Dynamics of silica cell wall morphogenesis in the diatom *Cyclotella cryptica*: Substructure formation and the role of microfilaments

Benoit Tesson, Mark Hildebrand *

Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0202, USA

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A B S T R A C T

Diatoms are unicellular algae that make cell walls out of silica with highly ornate features on the nano- to microscale. In addition to their silica structures, diatom genomic sequencing (Armbrust et al., 2004; Bowler et al., 2008) and genetic manipulation (transgenic) capabilities (Apt et al., 1996; Dunahay et al., 1995; Hildebrand, 2008; Kröger and Poulsen, 2008) have rendered diatoms as model organisms for the study of nanoscale pattern formation and silica mineralization at the single cell level. The cell biology of diatom silicification has a stand-alone interest; however, because diatoms are capable of making structures that exceed those possible using current materials synthesis approaches, they are being looked at as a possible biologically-based system to generate nanomaterials for technological applications (Hildebrand, 2008; Losic et al., 2009; Sandhage et al., 2005). This could entail either the direct use of materials derived from diatom silica (Sandhage et al., 2005), or the application of lessons learned from the study of diatom silicification to biomimetic approaches (Losic et al., 2009). In either case, it will be essential to understand at the molecular level the processes by which diatoms control the formation of silica from the microscale to the nanoscale.

A primary concept in biominalization is the need to generate a defined space in order to control the growth and shape of the structure being produced. This is particularly true with amorphous minerals such as silica. By definition, amorphous minerals do not possess structural order at the molecular level, which implies that there is no preferential growth direction (Addadi and Weiner, 1992). In the case of silica, its polymerization can be induced relatively easily (Iler, 1979), but forming a specific shape requires control at a different level.

Some basic concepts and components involved in diatom cell wall formation have been described. There are two major structural features comprising the diatom cell wall (also called the frustule), which has the general structure of a petri dish. The valves are the distinctive structures characteristic of a given diatom species and form the extreme upper and lower portions of the wall, and the girdle bands are most commonly thin strips of silica which encircle the cell, and constitute the side walls and provide overlap...
between the two halves of the cell. Observational studies have identified three scales of structure formation in diatoms (Davis and Hildebrand, 2007; Hildebrand et al., 2006; Pickett-Heaps et al., 1990); (1) the nanoscale, which involves the initial polymerization of silica from soluble precursors and which manifests as silica structures with nanometer-sized features, (2) the microscale, which relates to the overall shape of the silica structure formed within the confines of the silica deposition vesicle (SDV), which is a membrane-bound intracellular compartment wherein silification occurs (Drum and Pankratz, 1964; Pickett-Heaps et al., 1990), and (3) the mesoscale, in which nanoscale polymerization products are organized at a higher level within the confines of the SDV to form intermediate-sized silica structures. Mesoscale structures are commonly the features most distinct between species.

Molecular components involved in polymerization and structure formation have been described for the nano- and microscales. At the nanoscale, these include long chain polypeptides (LCPAs), which catalyze silica polymerization (Kröger et al., 2000), silaffins, which are highly post-translationally modified (poly)peptides that play either a catalytic or regulatory role in silica polymerization by organizing LCPAs via electrostatic interactions (Kröger et al., 1999, 2001, 2002), and silacidins, which are polyanionic polypeptides that are also involved in the organization of silica polymerization determinants (Wenzl et al., 2008). Nanoscale silica morphology is affected by the type of silaffin and ratio of silaffin to polyamine to silacidin, however well-organized higher order structure does not spontaneously form from these molecules (Kröger et al., 2000, 2002; Wenzl et al., 2008). On the microscale, the SDV membrane (the silicalemma) plays a critical role in silica structuring, having been shown to be shaped and molded both actively and passively to form structure (Pickett-Heaps et al., 1990). To date no organic components definitively shown to be part of the silicalemma have been characterized. A close association has been shown between the SDV and the cytoskeletal elements actin and microtubules, and these protein assemblies play a critical role in shaping microscale structure (Pickett-Heaps et al., 1990; van de Meene and Pickett-Heaps, 2002, 2004). Inhibitors of cytoskeleton formation can generate substantial changes in diatom silica structure (Cohn et al., 1989; Schmid, 1984). Direct visualization has shown that microtubules are generally found along rib structures in diatom silica, whereas actin is associated with the active front of silification (Pickett-Heaps et al., 1990; van de Meene and Pickett-Heaps, 2002, 2004). Fluorescence microscopy to localize actin and microtubules has only been done on two diatom species, and in both cases, microtubules were seen aligned in the direction of growth of the SDV just ahead of the silification front, and actin formed a ring structure that defined the diameter of the SDV, and expanded in diameter with increasing expansion of the SDV (van de Meene and Pickett-Heaps, 2002, 2004).

From what has been described, it is clear that diatom silification involves both “bottom up” (generation of larger-scale structure from assembly of smaller precursors), and “top down” (shaping of smaller scale structure from a larger scale) processes to form the myriad of structures possible. The scale at which the bottom up and top down processes interact is the mesoscale, which is the least well characterized scale of diatom silification. There are several models for how mesoscale structure may form. It has been proposed that (1) organic molecules serve as organizers of polymerization determinants inside the SDV (Hildebrand, 2008; Hildebrand et al., 2009a; Robinson and Sullivan, 1987), (2) the process occurs through diffusion limited aggregation where the role of organics is limited to providing a confined space (Parkinson et al., 1999), or (3) self assembly of silaffins and polyamions and silacidins (Sumper and Kröger, 2004; Wenzl et al., 2008) or phase separation processes involving polyamine droplets (Lenoci and Camp, 2008; Sumper, 2002) generate higher order structure. Observational data has resulted in the generation of models for mesoscale formation in which cytoskeletal elements located outside the SDV interface with membrane proteins spanning the silicalemma that position silica polymerization determinants inside (Pickett-Heaps et al., 1990; Robinson and Sullivan, 1987). These models not only describe a possible mechanism for mesoscale structure formation, but explain why the forming valve is anchored to one side of the SDV and only expands on the other side, which is commonly seen (Hildebrand et al., 2006; Schmid and Schulz, 1979; Schmid and Volcani, 1983). It is important to consider that, given the complexity of diatom silica structure, multiple mechanisms may be involved in formation of mesoscale structure, indeed in a recent report, both internal templating and confinement models could be used to explain formation of two different structures in *Thalassiosira pseudonana* (Hildebrand et al., 2009a).

Regardless of the model proposed, the predicted process of structure formation must correspond with observational data. Thus, there is continuing value for detailed microscopic analysis of diatom silica structure formation, especially with the application of advanced high resolution imaging techniques. Since silification is a non-equilibrium process, knowledge of the dynamics of the phenomenon and characterization of intermediate stages during wall formation is essential for placing the role of different components in the correct context.

Recently, a detailed examination of different stages in formation of the valve of *T. pseudonana* was presented (Hildebrand et al., 2006). After formation of a “base layer” consisting of deposition of radially-oriented ribs of silica to form the outline of the valve, additional deposition occurred only on one side of the ribs (the distal side) resulting in rigidification of the structure (Hildebrand et al., 2006). The *T. pseudonana* valve has a relatively simple structure, and yet characterization of its formation indicated a carefully controlled assembly process. Examination of structure formation processes in species with valves having increasingly complicated shapes should be beneficial for understanding the underlying general design and assembly principles that diatoms utilize. *Cyclotella cryptica* is an attractive next species for this purpose because of its distinctive valve structure (described herein), its relatedness to *T. pseudonana* in the same class of the *Thalassiosirales*, and the fact that it is genetically manipulable (Dunahay et al., 1995), which should enable application of transgenic techniques to study silica structure formation. In this work we describe in detail the dynamics of formation of different components of the frustule of *C. cryptica* using SEM, AFM, and fluorescence microscopy. Our results highlight the apparent pre-positioning of organic components prior to complete silification, and the role of the cytoskeleton in both positioning and in the dynamic growth of silica structure.

2. Materials and methods

2.1. Diatom culture conditions

*Cyclotella cryptica* strain CCMP332 was cultured in NEPC medium (http://www.botany.ubc.ca/cccm/NEPCC/esaw.html) at 18 °C under continuous light. Cultures were synchronized using a silicon starvation/replenishment procedure developed for *T. pseudonana* (Hildebrand et al., 2007), and although the cell cycle arrest point resulting from silicon starvation differed from *T. pseudonana*, cells undergoing valve synthesis were still enriched. When used, cytochalasin D (200 µM) and colchicine (12.5 mM) were prepared in DMSO and added to the medium at 3 and 50 µM, respectively. Oryzalin (2.9 mM) was dissolved in water and added to the medium at 0.2 µM.
2.2. Sample preparation for SEM and AFM

Diatom frustules were cleaned by acid treatment. Ten milliliters of diatom culture was harvested by centrifugation at 4000 rpm for 4 min, rinsed once with 2.3% NaCl and frozen at -20 °C. Acid treatment of cell walls was done by boiling cells in 1 ml concentrated sulfuric acid for 10 min, cooling, and then adding 20 mg KNO3, then boiling an additional 10 min. Frustules were then washed using centrifugation three times with ultra pure water. For observation of frustules in cross-section, cleaned frustules were sonicated two times for 30 s each in pulsed mode (Vibra cell, Sonics and Materials, Newtown, CT, USA).

For SEM examination, samples were coated with gold/palladium and observed with an FEI Quanta 600 (FEI Company, Hillsboro, OR, USA) scanning electron microscope at the Scripps Institution of Oceanography Unified Laboratory Facility. High resolution imaging was performed on a Philips XL 30 ESEM (UCSD, Calit2 Nano3 facilities).

For AFM imaging, frustules were mounted on poly-l-lysine coated slides and imaged in air. Images were acquired in AC mode using a silicon cantilever with a spring constant of 30 N/m (AC160TS, Olympus) on an Asylum MFP-3D-BIO Atomic Force Microscope coupled with an Olympus inverted fluorescent microscope. AFM images were processed using WSxM 4.0 software (Horcas et al., 2007).

Hydrofluoric acid etching was performed on acid cleaned frustules by suspending in 0.05 M HF for 5–10 min and then washing by centrifugation several times with ultrapure water.

2.3. Sample preparation for fluorescence microscopy

Silica incorporation was visualized by addition in the culture medium of 100 ng ml⁻¹ of PDMPO ([2-(4-pyridyl)-5-(4-(2-dimethylaminoethylamino-carbamoyl)methoxy)phenyl]oxazole (Invitrogen Corp., La Jolla, CA) prior to silicate replenishment (Shimizu et al., 2001). Actin staining followed the procedure of van de Meene and Pickett-Heaps (2002), where cells were fixed in 2% formaldehyde prepared in Actin Stabilizing Buffer (ASB – 10 mM Pipes/10 mM EGTA/5 mM MgSO4/pH 6.9) and containing 100 µM MBS (m-maleimidobenzoyl N-hydroxysuccinimide ester) and 1% NaCl at 4 °C. Rinsed twice in ASB buffer and stained with rhodamine phalloidin (Invitrogen Corp., La Jolla, CA), diluted at 1/100 in ASB buffer overnight at 4 °C. Samples were then rinsed twice in ASB prior to observation using a Zeiss AxioObserver inverted microscope equipped with an Apotome (Carl Zeiss Microimaging, Inc., Thornwood, NY, USA). The filter set used for PDMPO was Zeiss #21HE (Ex 387/15 nm, FT 409, Em 510/90 nm) and for rhodamine phalloidin, Zeiss #43HE (Ex 550/25 nm, FT 570 nm, Em 605/70 nm), respectively. With these filters and under the exposures used, no chlorophyll autofluorescence was visible. Chlorophyll was imaged using Zeiss filter set #16 (Ex 485/20 nm, FT 510 nm, Em 515 nm LP). Images were acquired with 63×/1.4 objective oil immersion plan APO and treated using Axiovision 4.7.2 software. Presented images are from 3D reconstructions (except when specified), and presented in girdle band view.

3. Results

3.1. Characteristics of the valve and its formation

The valve of C. cryptica is circular and averages 7–8 μm in diameter. The distal valve surface (away from the cell interior) consists of a radially-oriented pattern of alternating wide ribs and grid-like arrangements of pores (Fig. 1a and b). The central portion of the valve (Fig. 1c), constituting about half of the valve diameter, has low relief features and is relatively flat compared with the grid-like portion at the periphery of the valve (Fig. 1b). The overall relief shape of the valve in side view is undulated near the edges, with pore fields being rounded up above the flat ribs and central region (Fig. 1d). Fultoportulae are the moderately extended tubular structures located on the valve rim at the ends of the wide ribs and in between the pore fields (Fig. 1d, arrows). Most, but not all, of the wide ribs contain fultoportulae (Fig. 1e and f). Located in the central portion of the valve are one or two fultoportula which have an internal structure similar to the fultoportulae on the rim of the valve (Fig. 1e), but protrude less from the distal valve surface (Fig. 1a and c, arrows). The central fultoportula is more easily visible in the proximal surface valve view (Fig. 1e, arrow). The view of the proximal surface highlights the predominance of the wide rib structures, and the location of the fultoportulae along the rim (Fig. 1e and f). The wide ribs are typically narrower near the center of the valve, and widen towards the radius (Fig. 1e and f). The wide ribs are most commonly unit structures, but as shown in Fig. 1f, they can be bifurcated and then rejoined at the portula. In between the wide ribs in the region corresponding to the pore field are elongated narrow silica ribs (Fig. 1f). Generally 2–4 narrow ribs are present between adjacent wide ribs. A cross-sectional view of the valve (Fig. 1g) shows the relative relief of the fultoportula and the wide ribs, which become exponentially taller towards the rim of the valve, and have a buttress-like appearance.

Cross-sectioning, AFM, and HF dissolution of the valves revealed their internal structure and stages in their formation (Fig. 2). The thickness of the mature valve measured from SEM cross-sections varies depending on the location of measurement. The central region averaged 168.8 ± 3.5 nm (n = 15), and the rib and pore region averaged 128.0 ± 2.6 nm (n = 31). The narrow ribs appeared to be slightly less thick (by 10%) than the wide ribs (Fig. 2b). In all regions of the valve, larger silica structures were formed from an amalagamation of smaller irregularly-shaped silica particles of a few nanometers (Fig. 2). In the central region, the amalgamation was relatively uniform (Fig. 2a), but in the rib and pore region the smaller particles were assembled into larger particles that constituted the wide and narrow ribs (Fig. 2b and c). Spaces in between the larger particles constituted the pores. The narrow ribs were somewhat oval in cross-section, but were generally more flattened at the proximal surface, and rounded at the distal surface (Fig. 2b and c). This results in a trumpet shape to pores that span the cross section (Fig. 2b and c, arrows). Imaging of the ribs during their formation by AFM showed that initially deposited silica was particulate, but the surface became smooth with maturation (Fig. 2d). Measurements of roughness in these two regions yielded RMS values of 2.77 for the initial deposition, and 1.88 for the mature region. These data suggested that the initial structure was formed from particulate silica in which either the surface of the initially-formed particles became flattened or the spaces in between them became filled in. As measured by AFM, the thickness of the wide ribs at an initial stage of their formation was approximately 50 nm, and the narrow ribs were 30 nm (data not shown) – about one-third of that measured for the same structures in the mature valve, which is consistent with thickening.

Application of a synchronized growth procedure (Section 2) enabled examination of valve development using SEM and fluorescence microscopy and revealed distinct stages of formation (Fig. 3). Fluorescence microscopy of PDMPO-stained cells (Fig. 3a) was used to identify samples used for further examination by SEM and cells imaged by AFM. Observed at the earliest stage was a fractally-branched structure approximately 4.5–5.5 μm diameter (Fig. 3b and c), which corresponded to the diameter of the central region in the mature valve. Precursors to the wide ribs were visible radiating out from the center (Fig. 3b and c). At this stage the ends of the ribs did not have portulae.
Fig. 1. SEM of the valve of Cyclotella cryptica. (a) Overall view of the distal valve surface. Arrow denotes the location of a fultoportula offset from the valve center. (b) View of the distal surface, near the outer edge of the valve. Wide ribs with pore fields in between are evident. (c) Central portion of the distal surface. The fultoportula is located with an arrow. (d) Edge-on view of the distal surface, showing the raised pore fields, and location of fultoportulae along the upper rim (arrows). (e) Proximal view of the valve. Wide ribs are predominant and a fultoportula is located by an arrow. (f) Proximal view of the valve rim. Wide ribs are predominant and most contain fultoportulae located on the valve rim. One wide rib, located by the arrow, is bifurcated. (g) View of proximal surface in a broken valve. This view emphasizes the relief on proximal structures such as the wide ribs and portulae on the valve edge and in the central region (arrow).
The narrow ribs either branched directly off of the wide ribs, or branched off of nodes from the wide ribs (Fig. 3c and d). At a later stage, the wide and narrow ribs extended from the central region, the pore fields were initiated, and the portulae began to form (Fig. 3e). The wide ribs increased in width radially from the center, incorporated the fultoportulae at the upper valve rim, and then became narrower and fused with the lower valve rim (Fig. 3f and g). The edges of the wide ribs contained projections...
which cross-connected to the narrow ribs (Fig. 3g) – eventually forming the distal grid structure. Cross-connections between the narrow ribs were visible on the distal surface (Fig. 3g), but only the ribs were predominant in the proximal view (Fig. 3h). The rim of the valve was formed from the region under the portulae, prior to complete extension of the narrow ribs and pore fields (Fig. 3f). At the final stage the narrow ribs were completely incorporated into the curvature at the valve rim.

**Fig. 4.** Nanopore structure and arrangement. (a) Deflection AFM of the proximal valve surface highlighting the relatively smooth texture of the wide and narrow ribs, and the circular nanopores in between. (b) Deflection AFM of the central region of the distal valve surface. (c) SEM of HF-treated (see Section 2) proximal valve surface, highlighting the narrow ribs and circular nanopores. (d) Height AFM of HF-treated distal valve surface, showing the lower layer circular nanopore region, and the upper layer deposition to form the grid structure.

**Fig. 5.** Formation and structure of fultoportulae along the valve rim. (a) SEM of initial stage in fultoportula formation. Horseshoe-like structures are visible (green arrows) forming at the end of bifurcated wide ribs; in the upper central region is a rib that bifurcated well in advance of the forming fultoportula, and towards the lower right is a rib that bifurcated closer to the forming fultoportula. (b) and (c) Deflection AFMs of forming fultoportulae. (b) is an earlier stage where the initial form of the portula is being defined, and (c) is a later stage where rib silica deposition has moved past the location of the portula, and growth of the portula in the z-axis is occurring. (d) SEM of forming portulae showing circularization of the structure. (e and f) SEM of portulae where circularization is complete and initial stages of overgrowth of silica to form the outer chamber is occurring. (g) SEM of completed fultoportulae along the rim of a cross-sectioned valve. The projections of the portulae above the proximal and distal surfaces are visible. The inner and outer projections are not precisely aligned (arrows). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Pores on the proximal and distal valve surfaces had a distinct appearance (Fig. 4). On the proximal surface, they had a small opening (18 nm) which was circular and conserved in size between different pores (Fig. 4a). On the distal surface in the central region of the valve, the nanopores appeared to have more highly raised rims (Figs. 1c and 4b), but still were relatively circular and consistent in size. In the pore fields between the wide ribs, openings were larger, and irregular in size and shape, with a tendency towards being rectangular (Fig. 1b). Partial etching of valves by hydrofluoric acid (HF) treatment clarified the structure and arrangement of the pores and the valve in general. In the proximal view the circular nanopores were predominant near the valve center (Fig. 4c). Radially towards the periphery of the valve, the narrow ribs were visible, and in-between the ribs were both larger and smaller pores (Fig. 4c). The cross-connections were not visible on the proximal surface even though the narrow ribs were (Fig. 4c). AFM of the HF-etched distal surface revealed both the grid-like structure and the nanopores at a lower depth (Fig. 4d). From these images it is apparent that the grid-like structure was a distinct layer made of particles of silica raised above the base level of the nanopores (Fig. 4c and d).

### 3.2. Portula formation

Fultoportulae on the rim of the valve were formed by separation of the wide ribs into two parts at the margin of the growing valve (Fig. 5). This could involve a branching of the wide ribs either close to, or well in advance of, the forming portula (Fig. 5a). The initial stage of portula formation was distinguished by the appearance of a horseshoe-shaped structure (Fig. 5a), which was clearly dis-
tinct from the wide rib structure. After its initial definition in the \( x/y \) plane, the portulae grew in the \( z \)-axis direction distally, as can be seen by AFM in Fig. 5b and c. The ribs continued to extend past the portulae (Fig. 5b and c) during \( z \)-axis growth. The rim of the portulae circularized (Fig. 5c and d), and eventually became completely closed (Fig. 5e). Subsequently, there was an overgrowth from the wide ribs around the portula rim (Fig. 5e and f), which indicates additional silica deposition on a higher proximal plane in the \( z \)-axis direction. The overgrowth eventually closed, which generated a chamber for the portula that was external to the base layer of the valve (see Hildebrand et al., 2006). The portula opening grew slightly more to generate an extended tube (Fig. 5g). The inner and outer projections of the portulae were not precisely aligned (Fig. 5g, arrows).

The mechanism of the central fultoportula formation had similarities and differences compared to the valve rim fultoportulae (Fig. 6). At a very early stage of wide rib formation, the outline of the fultoportula structure was defined by an area of inhibition of silica deposition (Fig. 6a, arrow). In addition to formation of a large central pore, 2–4 smaller peripheral pores surrounding this were present (Fig. 6b and c). The peripheral pores were also present on the valve rim fultoportulae, but were difficult to see because of the angle. On the central fultoportula, the tubular structure grew in the \( z \)-axis plane on the proximal valve surface (Fig. 6d). The tubular structure did not extend appreciably on the distal surface (Fig. 6e and f), which is in distinction to the valve rim fultoportulae, and suggests that there is no overgrowth of silica. A cross-section

![Fig. 8. Rhodamine phalloidin staining of actin and PDMPO staining of silica associated with valve formation. Sets of images are presented with actin staining (purple), combined actin and silica (green) staining, or actin, silica, and chlorophyll autofluorescence (red). Scale bars are 4 \( \mu m \). (a and a') Early stage of valve formation. Prominent outer rings of actin are visible in the location of the outer edge of the two daughter cell valves, and inner actin filaments are also visible associated with the initially deposited silica. (b and b') Side view of the outer actin ring (o) and the inner filaments (i), which are positioned at the leading edge of silicification. Yellow arrows locate regions where the actin and silica are interdigitated. (c and c') Later stage in valve formation. The outer actin ring remains in the same position but the inner filaments have expanded in relation to the growing diameter of the valve. The inner and outer filaments have become in close proximity relative to (a). Yellow arrows locate positions of interdigitation between actin and silica. (d and d') Actin remaining associated with the newly formed valve after daughter cell separation. Yellow arrow locates a loop of actin that mirrors the position and structure of the raised pore field region. (e, e', and e'') Possible initial stage in valve (V) formation in which an actin ring surrounds the deposited silica. The ring is distinct from those in (a–c) because it is not the full diameter of the valve. (e) The actin rings in adjacent daughter cells. (e') Actin and silica, the large loop of silica outside of the upper actin ring is a labeled girdle band (GB). (e'') Image of the same cell in (e) and (e') (in 2D, central region) showing the position of the actin rings relative to the girdle band (which is the extreme outside extent of the cell).](image)

![Fig. 9. Rhodamine phalloidin staining of actin and PDMPO staining of silica associated with girdle band formation. In these images, girdle bands in two adjacent daughter cells that have not yet separated are visible. Actin is colored purple and newly deposited silica is green. Actin forms a ring at the front of silicification. Scale bars are 4 \( \mu m \).](image)
of the fultoportula is visible in Fig. 6f, showing the inner chamber
and extension from the proximal and distal valve surfaces.

3.3. Girdle band formation

The detailed structures of girdle bands are rarely imaged (Hildebrand et al., 2006, 2009b), due to the difficulty of visualizing these thin structures. It is not clear whether the images presented in Fig. 7 represent distinct stages in girdle band formation, but they do highlight distinct features. The girdle bands in C. cryptica were similar in structure to those of T. pseudonana (Hildebrand et al., 2006), and consisted of a relatively featureless portion that overlapped with adjacent girdle bands, and a more structured part containing nanopores. One edge of the featureless portion was smooth and appeared to have a lip, whereas the other edge was serrated (Fig. 7a). A cross-hatched silica grid structure was observed adjacent to the featureless portion (Fig. 7b) and the ribs were apparently precursors to the nanopores (Fig. 7c). AFM revealed the nanoscale features of the girdle bands (Fig. 7d), which consisted of particulate silica even in the relatively featureless portion, and ribs that were composed of organized agglomerates of particulate silica.

3.4. Actin localization

To examine the involvement of the cytoskeleton in cell wall formation in C. cryptica, actin was located in relation to newly formed silica by fluorescence microscopy using rhodamine phalloidin and PDMPO as stains, respectively. Although actin was seen to form a diffuse network inside the cell, it was evident that a substantial accumulation of actin was located in the proximity of the SDV during silica polymerization (Fig. 8). In Fig. 8a is a cell at an early stage

Fig. 10. SEM of the effects of cytoskeletal inhibitors on valve structure. Treatment with the microtubule inhibitor colchicine, distal (a) and proximal (b) views. Note the misalignment of ribs and mispositioning of portulae. Treatment with the microtubule inhibitor oryzalin, distal (c) and proximal (d) views. Ribs are also misaligned and portulae are mispositioned. Inset in (c) shows that the pore field region blends into the wide ribs under this treatment. (e and f) Treatment with the actin inhibitor cytochalasin D, both distal views. The overgrowth of silica in fultoportula formation is inhibited (e), and portions of the valve are not silicified (f). In addition, the normally raised pore field region (Fig. 1d), is not raised after cytochalasin D treatment.
of valve formation. There are two (one for each daughter cell valve) intensely stained rings of actin located where the periphery of the valve would be expected (Fig. 8a). In between the two rings were inner filaments of actin, which were associated with newly deposited silica (green fluorescence, Fig. 8a, right). The inner filaments and outer ring appeared to be connected (Fig. 8a). Examination of actin in relation to new silica deposition in another cell confirmed the connection between the ring and filaments and indicated that the inner filaments were located at the leading edge of silica deposition, and that some portions of the silica and actin were interdigitated (Fig. 8b, yellow arrows). At a later stage of valve formation (Fig. 8c) the silica-associated actin was seen to have expanded (the inner filaments and outer ring are closer together than in Fig. 8a) and yet maintained its peripheral association with the silica. In addition, strands of actin were seen to interdigitate between the wide rib/fultoportula portion of the valve (Fig. 8c, yellow arrows). In some cases, the interdigitiation was seen to persist even after valve completion and daughter cell separation (Fig. 8d, yellow arrows). In several cells we saw what could have been the earliest stage in valve formation in which the actin ring was not positioned at the extreme periphery of the cell (Fig. 8e). It also was in the same plane as the deposited silica, and not at the curved edge of the completed valve (Fig. 8e). Based on the initial stage structure identified in Fig. 3, this stage could represent formation of the central fractally-organized portion of the valve. If so, the data in Fig. 8 would indicate a dynamic movement of the actin ring from its initial to final position.

Actin was also visualized associated with forming girdle bands. Cyclotella cryptica starved for silicon can arrest in the G2 phase of the cell cycle (unpublished observation), and upon silicon replenishment, can complete valve synthesis and separate. In Fig. 9 is an image of cells that are in the process of separation, synthesizing new girdle bands. In both daughter cells, silica in the girdle bands is associated with an actin ring (Fig. 9). The actin ring is located at the edge of the girdle band furthest away from the valve, which corresponds to the pore region of the girdle band (Fig. 2).

We attempted without success to localize microtubules in relation to forming silica structures, using both tubulin antibodies and the microtubule-specific dye Oregon Green paclitaxel (Lillo et al., 2002). As an alternative approach, we examined the effect of two inhibitors of microtubule polymerization, colchicine and oryzalin, and also the actin inhibitor cytochalasin D, on valve formation (Fig. 10). For all inhibitors, a titration was performed to determine the concentration at which cell growth was reduced but not stopped, and cells from that treatment were isolated and purified for SEM. Although these inhibitors could have multiple cellular effects depending on the specific process an individual cell was undergoing, several consistent trends on alteration of valve structure were observed. Both microtubule inhibitors (which have similar modes of action) affected the positioning (but not necessarily the structure) of the fultoportulae, which were frequently located short of their normal location at the valve edge (Fig. 10b–d). The microtubule inhibitors also disrupted both the width and the radial orientation of the wide ribs, leading to a less organized and less linear arrangement (Fig. 10a–d). Close examination showed that in regions where the wide ribs were diminished, the pore fields extended into them (Fig. 10c, inset), and in general there was an increase in the numbers of thin ribs. The actin inhibitor cytochalasin D did not affect the positioning of the fultoportulae, but did affect the “overgrowth” of silica involved in forming the outer chamber of the fultoportula (forming an elongated slit rather than a circular opening), and had a slight effect on the linearity of the wide ribs (Fig. 10e and f). In some instances, portions of the valve structure were entirely compromised (Fig. 10f). The pore field regions were also affected, and frequently were not well formed or were not raised as high above the valve surface as usual (Fig. 10e).

4. Discussion

The present study on cell wall structure formation in C. cryptica revealed differences in structure formation compared with a previous analysis on T. pseudonana (Hildebrand et al., 2006, 2007). The Cyclotella cryptica valve has a distinct three-dimensional component, with pore fields raised above the plane of the rest of the valve (Fig. 1d) and has two distinct forms of ribs, both wide and narrow, whereas T. pseudonana has a flat valve and only consistently-sized narrow ribs (Hildebrand et al., 2006). The differences in structure relate to differences in structure formation processes in these two species.

Valve formation in C. cryptica demonstrates a fractal to path-dependent transition comparing the central region with the wide rib and pore field region (Fig. 3). At a fundamental level this suggests a transition from a relatively static self-organizing process in the central region, to a dynamic directional growth process to form the wide ribs and pore fields (Fig. 3). The change in position of actin in concert with the growing silica polymerization front (Fig. 8) suggests that movements of actin are involved in the directional growth process. In this scenario, initial silica deposition is confined within a space delineated by an actin ring (Fig. 8e) which corresponds to the primary silicification site (PSS) identified in other diatom species (Pickett-Heaps et al., 1990). There are apparently two stages of expansion of actin after the initial silica deposition. The first involves the expansion of the ring to its final position at the edge of the valve (compare Fig. 8e and 8a), and this expansion does not include the substantial deposition of silica. Recent work in T. pseudonana also indicated that the SDV was fully expanded prior to complete silica deposition, and showed an association with actin-sized filaments (Hildebrand et al., 2009a). The reason for the two-step positioning of the actin ring in C. cryptica is not clear, however we can speculate that it may have something to do with enabling formation of a flat-on-the-top (initial stage) but curved-on-the-edge (later stage) valve structure where the rigid flat structure provides an anchor point to enable curvature at the rim. Detailed investigation of other species that make distinctly-shaped valves is needed to provide further insight into this. The second process of actin expansion is seen in the movements of the actin filaments closely associated with the silica (Fig. 8a–c). Association of filamentous actin with the leading edge of silicification has been shown by TEM in a variety of diatom species (Pickett-Heaps et al., 1990; van de Meene and Pickett-Heaps, 2002, 2004), indicating a conserved and essential functional role. The filamentous actin may play an important role in shaping or maintaining the integrity of the SDV membrane, as will be discussed in more detail below.

Cross-sectioning and AFM revealed that all structures in C. cryptica are formed from nanoparticulate silica (Fig. 2), and these assemble into larger particles which are organized on an even higher level to form mesoscale structure. Nanoparticulate silica has been observed previously in diatom valve cross-sections and on surfaces (Crawford et al., 2001; Hildebrand et al., 2006; Losic et al., 2007; Noll et al., 2002). In some cases, these were shown to have species-specific average particle diameters (Crawford et al., 2001), which could result from the properties of silica polymerization determinants such as silaffins, LCPAs, and silacidins (Kröger et al., 2000, 2002; Poulsen and Kröger, 2004; Wenzl et al., 2008) unique to the species. Particle formation and association is a thermodynamically-favored aspect of silica polymerization chemistry (Iler, 1979). In diatoms, particle formation is catalytically promoted by the interaction between silaffins, LCPAs, and silacidins, however the silica particles do not spontaneously self-organize into a higher order (mesoscale) structure, although their assembly can reflect nanoscale textures seen in diatom frustules (Kröger et al., 2000, 2002; Poulsen and Kröger, 2004; Wenzl et al., 2008). Thus, in diatoms other organizational determinants...
must be involved in the formation of mesoscale structure (Davis and Hildebrand, 2007; Pickett-Heaps et al., 1990; Robinson and Sullivan, 1987). The mesoscale assembly pattern of particles as seen here (Fig. 2) is consistent with either confinement of silica polymerization within a defined space and/or organization of silica polymerization determinants on a template with numerous nucleating sites. Recent work in T. pseudonana is consistent with the concept of confinement in the formation of valve structure, and templating with the girdle bands (Hildebrand et al., 2009a).

An interesting observation is the smoothing of the surface of structures as they mature (Fig. 2). The cross-sections (Fig. 2b and c) reveal that part of the smooth surface morphology, especially on the proximal valve face, is intrinsically generated during silica particle formation, perhaps due to close oppression of the polymerizing silica with the SDV membrane (Crawford et al., 2001; Hildebrand and Wetherbee, 2003; Pickett-Heaps et al., 1990). On the distal surface, this is not the case (Fig. 2b). This is consistent with a closer association of one side of the membrane with the silica, and also additional deposition after initial particle formation on the other side. It is well established in other diatom species that at initial stages of deposition, silica is most closely associated with one side of the SDV, and that the SDV expands in the z-axis only in one direction towards the other side (Hildebrand et al., 2006; Schmid and Schulz, 1979; Schmid and Volcani, 1983). In some cases, the morphology of silica formed during z-axis expansion is dramatically different from the morphology of initially deposited silica (Hildebrand et al., 2006). In C. cryptica the z-axis expansion is apparently minimal.

On the proximal valve surface, circular nanopores (18 nm diameter) are visible in-between the ribs in the pore field region (Fig. 4a). On the distal surface, circular pores with similar characteristics are visible in the central region of the valve (Fig. 4b), but in the raised pore field region pores tend to be larger in diameter and elongated in the radial direction – some having a rectangular shape (Fig. 1b). The pore field regions (and wide and narrow ribs) constitute a distinct silica layer from the nanoporous region as can be seen in Fig. 4c and d. The distinct layer is not clearly evident in the cross-sections of Fig. 2a–c, which could be due to (1) the precise location of the viewed image, (2) disruption of the additional layer during cleavage, or (3) complete integration of the additional layer in the fully mature structure. From the proximal view, the narrow ribs are seen as linear structures aligned radially on the valve (Figs. 1f and 4c). On the distal surface the radially-oriented ribs are still visible, but much more noticeable are cross-connections between the ribs (Fig. 1b). The lack of cross-connections visible from the proximal side is consistent with a distinct silica layer obscuring the view, and the height difference between the nanopores and ribbed structures visible by AFM in Fig. 4d, directly shows the layers. The cross-connected rib structure is formed at an early stage during the expansion of the valve from the central region (Fig. 3g), and the nanopore layer is apparently formed later.

SEM and AFM provide a detailed look at fultoportulae formation (Figs. 5 and 6). The horseshoe structure of the initial form of the portula is well defined, and has the appearance of a pre-structured feature around which silica is deposited as the wide ribs are forming (Fig. 5a–d). A reasonable hypothesis is that the fultoportulae are formed from an organic template that is pre-positioned at specific locations where the wide ribs will propagate and form prior to silica deposition around the complex (Fig. 11). We interpret the differences in diameter of the forming portulae in Fig. 5b and c as being due to destruction of an organic complex at the earlier stage in Fig. 5b resulting from acid extraction. Recently, Davis and Hildebrand (2007) have suggested a model where silica polymerization determinants are anchor to the silicalemma via interaction with transmembrane proteins and the cytoskeleton, the same mechanism could be involved in the pre-positoning of the portulae complex. The results of the inhibitor experiments (Fig. 10), suggest that microtubules are involved in this positioning. To our knowledge, no TEM cross-sectional imaging of organics associated with the portulae during their formation have been published (although images of the complete portula structure have been – Herth, 1979, 1979), however other organic complexes have been seen associated with other silica structures projecting from the valve plane (Li and Volcani, 1985; Pickett-Heaps et al., 1990), suggesting that a proposed fultoportula complex is reasonable. The growing fultoportula forms both distal and proximal extensions from the valve surface, and in the case of the distal extensions, involve an overgrowth of silica from additional deposition on the wide ribs to form a projecting chamber (Fig. 5e–g; and Hildebrand et al., 2006). Such overgrowth is minimal in the central fultoportula (Fig. 6f), perhaps because growth is not path-dependent in that region.

We cannot rigorously establish whether the data in Fig. 7 represent distinct stages in girdle band formation. However, the localization of actin in Fig. 9 and the previous localization of actin always at the leading edge of silicification (Fig. 8 and Pickett-Heaps et al., 1990; van de Meene and Pickett-Heaps, 2002, 2004) is consistent with the relatively unstructured portion of the girdle bands being synthesized first, followed by the nanopore region. The unstructured portion is the region of girdle band that “underlaps” with the adjacent girdle band, and it makes logical sense that this would be synthesized first to maintain structural integrity. Recent AFM data (Hildebrand et al., 2009b) coupled with these images (Fig. 7) indicate that girdle bands exhibit similar complexity in their structure and formation processes as some valve structures.

Our results indicate a significant role for actin in the formation of both valves and girdle bands (Figs. 9 and 10). In both structures, actin is located at the leading edge of silicification (Figs. 9 and 10) suggesting that it is a critical determinant of silica formation. It has been proposed that actin filaments are involved in the extension and morphogenesis of the SDV through association with the growing edge of the silicalemma (Pickett-Heaps et al., 1990). Detailed observations of actin localization by fluorescence microscopy have been done in the centric diatoms Proboscia alata and Rhizosolenia setigera (van de Meene and Pickett-Heaps, 2002, 2004). These species have elongated and conical valve shapes, in contrast to the relatively flat-on-the-surface but curved-at-the-perimeter valve of C. cryptica and many other centric species. All three species have substantial actin rings, and in P. alata and R. setigera the rings have

![Fig. 11. Model for fultoportula formation. (a) Detailed view of proximal portion of a valve rim fultoportula showing the large central pore flanked by three accessory pores. (b) Schematic of how proposed organic complexes positioning the central and accessory pores control the molding of forming silica around them.](image-url)
been shown to be dynamically moved along with the silicification front and expand in diameter with the increase in diameter of the valve during its formation (van de Meene and Pickett-Heaps, 2002, 2004). In *C. cryptica* there appear to be two positions for the actin ring; the first defines the location of initial deposition, and the second the edge of the completed valve (Fig. 8). At the second position, the ring is not associated with silica (Fig. 8). The additional actin network associated with the silicification front in *C. cryptica* (Fig. 8) apparently plays a similar dynamic role as described for actin in the other species at the leading edge of silicification.

There are different possible functional roles that actin could play in the silicification process. One is as a stabilizer of the SDV membrane to prevent osmotically-induced swelling and breakage. Such a role has been documented in the maintenance of the Golgi apparatus in its stacked form – actin networks maintain the flattened membrane shape and prevent swelling resulting from accumulation of high concentrations of osmolytes (proteins and carbohydrates) in the Golgi lumen (di Campli et al., 1999; Egea et al., 2006). Osmotic stress should also be high in the SDV considering the accumulation of solid silica material inside the vesicle, which should induce swelling. One possibility is that actin stabilizes the SDV membrane until silica is deposited, at which point the membrane becomes anchored to the mineral and actin is no longer needed. A recently described close association of the SDV membrane only where silica was deposited is consistent with this (Hildebrand et al., 2009a). The effect of cytochalasin D on the overgrowth of silica during fultoportula formation but not the positioning of the ribs or portulae (Fig. 10e and f), suggests that actin may play a minimal role in the initial deposition of ribs, but a more substantial role in the subsequent expansion of the additional silica layer (Fig. 4). A second possible role for actin is in membrane shaping, in which actin is known to be involved in other systems (Egea et al., 2006; Lanzetti, 2007). Considering the interdigitation between actin and the silica adjacent to the raised pore field regions (Fig. 8), the hemispherical shape of actin observed in Fig. 8d, which has the same curvature as the silica in the pore field region, and the flattening of the pore field region in the presence of the actin inhibitor (Fig. 10e), actin could be involved in shaping the SDV membrane prior to deposition in this region. However, an alternate explanation is that actin is only playing a membrane stabilizing role, and conforms to the membrane shape derived from other forces. A third possibility relates to previous studies (Pickett-Heaps et al., 1990) and our results (Fig. 8) that indicate an involvement of actin in the microscale overall shaping of the forming silica structure. Models have been presented (Davis and Hildebrand, 2007; Robinson and Sullivan, 1987) whereby cytoskeletal elements organize silica polymerization determinants via interaction with proteins spanning the silicalemma, similar to the actin/spectrin/integrin system (Branton et al., 1981), thus actin could be involved in organizing silica polymerization determinants in the SDV, and play a role in structure formation at the mesoscale.

The mechanisms involved in formation of the raised pore fields relate to how diatoms may form particular three-dimensional structures. The raised regions must require extra addition of SDV membrane in their locations. In *C. cryptica*, the wide rib, fultoportulae, and valve rim (which may form an inflexible framework) are formed prior to completion of the raise pore fields (Fig. 3). As the pore field regions are developed, SDV membrane components could be added in excess in these regions, which could result in a need to bend the structure above the plane of wide rib deposition. Why would it bend up instead of down? Because of the directionality of membrane trafficking, membrane material must be added either on the proximal side of the SDV or at the leading edge of silicification. If the proximal membrane is relatively “fixed” by positioning of silica polymerization determinants or polymerized silica, then excess “free” membrane material would accumulate on the distal surface, enabling the structure to bend that way. Another contribution could be “interactive division” (Mann, 1984), in which adjacent daughter cell valves are closely appressed, and non-planar structures result in protrusions in one valve that form depressions in the other. We observed such an alignment between adjacent daughter cell valves in *C. cryptica* (Fig. 12). Interactive division probably only lowers the energy requirements for formation of such structures; however, and does not dictate them because there is no previously fixed structure in the formation of initial cells after sexual reproduction.

Our data (Figs. 8–10), coupled with previous observations (Pickett-Heaps et al., 1990), suggest several underlying concepts regarding valve formation in *C. cryptica*, and perhaps in general for centric diatoms with circular valves. Microtubules have been universally associated with rib structures in diatoms (Pickett-Heaps et al., 1990), and our data are consistent with microtubules defining the location of the wide ribs and positioning the fultoportulae (Fig. 10). Previous work on *P. alata* suggested that microtubules
were involved in the radial elongation of the valve (van de Meene and Pickett-Heaps, 2002). Actin is universally associated with the silica polymerization front (Pickett-Heaps et al., 1990; van de Meene and Pickett-Heaps, 2002, 2004, and Figs. 8 and 9), and although there may be multiple reasons for this (see previous paragraph), one consistent observation is that actin defines the diameter of the circular valve, and expansion of actin results in expansion of the silicification front. It is possible that actin interacts with microtubules to either help define the shape of the SDV in a static sense or enable dynamic movement of the silicification front during expansion. In the presence of microtubule or actin inhibitors (Fig. 10), patterning was affected but silicification still occurred. This indicates that additional components besides the cytoskeleton are involved in mesoscale structure formation, but suggests that an interaction between these components and the cytoskeleton are essential for correct pattern formation.

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References


