \text{ to } T = 6) \), the mean CFW intensity of cells increased (shifted upward). The CFW intensity of G2/M cells at \( T = 10 \) was at the same level (approximately 2200) as that at \( T = 6 \). The increase of CFW signals at \( T = 10 \) was caused by the increased percentage of G2/M cells from approximately 30% to approximately 70%.
Figure 2. Cellulose biosynthesis in normal C. cohnii cell cycle. A, Flow cytograms of propidium iodide (PI)-stained synchronous C. cohnii. T, Time (hour) that the samples were harvested after cell cycle synchronization. Cellulose deposition of C. cohnii is a stepwise process within a cell cycle. The CFW:forward scatter (FSC) ratio increased mainly in G1 phase and peaked at T = 6. The ratio decreased when the cells entered G2/M phase (T = 7 to T = 10). B, G1 and G2/M cells are marked on the density plot with CFW plotted against PI. Percentage of G1 and G2/M cells were determined by using WinMDI. Corresponding CFW intensities are written in brackets. C, Photomicrographs of CFW-stained cells (same exposure time, 4 s) corresponding to the specific time points after cell cycle synchronization. Scale bar = 10 μm. D, Fluorescence photomicrographs of CFW-stained cells (focusing on the cell surface) corresponding to T = 6 and T = 8. The white arrows indicate the cellulosic thecal plates. At T = 6, individual thecal plates were clearly demarcated. At T = 8, the margins between individual thecal plates became blur. Scale bar = 10 μm. E, Cell growth is coupled to CS. Cells growing at 32°C are larger than control at T = 4 (G1 phase) with more cellulosic contents. No significant difference in CFW:FSC ratio was observed for the cells growing at 32°C compared with control at T = 4. F, Line regression analyses of the CFW fluorescence intensities (determined by flow cytometry) and cellulose amount (determined by acetic-nitric cellulose assay) yield a significant positive correlation. Dotted line represents the regression line including the data obtained from T = 12 (R² = 0.6248, P = 0.0344) whereas the solid line represents the regression line excluding the data obtained from T = 12 (R² = 0.9797, P = 0.0002). Data were obtained from a time course experiment.
CS Is Coupled to an Increase in Surface Area in G1 Phase

If CS is coupled to cell growth, it should be temperature compensated. As compared with control, cells incubated at a higher temperature (32°C) exhibited larger cell size and possessed more cellulose at $T = 4$ (G1 phase; Fig. 2E), with a higher CFW:FSC ratio (data not shown). The CFW:FSC² (related to cell surface area) ratio for the cells grown at 32°C was analyzed, and no significant difference was observed as compared with control (Fig. 2E). This suggests that at faster growth rate, cells in G1 phase manufacture more cellulose to cover the requirement of the whole cell surface, keeping the CFW:FSC² ratio constant. This demonstrates that CS is coupled to an increase in surface area during cell growth in G1 phase.

DCB Delays Cell Cycle Progression of C. cohnii Transiently at G1 Phase, But Not at G2/M Phase

To examine whether CS is required for the progression of the G1 phase or G2/M phase, cells were synchronized and DCB (either 25 or 100 μM) was added either at G1 phase ($T = 0$) or at early G2 phase ($T = 7$). Dimethyl sulfoxide (DMSO; 0.0625% [v/v]), the vehicle used to dissolve DCB, had no adverse or cell cycle effects on C. cohnii, as shown by the negative control. The variation of CFW fluorescence intensity in cell cycle progression (Fig. 3C) shows that DCB perturbed CS in a concentration-dependent manner. CFW fluorescence intensity of DCB-treated cells was lower than that of the control throughout the cell cycle, especially at $T = 4$ and $T = 10$. According to Figure 3A, untreated synchronized cells finished a cell cycle within 12 h. G1 phase was delayed in cells with DCB added at $T = 0$. For cells treated with 25 μM DCB, there was a 2-h lag for the cells to enter S phase. For cells treated with 100 μM DCB, the delay was even more prominent. When DCB was added at $T = 7$ (Fig. 4A), when the cells just entered G2 phase, the cells still finished the cell cycle on time, that is, within 12 h, suggesting that CS is coupled to G1 phase, but not G2/M phase. When the MLH medium containing DCB was replaced with fresh MLH medium at $T = 10$, CS resumed and cell cycle progression reinitiated (Fig. 4A). The effect of DCB on C. cohnii cell cycle progression was reversible.

Increase in cell volume was reported in DCB-treated red microalgae (Cohen et al., 1998). However, the FSC (Fig. 4B) of DCB-treated cells were generally smaller (slower growth rate) than the control throughout the cell cycle. CFW:FSC² ratio of G1 cells ($T = 4$) was analyzed from flow cytometric data, and DCB-treated cells were observed to have a lower value (Fig. 3D). These results suggest that DCB not only inhibited CS but also prohibited cell growth in G1 phase (Fig. 3A).

The effective DCB concentration needed to delay the C. cohnii cell cycle was 100 μm, which is a high concentration when compared with that found in plant systems (mostly around 10 μm). Unlike the situation in plants and algae, in the dinoflagellate, DCB needs to penetrate through different layers of the amphiesma to reach the cellulose-synthesizing enzymes, which might account for the requirement of high DCB concentrations. Another possible reason is that the cytokinesis process of C. cohnii does not involve the formation of the cell plate, which would be more sensitive to the effects of DCB.

CS Is coupled to C. cohnii Cell Cycle Progression

Because cell cycle progression is coupled to CS at G1, it is possible that CS is also coupled to the progression of the cell cycle. We next used hydroxyurea (HU), an inhibitor of ribonucleotide reductase, to arrest cells at the G1/S phase and monitored CS in a delayed cell cycle. As shown in Figure 5A, there was a long delay in G1/S phase, and HU-treated cells took 18 h to finish the cell cycle. During the period of S phase delay, CFW intensity of HU-treated cells remained nearly constant (Fig. 5B). However, the cell cycle arrest induced by HU was only transient. Because the cells eventually exited S phase at $T = 12$, the CFW value was similar to the control (no difference between $T = 8$ to $T = 12$). It is possible that HU treatment caused DNA damage in C. cohnii cells, which caused a G2/M delay. During the delayed G2/M phase, the CFW intensity of the HU-treated cells increased about three times and peaked at $T = 18$, before the emergence of the daughter cells. One prominent effect of the cell cycle delay was the larger cell size attained than the control (Fig. 5C) after a 10-h extension of cell growth. After the substantial increase of CFW signals after G2/M phase, however, we recorded a decrease of CFW:FSC² ratio for the HU-treated cells at $T = 20$ (Fig. 5D). HU-treated daughter cells became oversized ($T = 20$) with a reduction in cellulosic contents per unit surface area. CS was not coupled to G2/M phase, thereby producing a cell with lower CFW:FSC² ratio. These results implicate that CS is coupled to G1/S progression, during which the production of cellulose is proportional to the increase in cell surface area but not to G2/M.

To further investigate the effects of cell cycle perturbation on CS, we monitored the effects of nocodazole, a microtubule inhibitor that has been shown to transiently prolong the G2/M phase in cell cycle of C. cohnii (Yeung et al., 2000). When nocodazole was added to the culture at $T = 7$, CFW intensity increased at $T = 8$, which was 2 h earlier than that of control (Fig. 6B). This was followed by a decrease in CFW intensity from $T = 10$ to $T = 12$, whereas the cells were arrested in late M phase (Fig. 6A). Neither a normal cell cycle pattern of CFW intensity nor
reduced level of cellulosic contents was observed in nocodazole-treated cells, confirming that CS is not coupled to G2/M phase in C. cohnii cell cycle.

**DISCUSSION**

The cell surface represents the margin of the cell and defines its size. It is conceivable that CS has to be tightly and synchronously regulated to generate a uniform cell wall during the active process of cell growth. In a normal cell cycle, the cell doubles its diameter before cytokinesis. It is expected that a 4-fold increase in total surface area would result in a 4-fold increase in cellulosic contents, assuming that cellulose was synthesized and deposited uniformly.

**Figure 3.** DCB-arrested C. cohnii cells at G1 phase. A. Flow cytograms of PI-stained synchronous C. cohnii cells treated with different concentrations of DCB. T, Time (hour) that the samples were harvested after cell cycle synchronization. Note that there is a G1 arrest for cells treated with 100 μM DCB. B, DCB-treated cells are smaller in cell size than the control. At T = 4, FSC data from DCB treatment (100 μM) were extracted and overlain on the control. Photographs (same exposure time) of CFW-stained cells corresponding to the time point T = 4 under different treatments: I, control; ii, 25 μM DCB; and iii, 100 μM DCB. Scale bar = 10 μm. C, At both T = 4 (G1 phase) and T = 10 (late G2/M phase), significant (P < 0.05) differences were observed for DCB concentrations above 25 μM. D, DCB dose dependently reduced the CFW:FSC² ratio.
to the cell surface. From our results, the total surface area calculated from FSC<sup>2</sup> increased 4.2 times from T = 0 (G<sub>1</sub>) to T = 10 (G<sub>2</sub>/M). A 4.8-fold increase in CFW intensity (cellulosic contents) within the same time interval was recorded by flow cytometric measurement. The presence of both the mother cell wall and daughter cells' cellulosic precursors at this late stage of the cell cycle might provide a plausible explanation for the slightly higher CFW intensity as compared with the corresponding increase in surface area. These results demonstrate flow cytometry as an accurate and rapid method to measure the cellular cellulosic contents concurrently with other cell cycle-related parameters. Furthermore, this reveals a strong relationship between CS and cell size increase. Cell size increase can be interpreted as cell mass increase, cell volume increase, or cell surface increase. It is unlikely that cells actually measure their cell size to affect a downstream event. Morris and Homann (2001) suggested that regulation of cell surface area and membrane tension might be important in coordinating cell growth and wall synthesis, thereby regulating cell size in animal and plant cells. We postulate that CS in dinoflagellate cells might play a critical role in the maintenance of membrane strength and, as a consequence, affect surface area increase. The fact that CFW:FSC<sup>2</sup> is constant in faster growing G<sub>1</sub> cells implicates the significance of surface area or cell wall integrity in the coupling of cell growth and cell cycle progression. Inhibition of CS by DCB might have perturbed the membrane tension, resulting in a G<sub>1</sub> delay. CS stopped when C. cohni cells were arrested by HU at G<sub>1</sub>/S transition. In Dictyostelium discoideum, an outside-in signaling pathway that involves cell surface proteins has been reported (West et al., 2002). In yeast (Saccharomyces cerevisiae), a compensatory delay (G<sub>2</sub>) of nuclear division is introduced under osmotic stress or actin perturbation at the morphogenesis/cytoskeleton checkpoint (Lew, 2000).

Because cell growth in C. cohni mainly occurred in G<sub>1</sub> phase, the increase of the CFW:FSC<sup>2</sup> ratio indicated more cellulose per unit surface area was synthesized and deposited to the thecal plates. In late G<sub>1</sub> to early S phase, deflagellation occurred and the cells became immotile. This might involve thickening and fusion of the existing thecal plates, as described by Kubai and Ris (1969). A decrease in cellulosic contents was recorded from T = 6 to T = 8 (Fig. 2A). A possible reason for explaining the decrease of CFW fluorescence intensity from T = 6 to T = 8 is that cellulose was retrieved back either from the thecal plates or pellicular layer. In photomicrographs of CFW-stained cells collected at T = 6 and T = 8 (Fig. 2D), margins of thecal plates became less defined as the cells progressed through G<sub>2</sub>. Thecal plate dissolution or a reduction of the pellicle thickness was reported in other dinoflagellate species before ecdisis (Dürr, 1979; Morrill and Loeblich, 1984). It is possible that dissolution of C. cohni mother cell wall occurred in S, and early G<sub>2</sub>/M phase (T = 6 to T = 8) provides precursor molecules or materials for future cell growth in G<sub>2</sub> and for the manufacture of daughter cell walls. This can represent an economical use of resources because cellulose deposition in the mother cell wall is a major investment of biological materials (mainly Glc), which is shed at the release of the daughter cells. This drop of cellulosic content in the cell cycle is a novel observation and is being further investigated further in our laboratory. After the drop of CFW signals, cellulosic contents accumulated in G<sub>2</sub>/M phase because new daughter cell walls were formed before the cells emerged from the old mother cell wall. There was detectable CFW fluorescence...
intensity in newly released motile cells, suggesting that the daughter cells had already synthesized some cellulose when they were still encased inside the mother cell wall. Our findings parallel Wetherbee's (1975) hypothesis that the cells have developed their new covering system before parental theca is discarded.

Cellulose is the major constituent of thecal plates. However, cellulosic amorphous layer has also been reported in the pellicle of C. cohnii (Kubai and Ris, 1969). Continuous pellicular layer was completed before cytokinesis, whereas newly synthesized thecal plates were not observed until the daughter cells emerged from the mother cell wall. We speculate that newly synthesized cellulose is mainly incorporated in the thecal plates during G1 phase. In G2/M phase, formation of the daughter cells' pellicle might be responsible for the increase of CFW intensity between T = 8 to T = 10. Based on this assumption, CS in G1 phase is mainly related to the formation and thickening of thecal plates, whereas cellulose in G2/M phase is mainly related to the new pellicle formation. At the same time, this also provides a plausible explanation why CS in the daughter cells is not coupled to G2/M phase.

Biochemically, cellulose is a carbohydrate. Cell growth has to be coupled to carbohydrate metabolism. As demonstrated by incubating the cells at different temperatures, a higher rate of CS was measured in cells growing at higher temperature (Fig. 2C). A sugar-sensing mechanism might be present in the dinoflagellate C. cohnii, comparable with the sugar-sensing mechanism in budding yeast (Roland et al., 2001). In budding yeast, metabolism is dictated by the availability of Glc, which is the preferred carbon source. Sugar availability has also been demonstrated to control cell cycle in plants by controlling expression of cyclin D2 and D3 genes in G1 phase of Arabidopsis (Riou-Khamlichi et al., 2000). The sugar-sensing system may signal the cell to synthesize more cellulose as growth rate increases. When CS is inhibited, cell growth ceases and a G1 cell cycle delay will be introduced.
Plant cells possess sensors that monitor alterations in cell wall structure or components, and compensatory mechanisms would be turned on to counter the perturbation. For instance, it has been reported that repression of lignin synthesis leads to an increase in CS (Huang et al., 1999). Furthermore, expression of a cell wall-related enzyme, endoxyloglucan transferase, was demonstrated to be controlled by cell wall integrity signals in tobacco (Nicotiana tabacum) Bright-Yellow 2 cells (Nakagawa and Sakurai, 2001). Stresses in plant cell walls could be detected by a stress-receptive portion of the cellulose microfibrils, thereby orienting microtubules via interactions with cell wall-linked transmembrane proteins (Williamson, 1990). Mechanical stress to a cell can be generated from an external source or internally by cell growth. There is evidence to relate cell wall integrity to cell growth in yeast (Valentini et al., 2002). The presence of such coupling mechanisms could allow the cell to maintain cell size homeostasis. We postulate that the coupling between CS and cell cycle control, identified in the present study, is part of the signal transduction pathway linking cell wall integrity to a cell size checkpoint (Fig. 7B).

**MATERIALS AND METHODS**

**Cell Culture and Cell Cycle Synchronization**

_Cryptothecodium cohnii_ Biechele strain 1649 was obtained from the Culture Collection of Algae (University of Texas, Austin), maintained in MLH liquid medium (Tuttle and Loeblich, 1975), and incubated at 28°C in the dark. _C. cohnii_ is a heterotrophic, armored species with relatively thin thecal plates of about 15 to 20 nm (Kubai and Ris, 1969). The complex cell cycle of _C. cohnii_ has been described (Bhaud et al., 1991). In _G_1 (the swimming stage), the _C. cohnii_ cells were motile. When they entered _G_2 phase, they became spherical, lost their flagella, and became immotile. Cell growth occurred in both _G_1 and _G_2, but mainly in _G_1. Highly synchronized _C. cohnii_ cells were obtained through colony release and filtration (Wong and Whiteley, 1996). To investigate the coupling of CS with cell growth, synchronized _C. cohnii_ cells were incubated at a higher temperature (32°C) to observe whether the CS and cell growth are temperature compensated. Flow cytograms of _C. cohnii_ cells treated at 32°C suggested that they are at _G_1 phase of the cell cycle at _T_ = 4, as in the control cells (data not shown).

**Chemical Inhibitor Treatments**

**DCB**

The herbicide dichlobenil (DCB) is an effective and specific inhibitor of CS in higher plants (Delmer et al., 1987; Delmer and Stone, 1988; Delmer and Amor, 1995) and red microalgae (Arad et al., 1994). DCB was shown to block formation of sitosterol-β-glucoside, the primer for cellulose biosynthesis in plants (Peng et al., 2002). DCB has no or little effect on the biosynthesis of non-cellulosic polysaccharides (Montezinos and Delmer, 1980), DNA synthesis, and nuclear division (Meyer and Herth, 1978), as well as protein synthesis (Gaithri and Shields, 1982). Stock solutions of DCB (D6755-8, Aldrich, Milwaukee, WI) were prepared in DMSO, with a final concentration at 2 μM. DCB-treated cells were harvested (1,500 g for 10 min) every 2 h after synchronization and analyzed by flow cytometry for DNA content, relative cell sizes (FSC), and cellulose content.

**Cell Cycle Blockers**

HU, an inhibitor of ribonucleotide reductase, was used to arrest cells at the _G_2/S interface. Stock solution of HU (1 μM) was prepared in MLH medium and added to the synchronized cell culture at _T_ = 0, with final working concentration at 10 mM. Nocodazole [methyl-(5-[2-thienylcarbonyl]-1H-benzoimidazol-2-YL) carbamate], a microtubule inhibitor that affects the dynamics of tubulin polymerization, is known to cause a _G_2/M cell cycle delay in _C. cohnii_ cells (Yeung et al., 2000). Nocodazole was first dissolved in DMSO and added to the synchronized cell culture at _T_ = 6 to final concentration at 2 μg mL⁻¹. For both treatments, cells were collected every 2 h and fixed for flow cytometric analysis.

**Flow Cytometry**

At defined time intervals, cells were harvested by low-speed centrifugation (1,500 g for 10 min), fixed in 70% ethanol, and kept at 4°C until analysis. The ethanol was replaced with phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M Na₂HPO₄, and 0.01 M KH₂PO₄ [pH 7.4]) containing 5 μg mL⁻¹ RNase and incubated at 37°C for 60 min. For DNA content analysis, the cells were stained with 2 μg mL⁻¹ PI.
Figure 7. Schematic diagram illustrating the variation of amphiesma in the C. cohnii cell cycle. A, Ultrastructure of the variation of C. cohnii amphiesma was drawn based on Morrill and Loeblich (1984). i, Cellulosic thecal plates are absent in newly emerged daughter cells. As the cell grows in G1 phase, cellulose accumulated and thecal plates thickened (ii). iii, Small prethecal vesicles formed beneath the cytoplasmic membrane after the cells shed the flagella. iv, Prethecal vesicles then enlarged and fused, with a new pellicle formed within. Only the cytoplasmic membrane remains and becomes the outermost membrane of the emerged daughter cell. New thin thecal plates are formed shortly after ecdysis. OM, Outermost membrane; OP, outer plate membrane; TV, thecal vesicle; TP, thecal plate; P, pellicle; CM, cytoplasmic membrane; PTV, prethecal vesicle; MCW, mother cell wall; DCW, daughter cell wall. B, Hypothetical model describing the bidirectional flow of information between cell wall integrity and cell cycle control in G1 phase of C. cohnii cell cycle. Our results demonstrated a novel link between CS and cell cycle progression.
Specificity of CFW Staining

CFW, a fluorescent brightening agent (UV excitation), has been used extensively to visualize the thecal plates of armored dinoflagellates (e.g., Fritz and Triemer, 1985). Carbohydrates in the dinoflagellate *Heterocapsa ni ei* include mainly cellulose, hemicellulose, starch, and soluble sugars by phenol-sulfuric acid reaction (Loeblich, 1977). A working stock solution of 0.1% (w/v) CFW was made with PBS according to the ratio: 0.001 g of CFW in 1 mL of PBS. Although thecal plates are basically cellulose (Loeblich, 1970), CFW can also potentially bind to chitin (Hayashibe and Katohda, 1976), callose (Hughes and McCully, 1975), and other β-linked polymers including carboxymethyl-cellulose and DEAE-cellulose (Maeda and Ishida, 1976). The specificity of cellulose staining with CFW was tested by acetic acid- and nitric cellulose assay as described by Updegraff (1980). The possible presence of callose and chitin in *C. cohnii* cells was also independently estimated by two additional fluorescent probes. Aniline blue (0.05% [w/v]), which is specific for callose (β-1,3-glucan), is widely used for callose staining in sieve plate pores (Eschrich, 1975), pollen mother cell walls (Heslop-Harrison, 1964), and cotton (Gossypium hirsutum) seed hairs (Maltby et al., 1979). Chitin (β-1,3-glucan) was assayed by staining *C. cohnii* cells with a chitin-specific stain, Chlorazol black (Kumagai et al., 2001). All flow cytometric analyses were carried out in a Vantage fluorescence-assisted cell sorter (Becton-Dickinson, Franklin Lakes, NJ).

FSC Analyses

FSC is flow cytometric measurement related to the relative cell size of the sample. Greater FSC corresponding to a cell with a large cross-sectional area can refract a larger amount of light on to the photosensor-positioned parallel to the laser beam. Based on the relationship of FSC and cell size, mean cell surface area and volume could be estimated roughly from FSC2 and FSC3, respectively. All flow cytometric data, including DNA content histograms, FSC and CFW intensity or dot plots, were analyzed by using the software WinMDI (version 2.8, The Scripps Research Institute, http://facs.scripps.edu/software.html) running “total” events (10,000). Percentage of G1 and G2/M cells were determined by using the “regions” command and style set as “polygon.”

Received December 9, 2002; returned for revision January 22, 2003; accepted January 22, 2003.

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