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Secretion in Unicellular Marine Phytoplankton: Demonstration of Regulated Exocytosis in *Phaeocystis globosa*

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Almost half of the global photosynthetic activity is carried out in the ocean. During blooms, *Phaeocystis* can fix CO_2 at rates up to 40 g C m^{-2} $month^{-1}$. Most of this carbon is released as polysaccharides. However, the cellular mechanism whereby this huge amount of organic material is exported into the seawater remains unknown. A vaguely defined process of "exudation" is believed responsible for the release of these biopolymers. Here we report the first demonstration that *Phaeocystis globosa* does not "exude", but secretes microscopic gels. Secretion is stimulated by blue light (λ = 470±20 nm), and it is transduced by a characteristic intracellular Ca^{2+} signal that precedes degranulation. The polysaccharides that form the matrix of these gels remain in condensed phase while stored in secretory vesicles. Upon exocytosis, the exopolymer matrix undergoes a characteristic phase transition accompanied by extensive swelling resulting in the formation of microscopic hydrated gels. Owing to their tangled topology, once released into the seawater, the polymers that make these gels can reptate (axially diffuse), interpenetrate neighboring gels, and anneal them together forming massive mucilage accumulations that are characteristic of *Phaeocystis* blooms. These gel masses can supply a rich source of microbial substrates, disperse in the seawater, and/or eventually sediment to the ocean floor.

Keywords: Exocytosis — Exopolymer — Granule — Light — Mucilage — *Phaeocystis*.

Abbreviations: ASW, artificial seawater; DOC, dissolved organic carbon; PBS, phosphate-buffered saline.

Introduction

One of the most dynamic and least explored pools of marine organic carbon on our planet occurs as polymer hydrogels (Wells 1998). Gels play a critical role in marine heterotrophic cycling by providing a rich source of nutrients for marine microbes (Kepkay 1994, Azam 1998, Wells 1998). The main source of marine and riverine water gels probably results from a dispersion ↔ assembly equilibrium of polymers found in the dissolved organic carbon (DOC) pool (Chin et al. 1998, Kerner et al. 2003). However, phytoplankton species are an important source of marine gels. About 13% of the total primary production of phytoplankton is released to the seawater as polymers, comprising a major primary supply of organic carbon to the microbial loop (Baines and Pace 1991, Azam 1998). Despite their critical role in the heterotrophic cycle, the mechanisms whereby marine phytoplankton release gels into seawater have been the subject of much speculation (Mague et al. 1980, Bjornsen 1988, Lignell 1990, Baines and Pace 1991).

Using *Phaeocystis globosa* as a model, we designed experiments to evaluate the hypothesis that the polymeric material produced by this Prymnesiophyte is secreted and released to the seawater by exocytosis. Secretion has been extensively investigated in animal cells and vascular plants in which the regulated export of cellular products takes place via exocytosis (Palade 1975, Verdugo 1990, Battey and Blackbourn 1993, Zucker 1996, Martin and Kowalchyk 1997, Thiel and Battey 1998, Jahn and Sudhof 1999). In secretory cells, exported products are transported through the Golgi apparatus and densely packed in a condensed polymeric matrix. The matrix and its content are encapsulated in membrane-bound secretory granules, and eventually actively released from the cell by exocytosis. A key feature in regulated secretion, found in both animal and vascular plant cells, is that exocytosis takes place as a response to a specific stimulus. Stimulation is relayed to the export machinery of the cell by a signal transduction mechanism in which cytosolic Ca^{2+} functions as a second messenger. An ubiquitous increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$) follows stimulation in all secretory cells; it elicits the transport and fusion of the granules to the cell membrane, and the formation of a secretory pore (Battey and Blackbourn 1993, Monck and Fernandez 1996, Zucker 1996, Martin and Kowalchyk 1997, Thiel and Battey 1998, Chin et al. 1998, Jahn and Sudhof 1999, Quesada et al. 2001). Upon the formation of a secretory pore the polymer matrix inside the granule undergoes an ion-exchange-triggered polymer gel volume transition, quickly swelling and releasing its stored products to the extracellular space. In some cases like in mucus secreting cells, the main secretory product is the polymer matrix (Verdugo 1984, Verdugo 1990, Verdugo 1991). In other instances, like endocrine cells, the secretory material includes the matrix plus low

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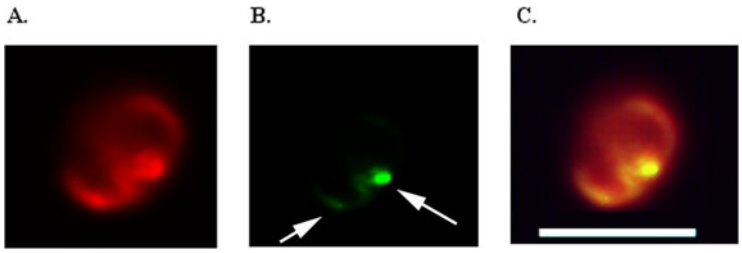

Fig. 1 Fluorescence excitation at λ_{ex} = 494 nm wavelength induces emission of both chlorophyll in the chloroplast that fluoresces bright red (Panel A), and emission of the secretory granules, labeled with a rabbit anti-*Phaeocystis* polymers antibody, that fluoresces in green as indicated by arrows (Panel B). Notice that the green granules are almost at the limit of resolution of the optical microscope. A few can be distinguished individually, and most of them are clustered inside the cell. Panel C is the merged image of (A) and (B), bar = 8 μm.

molecular weight active species including hormones, mediators, antibacterial peptides, etc., that remain entrapped in the condensed matrix while stored in the granule (Fernandez et al. 1991, Nanavati and Fernandez 1993, Marszalek et al. 1997). Upon exocytosis, the polymer matrix undergoes a characteristic high cooperativity polymer gel phase transition from condensed to hydrated phase accompanied by massive swelling and release of the entrapped active moieties (Tanaka 1981, Verdugo 1990, Verdugo 1994, Fernandez et al. 1991). Exocytic swelling is governed by the theory of swelling of polymer gels (Tanaka and Fillmore 1979), and—due to the polyanionic nature of the secretory matrices—it is driven by a Donnan equilibrium process (Katchalsky et al. 1951, Aitken and Verdugo 1989). These features provide a multiple set of specific criteria to rigorously verify if *Phaeocystis* functions as a secretory cell.

Results

The results described below were the outcome of experimental protocols designed to: (i) verify the presence of secretory granules in *Phaeocystis*; (ii) identify a specific stimulus that elicits *Phaeocystis* to secrete; (iii) search for a corresponding intracellular Ca^{2+} signal; and (iv) confirm that, as in other secretory cells, the swelling of *Phaeocystis* exocytosed material can undergo polymer gel phase transition and exhibit the characteristic features predicted by the theory of swelling of polymer gels (Tanaka and Fillmore 1979, Tanaka 1981, Verdugo 1994).

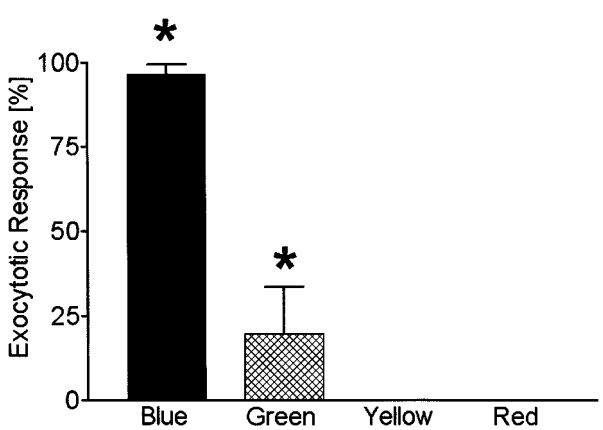

Fig. 2 Exposure of *Phaeocystis* to a photon flux of 480 μmol m^{-2} s^{-1} of blue light (λ = 450–490 nm) for 60 min induced secretion in 97±2.9% of the cells, while similar exposure to green light (λ = 500–540 nm) resulted in secretion in only 20±14% of the cells. Exposure to equal time/fluxes of yellow (λ = 560–600 nm) and red (λ = 640–720 nm) light fail to induce secretion. * Statistically significant, P <0.0001.

Verification of intracellular secretory granules in Phaeocystis

We used an immunocytochemical probe to unequivocally identify that *Phaeocystis* secretory products are stored inside the cell forming discrete structures. Fig. 1 is a typical image of *P. globosa* cells stained with a rabbit antibody that recognizes *Phaeocystis* polymers (Orellana et al. 2003). Note that the excitation wavelength of FITC (λ_{ex} = 494 nm) also excites chlorophyll that fluoresces bright red. The green antibody-labeled granules are almost at the limit of resolution of the optical microscope, a few can be distinguished individually, and most of them are clustered inside the cell, a pattern often found in secretory cells (Belan et al. 1996, Goodman et al. 1996, Petersen 1996, Titievsky et al. 1996, Jahn and Sudhof 1999).

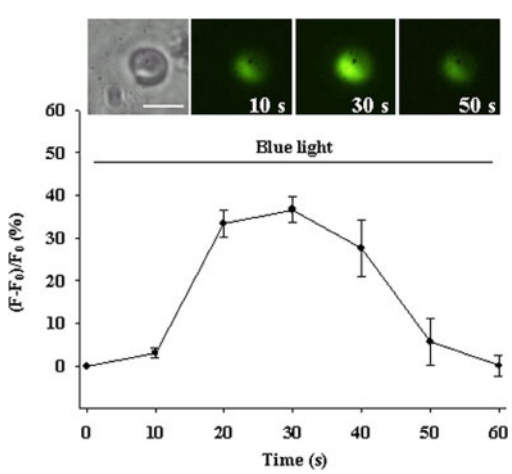

Fig. 3 Fluctuations of intracellular Ca^{2+} are a critical step in the signal transduction cascade of stimulus–secretion coupling. The role of intracellular Ca^{2+} in the signal transduction mechanism of light-stimulated exocytosis in *Phaeocystis* was investigated by loading *Phaeocystis* cells with the membrane permeant fluorescent Ca^{2+} probe Fluo 4-AM. As indicated by the increase in the fluorescence intensity ratio ($F - F_0/F_0$) of control (F_0) to post stimulated (F) emission, stimulation of *Phaeocystis* by 450–490 nm blue light resulted in the corresponding cytosolic Ca^{2+} increase that was followed by exocytosis. Each point corresponds to the mean ± SEM of five measurements.

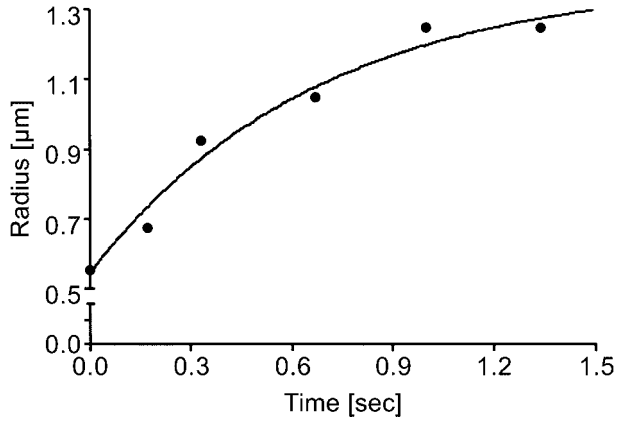

Fig. 4 As observed in other secretory cells, the radial expansion of the exocytosed granules follows typical first order kinetics, lending the process to be formalized and evaluated in light of Tanaka's theory of swelling of polymer gels. Video micrographs of exocytosis in a *Phaeocystis* cell were captured at 30 fields s^{-1}. Measurements performed by digitizing video microscopic records demonstrate that during product release the radius of the secreted polymer follows first order kinetics. The continuous line is a non-linear least square fitting of the data points to $r(t) = r_f - (r_f - r_i)e^{-t/\tau}$, where r_i and r_f are the initial and final radius of the granule, and τ is the characteristic relaxation time of swelling.

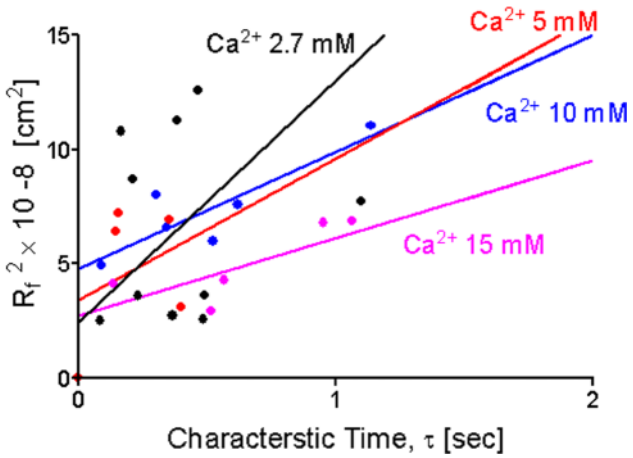

Fig. 5 *Phaeocystis* grown in F/2 marine medium and axenic conditions were equilibrated in ASW containing increasing concentrations of $[Ca^{2+}]$ from 2.7 mM to 15 mM at pH 8.2, 20°C, and stimulated to secrete by exposure to blue (450–490 nm) light. According to polymer gel theory, the relationship between characteristic time (τ) of the swelling kinetics, and the second power of final radius $(r_f)^2$ of the exocytosed granules exhibits a characteristic linear function with dimension of $cm^2\ s^{-1}$ and represent the diffusivity of the gel $D = (r_f)^2/\tau$ $[cm^2\ s^{-1}]$. In these experiments $(r_f)^2$ ranged from 0.98 at 2.7 mM Ca^{2+}, $N = 10$, to 0.86 at 5 mM Ca^{2+}, $N = 6$, 0.76 at 10 mM Ca^{2+}, $N = 6$, and 0.90 cm^2 at 15 mM Ca^{2+}, $N = 7$, yielding values of D that decreased from $1.1\pm0.1\times10^{-7}$ $[cm^2\ s^{-1}]$ in ASW containing 2.7 mM $[Ca^{2+}]$, to $3.4\pm0.5\times10^{-8}$ $[cm^2\ s^{-1}]$ in ASW containing 15 mM $[Ca^{2+}]$.

Photostimulation of exocytosis in Phaeocystis

Phaeocystis degranulation was stimulated by blue light and to a lesser extent by green light. At photon fluxes of 480 µmol m^{-2} s^{-1} *Phaeocystis* did not respond after 60 min exposure to yellow (λ= 580±20 nm) or red light (λ = 680±40 nm). However, 60 min exposure to blue light (λ = 470±20 nm) or to green light (λ = 520±20 nm) stimulated exocytosis in 100% and 20% of the cells respectively (Fig. 2). At a higher photon flux (2,700 µmol m^{-2} s^{-1}) blue-light induced secretion in about 20 s (data not shown).

An intracellular $[Ca^{2+}]_C$ increase follows photostimulation and precedes exocytosis

The role of fluctuations of intracellular $[Ca^{2+}]_C$ in the transduction of stimulus in secretory cells is now well established (Battey and Blackbourn 1993, Zucker 1996, Martin and Kowalchyk 1997, Thiel and Battey 1998, Jahn and Sudhof 1999, Nguyen et al. 1998, Quesada et al. 2001). One of the most reliable methods to monitor the dynamics of $[Ca^{2+}]_C$ is the use of fluorescent probes that change their emission with changes of $[Ca^{2+}]$ (Grynkiewicz et al. 1985, Nguyen et al. 1998, Simpson 1999). We investigated the presence of a Ca^{2+}-signal in photostimulated *Phaeocystis* by loading these cells with the membrane-permeant fluorescent Ca^{2+} probe Fluo 4-AM. As shown in Fig. 3, exposure of *Phaeocystis* to 450–490 nm blue light resulted in a characteristic increase in $[Ca^{2+}]_C$ that was consistently followed by exocytosis.

Swelling kinetics of exocytosed gels

In a broad range of cells in which secretion has been directly recorded, exocytosis results in a characteristic swelling of the granule matrix implying that the matrix polymer network must remain in condensed phase while stored inside the granule (for review see Verdugo 1991). The radial expansion of the exocytosed material exhibits the characteristic features of swelling of polymer gels. This process has been formalized and evaluated in light of Tanaka's theory of swelling of polymer gels (Tanaka and Fillmore 1979, Verdugo 1994). Measurements obtained by digitizing video microscopy recordings demonstrate that during product release the radius of the *Phaeocystis*' secreted polymer gels increases according to typical first-order kinetics. The continuous line in Fig. 4 is a non-linear least square fit of the data points to $r(t) = r_f - (r_f - r_i)e^{-t/\tau}$, where r_i and r_f are the initial and final radius of the granule, and τ is the characteristic relaxation time of swelling. As in gels from other secretory cells (Verdugo 1984, Verdugo 1991), the final radius of r_f of the exocytosed gel shows a typical linear relationship with τ^2, the second power of the characteristic time of swelling. The slope of this line $\tau = D\ (r_f)^2$ represents the diffusivity of the granule secretory matrix in the extracellular medium (Tanaka and Fillmore 1979, Verdugo et al. 1987). In the case of *Phaeocystis*, the diffusivity (D) of the secreted gels is $5.1\pm1.4\times10^{-8}$ $[cm^2\ s^{-1}]$ which is remarkably high considering the high concentration of Ca^{2+} found in seawater, and probably

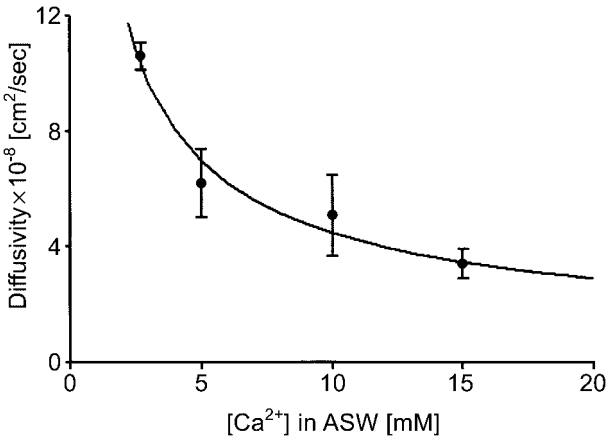

Fig. 6 Diffusivity (D) decreases with increasing ASW [Ca^{2+}] from 2.7 mM to 15 mM suggesting that Ca^{2+} must function as a crosslinker in *Phaeocystis* secreted gels. Each point corresponds to the mean ± SEM (N = 10, 6, 6 and 7 respectively).

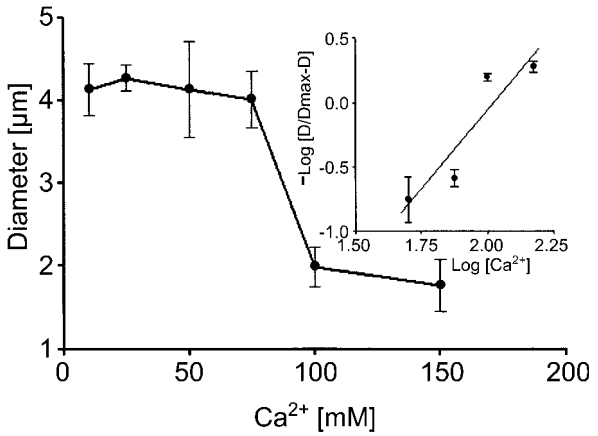

Fig. 7 The hydrated polymer matrix of exocytosed *Phaeocystis* gels undergoes a characteristic volume transition when exposed to conditions that mimic the intragranular environment. The diameter of exocytosed gels equilibrated in a buffer solution at pH 3, 20°C, containing increasing the [Ca^{2+}] from 10–150 mM with $CaCl_2$ shows a typical critical point steep inflexion between 60 and 100 mM $CaCl_2$ at which they undergo phase transition to the condensed phase. The process is reversible, and as shown by the Hill plot in the inset, it exhibits a characteristic high cooperativity found in phase transitions with a Hill coefficient >2.5.

reflects the strong polyanionic nature of the secreted polymers (Fig. 5).

Ca^{2+} crosslinking and ion-exchange properties of Phaeocystis gels

To further investigate the polyanionic nature of the polymer gel matrix and the role of cationic bonding in these granules, *Phaeocystis* grown in F/2 marine medium under axenic conditions were equilibrated in artificial seawater (ASW) containing increasing concentrations of [Ca^{2+}] from 2.7 mM to 15 mM at pH 8.2, at 20°C, and stimulated to secrete by exposure to blue light (450–490 nm). Results indicate that, as expected for a polyanionic gel, and consistent with swelling kinetics in the secretory matrixes of other cells (Aitken and Verdugo 1989, Verdugo 1991), the diffusivity (D) of the exocytosed gel is governed by a Na^+/Ca^{2+} ion exchange process (Katchalsky et al. 1951, Marszalek et al. 1997). D decreases from $1.1\pm0.1\times10^{-7}$ [cm^2 s^{-1}] in ASW containing 2.7 mM [Ca^{2+}], to $5.1\pm1.4\times10^{-8}$ [cm^2 s^{-1}] and $3.4\pm0.5\times10^{-8}$ [cm^2 s^{-1}] in ASW containing 10 and 15 mM [Ca^{2+}] respectively (Fig. 6).

Phase transition of Phaeocystis polymer gels

The characteristic intralumenal low pH and high [Ca^{2+}] found in secretory granules are responsible for keeping the secretory matrix in condensed phase (Fernandez et al. 1991, for reviews see Verdugo 1990, Verdugo 1991). Upon exocytosis the matrix undergoes a volume phase transition and decondenses forming a small hydrogel. However, this process is reversible, and as shown in Fig. 7, exposure of exocytosed *Phaeocystis* gels to conditions that mimic the intragranular environment can readily re-condense the exocytosed polymer matrix. Polymer gel phase transition is a characteristic property of all natural or synthetic polymer gels, regardless of their chemical composition. Gels secreted by *Phaeocystis* exhibit the typical features of critical phenomena found in all phase transitions. Namely, a critical point at which transition happens, in this case a critical [Ca^{2+}] at which reversible volume transition occurs (Fig. 7), and a characteristic high cooperativity of critical phenomena (Tanaka 1981), as reflected by a large Hill coefficient that in this case is >2.5 (see inset in Fig. 7).

Discussion

Our data provide multiple lines of evidence to support the idea that *Phaeocystis* functions as a secretory cell. First, immunocytochemical-labeling shows that a discrete cellular compartment that accumulates the polymeric material released by *Phaeocystis* is indeed present inside these cells (Fig. 1). Second, secretion is stimulated by blue light and to a lesser extent by green light at fluxes equivalent to those found during the summer (Mague et al. 1980). Moreover, *Phaeocystis* does not respond to illumination with other wavelengths suggesting that secretion results from the activation of specific photoreceptors (Fig. 2). Although the response to green light could give an idea of the spectral width of *Phaeocystis* photoreceptors, it probably has limited physiological significance since in nature blue radiation is ubiquitous and has better penetration than other wavelengths into the seawater column (Smith and Baker 1981). These findings are in agreement with previous reports of the light-dependent production of extracellular polymers and the association of mucilage accumulation with *Phaeocystis* blooms (Matrai et al. 1995). Third, results using the fluorescent Ca^{2+}-probe Fluo 4-AM provided direct verification of a

characteristic transient increase of cytosolic Ca^{2+} concentration that follows blue light stimulation and precedes the release of the granules (Fig. 3). Fourth, during exocytosis the polymer matrix inside the granule of a broad range of cells undergoes an ion-exchange-triggered polymer gel volume transition, quickly swelling and subsequently releasing the stored product to the extracellular space (Verdugo 1990, Marszalek et al. 1997). The exocytic swelling of the secretory matrix of *Phaeocystis* granules was no exception (Fig. 4). In agreement with the theory of swelling of polymer gels (Tanaka and Fillmore 1979), the increase of the linear (radius) dimension of exocytosed material exhibits a typical first-order kinetics (Fig. 4), and the second power of the final radius was found to be proportional to the characteristic time of swelling—the reciprocal value of the kinetic constant (Fig. 5). Notice that the slope of this linear function has the dimension of diffusion (D) [$cm^2\ s^{-1}$]. It corresponds to the diffusivity of the polymer matrix of *Phaeocystis* granules in seawater (10 mM Ca^{2+}, pH 8.2) yielding a value of $5.1\pm1.4\times10^{-8}$ [$cm^2\ s^{-1}$]. As expected for a polyelectrolyte network, the diffusivity of the secretory matrix increases in ASW containing lower [Ca^{2+}] (Fig. 6), and exposure of the *Phaeocystis* exocytosed polymer gels to Ca^{2+}-free ASW results in the immediate dispersion of the matrix with its polymeric component going into solution (data not shown). These outcomes imply that the polymers that make the granule matrix are polyanionic, and that they are not covalently crosslinked but held together by tangles and low energy Ca^{2+} bonds (Verdugo et al. 1987, Verdugo 1990, Verdugo 1994). A similar Ca^{2+}-bonded tangled topology is also found in the mucin matrix of secretory granules of goblet cells (Verdugo et al. 1987, Verdugo 1990, Verdugo 1994). These results agree with previous observation showing that Ca^{2+} can stabilize *Phaeocystis* mucus colonies (van Boekel 1992). The anionic bonding sites in *Phaeocystis* polymers seem to be highly specific for Ca^{2+} since in these experiments the Ca^{2+}-free ASW contained concentrations of Mg^{2+} equivalent to those found in the ocean (40 mM). Similar ion-selectivity also has been found in the heparin matrix of mammalian mast cells (Curran and Brodwick 1991, Fernandez et al. 1991). Fifth, exocytic swelling of the *Phaeocystis* secretory matrix suggests that these polymer networks must remain highly condensed during storage inside the granule. Exposing the exocytosed gels to conditions that mimic the intragranular environment can readily re-condense these gels, which further verifies the mechanism of condensation. As shown by the Hill plot (inset Fig. 7), re-condensation, like in other secretory matrixes, exhibits the typical high cooperativity of a polymer gel phase transition and exhibits a critical [Ca^{2+}] between 80 and 100 mM (Fig. 7). A characteristic high Hill coefficient as shown by the steep sigmoid relationship between changes of [Ca^{2+}] and gel diameter provides objective indication of the phase transition property of the gels released by *Phaeocystis* (Tanaka 1981, Verdugo 1991, Fernandez et al. 1991, Verdugo 1994).

Exocytosis had been previously suggested to take place in green algae (Domozych and Domozych 1993, Hofberger et al. 1995). However, the verification that *Phaeocystis* functions as a secretory cell, releasing its products by regulated exocytosis provides insight into conflicting speculations about the mechanisms of extracellular release in phytoplankton (Mague et al. 1980, Bjornsen 1988, Lignell 1990, Baines and Pace 1991). Although indirect morphological evidence suggesting that secretion could take place in marine phytoplankton had been published as early as 1971 (Aaronson 1971), later reports proposed passive permeation (diffusion across the membrane) via a mechanism that "exude" and release exopolymers in marine phytoplankton (Bjornsen 1988). Based on the low diffusion rate of high molecular weight molecules, Lignell (1990) argued that passive permeation fails to explain the release of high molecular-weight organic compounds that constitute the major portion of extracellular release (Lignell 1990). Estimates from the model of Baines and Pace (1991) also disagreed with a "passive diffusion" hypothesis (Baines and Pace 1991). Lignell (1990) speculated on "the existence of some form of control" in phytoplankton to mediate extracellular release. However, while ions and small molecules can indeed move through the cell membrane by diffusion across well-defined channels, there is no evidence to indicate that large molecules can diffuse across the plasma membrane. Conversely, the concept that cells export high molecular weight molecules across the cell membrane by exocytosis has been experimentally verified in a broad range of animal and plant cells (Verdugo 1990, Battey and Blackbourn 1993, Zucker 1996, Martin and Kowalchyk 1997, Thiel and Battey 1998, Jahn and Sudhof 1999). The evidence presented here strongly suggests that *Phaeocystis*, and perhaps other phytoplankton cells, release polymeric material via the regulated exocytotic pathway.

In gels containing a matrix with polymers woven in a tangled topology the translational diffusion of the polymer chains is constrained to a snake-like axial motion through the intertwining of surrounding polymer molecules (de Gennes and Léger 1982, Edwards 1986). This is known as reptational axial diffusion (Edwards 1986) and it allows polymer chains to walk out of the matrix and disperse, or to interpenetrate adjacent gels annealing them together. Therefore, the tangled topology of the polymer matrix of *Phaeocystis* granules explains both how these exocytosed microscopic gels can anneal together forming the characteristic large masses of mucilage found during blooms, and why over a longer time frame, these large gel masses can disperse into the DOC pool of the ocean (Orellana et al. 2003). Whether the polymer networks released by *Phaeocystis* remain in gel phase or undergo dispersion depends on environmental factors such as turbulence, temperature, or changes of pH or ionic concentrations inside the gel matrix. Small differences in those conditions can result in the shift between gel and dispersed phases (Tanaka 1981, Chin et al. 1998).

The polymer gel nature of *Phaeocystis* secretion has important implications for the fate of the organic carbon photosynthesized by these prymnesiophytes. These tangled gels form porous niches containing high concentrations of substrate with drastically higher biological availabilities than the free polymeric components that result from their dispersion (Kepkay 1994, Azam 1998, Chin et al. 1998, Wells 1998). Alternatively, the annealing process can increase the size of suspended gel particles, accelerating their sedimentation and removal from the water column (Chin et al. 1998). On the other hand, polymer photocleavage produced by ultraviolet radiation can shorten polymer chains making tangled networks unstable (Edwards 1986) and can readily disperse marine polymer gel matrices (Orellana and Verdugo 2003). These potential outcomes are consistent with field observations that accumulation of polymer gels does not always accompany *Phaeocystis* blooms (Davison and Marchant 1995, Matrai et al. 1995), and explain why microbial utilization rates of phytoplankton extracellular polymers can vary seasonally and between ecosystems (Baines and Pace 1991) as the balance between annealing, dispersion, and sedimentation of *Phaeocystis* gels should vary with time, temperature, and the local hydrodynamic conditions.

In summary, this is the first report indicating that *Phaeocystis* stores its photo-assimilated polymers in secretory vesicles and exports them via regulated exocytosis. Our data also indicate that, like in other secretory granules, the polysaccharides that make the matrix of *Phaeocystis* gels are polyanionic (Guillard and Hellebust 1971, Davison and Marchant 1995, Matrai et al. 1995, Solomon et al. 2003, Orellana et al. 2003). In the light of polymer gel theory (Tanaka and Fillmore 1979, de Gennes and Léger 1982, Edwards 1986, Verdugo 1994), our results suggest that *Phaeocystis* secretory granules contain a tangled polymer matrix stabilized by low energy Ca^{2+} bonds. These polymers are woven forming networks that are stored in condensed phase, which upon exocytosis undergo polymer gel phase transition to hydrated phase (Verdugo 1990, Verdugo 1994). Exocytosis is stimulated by blue light, and—as in other secretory cells—the stimulus is transduced by a characteristic increase of intracellular [Ca^{2+}]. Upon release the material swells, following characteristic first-order kinetics that results in the formation of microscopic hydrogels containing a tangled polymer matrix that is stabilized by Ca^{2+}-ion bonds. Although *Phaeocystis* secretion is discrete, quantal, and microscopic in nature, the tangled topology of these exocytosed microgels allow them to interpenetrate each other forming larger gels that result in the characteristic mucilage accumulations that serve as a major source of microbial substrate, eventually disperse as DOC, and/or get removed from the water column by sedimentation (Smith et al. 1991, Davison and Marchant 1995, DiTullio et al. 2000, Orellana et al. 2003, Solomon et al. 2003).

Materials and Methods

Cell culture

Batch cultures of *P. globosa* (CCMP629, Provasolli-Guillard marine phytoplankton culture collection, West Boothbay Harbor, MN, U.S.A.) were grown in F/2 marine medium (Sigma, MO, U.S.A.) on a 14 : 10 (light : dark) cycle at 100 μmol m^{-2} s^{-1} and 18°C under axenic condition. Growth phase of the culture was determined by cell counting with a hemocytometer. All experiments were conducted with stationary-state cultures.

Labeling of secretory granules with in situ immunostain

Phaeocystis cultures were transferred to poly-lysine-coated glass-bottom culture dishes (MatTek, Ashland, MA, U.S.A.). The cells attached to the dish bottom after 4–6 h. Excess cells were washed away with repeated rinsing with ASW (Sigma, MO, U.S.A.). The attached *Phaeocystis* cells were fixed by exposure to HistoChoice™ in ASW solution (Amresco, Ohio, U.S.A.) for 10 min. Cells were then washed first with phosphate-buffered saline (PBS) two times, and then equilibrated in blocking solution (PBS+1% bovine serum albumin) for 1–2 h at room temperature. Primary antibody (rabbit anti-*Phaeocystis* polymers antibody) was added to the blocking solution in a 1 : 100 dilution for another period of 4 h. The specificity of this antibody for the released polymeric materials from *Phaeocystis* was demonstrated in our previous report (Orellana et al. 2003). The samples were then washed three times with PBS to remove all buffer containing primary antibodies. Secondary antibody (goat anti-rabbit FITC-labeled antibody, Sigma, MO, U.S.A.) was diluted 1 : 500 in PBS buffer containing 1% goat serum. The cells were equilibrated for 4 h in this buffer followed by thorough washes with PBS. In situ localization of the fluorescently labeled antibody directed against *Phaeocystis* polymers was verified by photomicroscopy using a Nikon inverted fluorescence microscope at 600× magnification with a Nikon B-2A fluorescence filter set (excitation: 450–490 nm band-pass filter, emission: 515 nm long-pass filter).

Photostimulation and video recording of degranulation in *Phaeocystis*. Petri dishes containing cultured *Phaeocystis* were positioned under a Nikon Diaphot inverted fluorescence microscope and video recorded while illuminated—via the excitation port—for either 60 min, or 60 s to 480 μmol m^{-2} s^{-1} or 2,700 μmol m^{-2} s^{-1} respectively of blue (λ = 450–490 nm), green (λ = 500–540 nm), yellow (λ = 560–600 nm) or red (λ = 640–720 nm) light. Light was band-pass filtered and photon flux density was measured with an irradiance cosine sensor Li-Cor 191SA (Li-Cor Inc.). Exocytosis in *Phaeocystis*—like in other secretory cells—is a discrete quantal process. Degranulation is discontinuous, as secretory vesicles are released one at a time undergoing quick swelling whilst they come in contact with the seawater. Video-recordings of degranulation in *Phaeocystis* were captured at 30 fields s^{-1} using a progressive scan digital video camera (Canon Optura) attached to the inverted microscope using phase contrast optics with a magnification of 600×.

Swelling kinetics and phase transition of exocytosed Phaeocystis gels

Release of secretory material was readily observed after *Phaeocystis* was exposed to blue light. We assessed the swelling kinetics of newly released granules from *Phaeocystis* equilibrated in ASW by digitizing video microscopy images collected at a sample rate of 30 frames s^{-1}. Measurements of the radius of the released matrixes as a function of time were used to verify if the swelling of the secreted material followed the characteristic features of polymer gels. According to Tanaka's theory, the swelling of polymer gels follows a typical diffusive kinetics that is independent of the size, internal topology, or chemical composition of the gel (Tanaka and Fillmore 1979, Tanaka

1981). For spherical gels, like those observed in *Phaeocystis* exocytosis, the radial dimension increases following a characteristic first order kinetics of the form $r(t) = r_f - (r_f - r_i)\, e^{-t/\tau}$, where r_i and r_f are the initial and final radius of the granule respectively, and τ is the characteristic relaxation time of the swelling process (Verdugo et al. 1987, Verdugo 1990). The polymer network of gels diffuses into the solvent, in this case seawater, with a diffusion coefficient $D = (r_f)^2/\tau$ [$cm^2\ s^{-1}$].

Whereas the diffusivity (D) of gels made of neutral polymers is independent of the polyelectrolyte composition of the solvent, D in polyionic gels varies with the concentration of counterions in the swelling medium. In the case of secretory granules, it varies drastically with the concentration of Ca^{2+} in the medium (Verdugo et al. 1987, Fernandez et al. 1991). To evaluate this feature we measured the kinetics of exocytotic swelling of *Phaeocystis* gels in ASW (Sigma, St. Louis, MO, U.S.A.) containing Ca^{2+} concentrations ranging from 2.7 to 15 mM (Fig. 5).

Phase transition of Phaeocystis secretory granules

A ubiquitous feature of polymer gels is their ability to undergo phase transition (Dusek 1967, Tanaka 1981). We found that, like in other secretory granules, the polymer matrix of *Phaeocystis* gels also exhibits characteristic phase transition behavior. It remains in the condensed phase while stored in the granule and undergoes phase transition to the hydrated phase upon release (Verdugo 1991, Fernandez et al. 1991, Verdugo 1994). Polymer gel phase transition is reversible. Exposure of the hydrated gels resulting from exocytosed secretory matrixes of different types of cells to conditions that mimic those prevailing in most secretory granules, namely low pH and high Ca^{2+} (Nicaise et al. 1992, Quesada et al. 2003) results in re-condensation (Fernandez et al. 1991, Verdugo 1991, Verdugo 1994). To demonstrate volume phase transition, we equilibrated gels exocytosed by *Phaeocystis* in a buffer solution at 20°C, pH 3 (buffered with 10 mM Tris, MES (2-[*N*-morpholino]ethanesulfonic acid and citric acid), and containing 10–150 mM $CaCl_2$, while their size was continuously monitored by video microscopy.

Loading of Ca^{2+}-sensitive probes and detection of $[Ca^{2+}]_C$

Intact *Phaeocystis* cells were equilibrated for 60 min at 20°C in ASW containing 5 μM Fluo 4-acetoxymethyl ester (Molecular Probes, Oregon, U.S.A.) from a stock solution in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the ASW was 0.1%. The Ca^{2+}-sensitive dye, Fluo 4-AM, has correspondingly high Ca^{2+}-affinity (K_d = 345 nM) and is an appropriate choice for the low range Ca^{2+}-detection. One minor limitation of this protocol is that the excitation wavelength of Fluo-4 is 494 nm that can also stimulate secretion in *Phaeocystis*. To avoid this problem, measurements of fluorescence emission were performed in a chopped-sampling mode using pulses much shorter (300 ms) that those required to trigger exocytosis in *Phaeocystis* (20 s). Emission of Fluo-4 was collected with a 500–560 nm band pass filter to monitor the corresponding $[Ca^{2+}]$ fluctuations. Images were captured by a thermoelectrically cooled, low dark noise (1.3 photoelectrons s^{-1} $pixel^{-1}$ @ –36°C) digital camera with a 16-bit pixel resolution 336×243, CCD matrix, and a maximum readout rate of 100 Kpixel s^{-1} (Spectra Source Model 400, Westlake Village, CA, U.S.A.). All fluorescence measurements and analysis were conducted with digital-deconvolution algorithms developed previously for Ca^{2+} measurements (Nguyen et al. 1998).

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