Exploring Bioinorganic Pattern Formation in Diatoms.
A Story of Polarized Trafficking

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The world’s oceans cover 70% of the surface of our planet. The photosynthetic organisms living within its photic zone are responsible for about one-half of global primary productivity. The most successful organisms are thought to be photosynthetic prokaryotes (cyanobacteria and prochlorophytes) and a class of eukaryotic unicellular algae known as diatoms (Norton et al., 1996; Van Den Hoek et al., 1997; Falkowski et al., 1998).

Diatoms are likely to have arisen around 280 million years ago following an endosymbiotic event between a red eukaryotic alga and a heterotrophic flagellate related to the Oomycetes (Medlin et al., 1997, 2000). As a consequence, their only phylogenetic similarity to green algae and higher plants is derived from the primary endosymbiotic event, which is thought to have occurred at least 700 million years ago (Kowallik, 1992). Therefore, diatom cells have a range of features that make them highly divergent from the classical cellular structure of higher plants, including:

(a) The use of the brown carotenoid pigment fucoxanthin for light energy transfer within the light-harvesting complexes of photosystems I and II.

(b) The presence of four membranes around their plastids. The inner two membranes are equivalent to the membranes that normally surround higher plant chloroplasts, whereas the second membrane (as counted from the outside) is thought to be derived from the endosymbiont’s plasma membrane, and the outer membrane is continuous with the endoplasmatic reticulum of the host cell.

(c) The presence of a rigid cell wall composed largely of amorphous silica (i.e. glass). The exquisite lacework-like patterning of diatom cell walls (see example in Fig. 1) is reproduced with high fidelity from generation to generation and is species specific. For these reasons, it has been used since the last century for taxonomic classification.

Current phylogenetic trees place diatoms close to Alveolata lineages and far from the green and red lineages of other photosynthetic eukaryotes (Baldauf et al., 2000). This has been further confirmed by the analysis of diatom expressed sequence tags (ESTs), although more similarity than expected was found with Metazoan genes (S. Scala, N. Carels, A. Falciatore, M.L. Chiusano, and C. Bowler, unpublished data). The EST program has revealed the presence of genes encoding enzymes involved in cAMP metabolism, as well as genes encoding the components of the animal extracellular matrix, such as fibronectins, elastins, and tenascins, none of which appear to be present in higher plants. As a consequence, one could almost consider diatoms as photosynthetic animals rather than unicellular plants!

The study of diatom biology therefore promises to reveal many novel aspects (Scala and Bowler, 2001), and now that so much information has been generated about the “conventional” metabolism of model organisms such as yeast (Saccharomyces cerevisiae), Caenorhabditis elegans, mouse (Mus musculus), and Arabidopsis, it is possible to divert some attention to more recalcitrant experimental systems. Given the extreme ecological importance of diatoms, they should come close to the top of the list of priorities for plant biologists, and could become the system of choice for studying phytoplankton interactions with the marine environment. The range of molecular-level technologies that have now been established, such as genetic transformation (Dunahay et al., 1995; Apt et al., 1997; Falciatore et al., 1999; Fischer et al., 1999), protocols to study gene expression (Leblanc et al., 1999), and the availability of several reporter genes (Falciatore et al., 1999; Zaslavksaia et al., 2000) make them all the more attractive.

It is not surprising that the study of silica cell wall biogenesis has been a major focus of diatom research for the last 150 years. However, most information is descriptive, being based on microscopic observations, and only recently have biochemical and molecular techniques been incorporated, due largely to the pioneering work of Nils Kröger and colleagues (for review, see Kröger and Sumper, 1998). The current review aims to summarize the basic aspects of the silica biomineralization process and to indicate the major gaps in current understanding that can now be addressed using knowledge and tools derived from better studied organisms.
Diatoms lie within the Heterokont division of the class Bacillariophyceae (also known as Stramenopiles). They usually exist as single cells of between 5 μm and 5 mm, depending on the species, although some can form chains or colonies (Van Den Hoek et al., 1997; Lee, 1999). Each cell is surrounded by a unique type of cell wall (known as frustule) which consists of two halves of amorphous polymerized silica resembling a box with an overlapping lid (Fig. 2). The inner frustule is known as the hypotheca and the outer one is denoted the epitheca. In addition to the main valves (hypovalve and epivalve), each frustule often contains several ring-like silica structures denoted girdle bands (Fig. 2).

There are two major diatom groups: the centric and the pennate diatoms. These can be most simply distinguished from each other on the basis of cellular symmetry: Centric diatoms are radially symmetrical (resembling a petri dish), whereas pennate diatoms are elongated and bilaterally symmetrical.

Diatom cell division typically proceeds through asexual mitotic divisions. However, because the rigid siliceous cell walls largely preclude cell growth through expansion (except unilaterally by the addition of new girdle bands), the two daughter cells must be formed inside the parent cell (Fig. 3; Pickett-Heaps et al., 1984, 1990; Van Den Hoek et al., 1997). One sibling cell uses the epitheca of the parent cell as a size guide to generate a new hypotheca, whereas the other uses the parental hypotheca, which therefore becomes the epitheca of the daughter cell (Fig. 3). As a consequence, mitotically dividing populations decrease in mean size over time. In most species, size restoration occurs through sexual reproduction once a critical size threshold (typically 30%–40% of the maximum size) has been reached (Mann, 1993; Van Den Hoek et al., 1997).

The strict requirement for silica in frustule formation has led to the evolution of silica-dependent checkpoints in diatom mitosis, which presumably ensure that cell division does not occur unless there are sufficient bio-available silica for frustule formation. Checkpoints have been found to occur in diatoms at both the G1/S and G2/M transitions (Brzezinski et al., 1990; Martin-Jezequel et al., 2000) and it has been speculated that diatom DNA polymerases may be silica dependent. This has not yet been confirmed at the molecular level.

It is known that diatoms possess silica uptake transporters that are able to accumulate silica at intracellular concentrations up to 250 times higher than in the surrounding media (Martin-Jezequel et al., 2000). Where it is localized intracellularly is still open to debate (see “Transport to the SDV”). These transporters have novel primary structures, and by expression in *Xenopus laevis* oocytes it has been found that they are highly selective for the soluble form of silica, Si(OH)₄ (Hildebrand et al., 1997, 1998). Homologous sequences do not exist in the Arabidopsis genome, although it is possible that they will be found in rice, which is known to have silicified hairs (Raven, 1983).
epitheca on one side and the other bound by the hypotheca on the other side (Fig. 3). Each daughter cell must then synthesize a hypotheca ex novo before they can separate. This process has been observed extensively in numerous diatom species at the ultrastructural level, such that a rather complete description of the process is now available (Pickett-Heaps et al., 1990). The key events are shown in Figure 3 and can be summarized as follows:

(a) The nucleus of each daughter cell moves to the side of the cell where the hypotheca will be formed.

(b) A microtubule center (MC) positions itself between the nucleus and the plasma membrane above which the hypotheca will eventually be placed.

(c) A specialized vesicle known as the silica deposition vesicle (SDV) forms between the MC and the plasma membrane in a region that becomes the “pattern center.”

(d) The SDV elongates into a tube and then spreads out perpendicularly to eventually form a huge vesicle along one side of the cell.

(e) A new valve is formed within the SDV by the targeted transport of silica, proteins, and polysaccharides. During this process, the SDV becomes acidic as a result of the silica polymerization process (Vrieling et al., 1999). Some of the organic components eventually form a coat around the silica framework, whereas others are involved in silica deposition.

(f) Once valve biogenesis is complete, it is exocytosed by fusion of the SDV membrane (the silicalemma) with the plasma membrane. As a consequence, the inner face of the silicalemma is thought to become the new plasma membrane.

(g) Following separation, the daughter cells can expand unidirectionally along the cell division axis by the biogenesis of girdle bands. These structures are also formed within SDVs in a manner analogous to that described above.

These events are very similar in both pennate and centric diatoms, although the timing can be different (Pickett-Heaps et al., 1990; Van Den Hoek et al., 1997). Very little is known about the underlying mechanisms controlling these events. Most intriguing is how such intricate structures can be generated from a shapeless inorganic substrate. Although it has been proposed that much of the early biomineralization process can proceed via physico-chemical space filling of silica (Gordon and Drum, 1994), this is certainly not sufficient to explain the exquisite species-specific designs that are ultimately generated. The fact that frustule design is faithfully reproduced from parent to daughter cells indicates that there is a significant genetic basis underlying bioinorganic pattern formation.

One strategy that appears to be important in pattern formation is known as macromorphogenesis, whereby silica deposition is “molded” into a pattern by the presence of organelles such as mitochondria spaced at regular intervals along the cytoplasmic side of the SDV (Schmid, 1994). Therefore, these or-

**Figure 3.** Schematic overview of mitotic cell division and hypovalve and girdle band formation. For further details see text. N, Nucleus; MC, microtubule center; SDV, silica deposition vesicle. Drawing by Ian Nettleton.
ganelles are thought to physically restrict the targeting of silica from the cytoplasm, to ensure the laying down of a correctly patterned structure (for models, see Schmid, 1994).

The importance of macromorphogenesis will only be clear when it is known how silica and silica-associated proteins and polysaccharides are deposited into the SDV. This is thought to occur, at least partially, by their transport to the SDV in vesicles, which is described as membrane-mediated morphogenesis (Pickett-Heaps et al., 1990).

ORGANIC COMPONENTS OF DIATOM CELL WALLS

Much of the biochemical studies of frustule composition have been done in the pennate diatom *Cylindrotheca fusiformis* (Kröger and Sumper, 1998). This work has led to the discovery of novel peptides known as silaffins that may participate in the basic biomineralization process within the SDV (Kröger et al., 1999). These cationic peptides of various sizes are generated from precursor polypeptides and are characterized by the presence of Lys-Lys repeat elements that are covalently modified by the posttranslational addition of novel chemical units, such as oligo-N-methylpropylamines (Kröger et al., 2001). It is interesting that silaffins can promote the formation of nanoscale silica spheres in vitro (Kröger et al., 1999).

Other major organic constituents of diatom bio-
silica are putrescine-derived long-chain polyamines. Like the silaffins, these can also induce rapid silica precipitation in vitro (Kröger et al., 2000), forming spheres between 100 nm and 1 μm depending on the polyamine used. Different diatoms have different complements of silaffins and frustulins, which confer species-specific differences to in vitro silica precipitation. It is remarkable that some of these combinations generate silica blocks in addition to silica spheres. This observation infers that silaffins and polyamines therefore may be involved in generating the basic building blocks that are then modeled into diatom frustules, and that combinatorial interactions between them may be responsible for the underlying genetic basis of species-specific pattern formation. However, the fact that they are both bound tightly to the silica scaffold of diatom cell walls begs the question: “How did they get there?” If they are bound to a preformed silica framework, then how was it formed? On the other hand, if silaffins and polyamines are responsible for silica pattern formation, how are they themselves directed into a network that determines the final pattern? To find out, it is necessary to understand the mechanisms underlying the targeting of silaffins, polyamines, and silica to the SDV.

Other proteinaceous components of diatom cell walls include frustulins and pleuralins (formerly denoted HEP proteins; Kröger et al., 1997; Kröger and Wetherbee, 2000). Like the silaffins, pleuralins are also tightly bound to silica and can only be removed from diatom cell walls following the solubilization of silica with hydrogen fluoride. Pleuralins are encoded by a small multigene family and are characterized by the presence of repeated amino acid motifs. It is interesting that the localization of one of them, pleuralin-1, is precisely restricted to the most terminal girdle band (known as the pleural band) of the epitheca (Fig. 2), in the region of overlap with the hypotheca. During cell division it also becomes associated with the pleural band of the hypotheca, at a time when the parental hypotheca is functionally converted into an epitheca of one of the daughter cells (see Fig. 3; Kröger and Wetherbee, 2000). This is a very significant observation, because it demonstrates that hypotheca-epitheca differentiation is under strict developmental control.

Pleuralin-1 is not targeted to the SDV but is directly secreted into the cleavage furrow that forms between the two daughter cells (Kröger and Wetherbee, 2000). Association with the terminal girdle band of the hypotheca therefore occurs in the extracellular space. Secretion is thought to occur by general mechanisms of exocytosis, but it is nonetheless an intriguing example of polarized transport specifically toward the cleavage furrow. It will be interesting to determine how many other wall-associated proteins avoid the SDV during their secretion and incorporation into diatom frustules.

Frustrulins are much more loosely associated with diatom cell walls than are silaffins and pleuralins, and can be extracted with EDTA. To date, five different frustulins have been described, all of which are glycoproteins able to bind calcium due to the presence of EF hands (Kröger et al., 1994). They also contain characteristic acidic Cys-rich domains, the function of which is not yet known. Frustrulins are not thought to be involved in silica deposition, but constitute the outermost protein coat of the cell wall (van de Poll et al., 1999).

TRANSPORT TO THE SDV

Both the silaffins and the frustulins must be targeted to the SDVs. Because the SDV is such an unusual but fundamental structure for the diatom cell, understanding its biogenesis and dissecting the targeting mechanisms for silica and protein import are of major biological interest. Furthermore, because the frustule is formed on only one-half of the cell, it is a dramatic example of cell polarity.

Silaffins, frustulins, and pleuralins are all synthesized as precursor proteins containing aminoterminal presequences that resemble typical signal sequences for the co-translational import of proteins into the endoplasmic reticulum. Other unidentified sequences are likely to be present within the silaffin and frustulin precursors that target them from the
Golgi apparatus to the SDV. The silaffin precursor is cleaved into specific peptides that are found associated with silica in the frustule (Kröger et al., 1999). Therefore, it is conceivable that this maturation event occurs in a post-Golgi compartment (e.g. within the transport vesicles or in the SDV itself after specific targeting of the precursor protein to the SDV).

Bearing in mind the above considerations, it seems likely that the Golgi apparatus is connected to the SDV via transport vesicles. Such a system would also provide a convenient and economical means for silicalemma expansion. Although clouds of vesicles emanating from the Golgi have often been observed during cell wall deposition (Pickett-Heaps et al., 1990), vesicle fusion to the SDV has not been clearly established. However, because many specific mechanisms of vesicle targeting to organelles have been studied intensively in other eukaryotes, parallels can be easily searched for. One approach would be to look for the presence of specific protein pairs such as V-SNARE and T-SNARE, which are known to mediate vesicle docking (Chen and Scheller, 2001; Pelham, 2001). Furthermore, the use of drugs such as brefeldin A, which blocks forward vesicle trafficking from the Golgi apparatus (Lippincott-Schwartz, 1983; Pelham, 1991), could clarify the role of Golgi vesicles in SDV formation.

We envisage two possible mechanisms of vesicle targeting from the Golgi apparatus to the SDV. Analysis of the silaffin and frustulin sequences reveals stretches of amino acids highly enriched in acidic residues, which is also typical of granins, secretory proteins that accumulate in post-trans-Golgi network (TGN) secretory granules in neuroendocrine cells (Huttner et al., 1991). Therefore, these SDV-targeted proteins could follow a pathway similar to regulated secretion occurring at the plasma membrane of specialized secretory cells (Miller and Moore, 1990; Tooze, 1998). In this case, peptides would accumulate in the Golgi-derived vesicles by a process requiring protein aggregation, due to acidic and Pro-rich amino acid sequences (Benedum et al., 1987; Castle et al., 1992; Rindler, 1998), and would then be targeted to the SDV in response to a specific signal in a manner analogous to the regulated secretory pathway of mammalian cells. A report of small vesicles surrounding the nascent SDV, but not yet fusing (Li and Volcani, 1985), might support this model.

Another possibility would be a mechanism similar to the Man 6-phosphate receptor-mediated pathway that hydrolytic enzymes follow from the TGN to the lysosomes (von Figura, 1991). In this case, a transmembrane receptor protein should be present in the TGN, where it would bind to signals within the SDV-targeted proteins separating them from other proteins in the lumen and concentrating them in coated vesicles that would then be specifically targeted to the silicalemma. The fact that some Golgi-derived vesicles in diatoms seem to possess a coat (Gordon and Drum, 1994) could support this hypothesis.

Conversely, how silica is targeted to the SDV is also a mystery. In principle, it could follow the same route followed for cell wall-associated proteins or could be targeted independently. It is not known if it is already complexed with organic molecules during its transport through the cell or even whether it is sequestered into vesicles. The existence of silica transport vesicles has been proposed (Schmid and Schulz, 1979), although evidence is poor. What is clear is that if silica was already precipitated inside targeting vesicles, it would have been observed in electron micrographs. The fact that it has not (Pickett-Heaps et al., 1990; Martin-Jezequel et al., 2000) indicates that silica is only likely to encounter silaffins and polyamines once inside the SDV. Furthermore, if silica is packaged into vesicles, it must first be targeted into them. There is no evidence that this happens. Although both silica transporters and ionophores have been identified in diatoms (Bhattacharya and Volcani, 1983; Martin-Jezequel et al., 2000) it is not known whether they are localized on vesicles inside the cell. Furthermore, it is not clear why a specific vesicle transport mechanism should be needed to transport silica to the SDV because the presence of silica transporters on the silicalemma could be sufficient. The availability of antibodies against the silica transporters would make it possible to determine whether they are present on the silicalemma as well as on the plasma membrane. This is one of the most urgent questions that requires an answer.

If silica transporters are present on the silicalemma, they could be targeted to it by endocytic vesicles derived from the plasma membrane. This process would both increase the size of the SDV and create the conditions for concentrating silica within this organelle. Fusion of Golgi-derived vesicles carrying silaffins and polyamines with the SDV would then initiate the process of silica deposition.

**SDV FORMATION**

Based on the above considerations, it is possible that the SDV has an endocytic origin similar to the early endosomal compartment present in mammalian cells (Woodman, 2000). The initial formation of this organelle could be strictly linked (or driven) by the positioning of the MC between the nucleus and the plasma membrane immediately after cytokinesis. The fact that microtubule depolymerization affects valve formation supports a role for microtubules in SDV formation (see Pickett-Heaps et al., 1990, and refs. therein).

The observation that the acidity of the SDV increases with silica deposition (Vrieling et al., 1999) can be paralleled with the maturation hypothesis of mammalian cells, which postulates that late endosomes/lysosomes derive from maturation of early
endosomes (Mullins and Bonifacino, 2001). The identification of markers specific for early and late endosomes and lysosomes (e.g. Rab5, Rab7, and Rab9, respectively; Zerial and McBride, 2001) on the silica-lemma therefore could provide some clues to the origin of the SDV.

An additional, albeit speculative, connection between silica polymerization and lysosomes comes from the recent finding that a lysosomal cathepsin T-like hydrolase is the major silica binding protein within the silica-rich spicules of sponges (Shimizu et al., 1998).

**EXOCYTOSIS**

The final stage of the valve formation process is exocytosis of the SDV. There are at least four models for how this could occur (Pickett-Heaps et al., 1990; van de Poll et al., 1999). It is most likely that the SDV fuses at specific sites, perhaps the valve edge. To drive fusion at specific sites, landmarks for vesicle docking must be present. Furthermore, timing must be precise. In the case of mammalian and plant cells, specific vesicle fusion is driven by the pairing of V- and T-SNAREs (Chen and Scheller, 2001; Pelham, 2001). However, additional factors define cognate fusion and docking sites. One example is represented by the exoyct in yeast, a multiprotein complex involved in vesicle targeting and docking at the plasma membrane (TerBush et al., 1996). The exocyst is specifically localized to sites of active exocytosis and its proper localization is thought to be important for spatial regulation of secretion in yeast and mammalian cells (TerBush et al., 1996; Zahraoui et al., 2000). It has been found recently that the small GTP-binding protein Rh1 is involved in regulation of exocyst localization at the plasma membrane (Guo et al., 2001). It would be interesting to analyze whether a similar multiprotein complex exists at specific sites of the SDV or plasma membranes of diatoms.

**FUTURE PROSPECTS**

There is clearly a great need for the development of pharmacological, biochemical, molecular, and genetic tools to address the novel aspects of diatom frustule biogenesis such as SDV formation, targeting to the SDV, silica biomineralization, bioinorganic pattern formation, and exocytosis. A major bottleneck has been the inability to purify the SDV. However, a technique for fluorescently labeling the SDV in vivo has been described using rhodamine 123, which binds silica and which fluoresces brightly at the acidic pH of the SDV (Li et al., 1998; Brzezinski and Conley, 1994). It is possible that such labeling combined with modern fractionation techniques could be a useful approach. Furthermore, the use of green fluorescent protein to fluorescently tag SDV-destined proteins such as frustulins could reveal the fundamental features of their intracellular transport, and could reveal where different silica transporters are localized and whether they are mobile during SDV formation and silica deposition.

Of major importance is the identification of other components regulating SDV biogenesis, frustule formation, and exocytosis. One extremely valuable approach is the random sequencing of expressed genes to generate ESTs. Several thousand ESTs have been generated in the pennate diatom *Phaeodactylum tricornutum*, and sequences encoding proteins homologous to a range of GTP-binding proteins such as Rabs and Rhos have already been identified, as have COP coatamer proteins, dynamin, cathepsins, and clathrin adaptor proteins (S. Scala, N. Carels, A. Falciatore, M.L. Chiusano, and C. Bowler, unpublished data). Therefore, these provide important new substrates for initiating molecular and cellular studies in diatoms.

The species-specific patterns of silica nanofabrication indicates that there is a considerable genetic basis underlying pattern formation. The isolation of pattern mutants will be an important approach to develop insights into how this works. The two diatom species for which most molecular tools have been developed, *P. tricornutum* and *C. fusiformis* (Scala and Bowler, 2001), represent simple experimental systems that can be used for such approaches.

The eventual elucidation of the mechanisms used by diatoms to transform soluble silica into sturdy intricate structures at ambient temperatures and pressures that way exceed current human capabilities could eventually allow materials scientists to generate micrometer scale silica structures for a whole range of nanotechnological applications (Mann and Özin, 1996; Morse, 1999; Parkinson and Gordon, 1999). This, combined with the intrinsic scientific interest of understanding such a process, makes diatom cell wall biogenesis an attractive research topic that is in desperate need of intensive molecular and cellular-based studies.

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**LITERATURE CITED**

