The timely breakdown of the extracellular matrix by proteolytic enzymes is essential for development, morphogenesis and cell proliferation in plant and animal cells. Sporangin of the unicellular green alga *Chlamydomonas reinhardtii* that mediates breakdown of the sporangial cell wall to liberate the daughter cells after cell division is characterized as a subtilase-like serine protease. The sporangin gene is specifically transcribed during S/M phase in a synchronized vegetative cell cycle. In immunoblot analyses using a polyclonal antibody raised against the sporangin polypeptide, the enzyme is synthesized after mitotic cell division and accumulated in the daughter cells before hatching. Immunofluorescence analyses showed that sporangin is localized to the flagella of the daughter cells within the sporangial cell wall, and released into the culture medium. The data suggest that sporangin is released from flagella concurrently with the digestion of sporangial cell wall, and then the daughter cells are hatched from the sporangia in the *Chlamydomonas* vegetative cell cycle.

**Keywords:** Cell cycle • *Chlamydomonas reinhardtii* • Flagella • Hatching enzyme • Subtilase-like serine protease.

**Abbreviations:** ECM, extracellular matrix; EST, expressed sequence tag; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends.

The nucleotide sequence of sporangin cDNA has been deposited in the EMBL/Genbank database under the accession number AB303361.

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**Introduction**

Extracellular proteolysis is important in many biological processes, resulting in remodeling or disintegrating the extracellular matrix (ECM) of cells in both animals and plants (Basbaum and Werb 1996, Vierstra 1996). In the unicellular biflagellated green alga *Chlamydomonas reinhardtii*, there are at least two distinct proteolytic enzymes that function at specific stages of the life cycle in order to degrade the cell wall, a type of ECM unique to volvocine algae (Roberts et al. 1985, Sumper and Hallmann 1998): (i) gametolysin (gamete lytic enzyme, GLE) that mediates digestion of the cell walls of gametes of two mating types during mating as a necessary prelude to cell fusion (Claes 1971); and (ii) sporangin (vegetative lytic enzyme, VLE) that mediates breakdown of the sporangial cell walls, thereby allowing the liberation of daughter cells after mitosis in the mitotic cell cycle (Schlösser 1981).

Gametolysin (Matsuda and Kubo 2004), which is released into the culture medium by mating gametes concurrently with shedding of the cell walls, is a zinc-containing matrix metalloprotease with a molecular mass of 62 kDa (Matsuda et al. 1985, Kinoshita et al. 1992). The gametolysin gene is expressed in vegetative cells (Kubo et al. 2001), and the proenzyme with a molecular mass of 65 kDa is stored in the periplasm of cells until its release (Matsuda et al. 1987, Buchanan et al. 1989). Flagellar agglutination between gametes of opposite mating types triggers the activation of the proenzyme by a second enzyme termed p-lysinase.
Chlamydomonas show that sporangin is synthesized at a specific stage of the serine protease with a transmembrane segment. We also daughter cells within the sporangial cell wall.

Venning G2 period take place at 12–14 h to produce 4–16 phase) and then 2–4 rounds of S/M phase without an inter-

We present here the complete nucleotide and deduced amino acid sequence of the cDNA encoding sporangin, revealing that the Chlamydomonas hatching enzyme is, like the Volvox VheA (Fukuda et al. 2006), a subtilisin-like serine protease domain. The 143 kDa proenzyme has a transmembrane segment and is synthesized long before the hatching stage and stored throughout the life cycle.

Recently, a hatching enzyme, VheA of the multicellular volvocine alga Volvox carteri, which is evolutionarily related to the unicellular Chlamydomonas (Kirk and Harper 1986, Kirk 1998), has been purified and characterized by Fukuda et al. (2006). VheA, which is released into the culture medium concurrently with the liberation of juveniles from parental spheroids, is a 125 kDa glycoprotein with a subtilisin-like serine protease domain. The 143 kDa proenzyme has a transmembrane segment and is synthesized long before the hatching stage and stored throughout the life cycle.

We successfully carried out N-terminal sequencing of the 76 kDa polypeptide, yielding a 21 amino acid sequence, ALTLARTXRTTVVRELDXSTA. The N-terminal sequence thus obtained was used as a probe to screen the Kazusa Chlamydomonas expressed sequence tag (EST) library using the TBLASTN search program (Asamizu et al. 1999). A clone (MXL089e06) with a 3.8 kb cDNA insert was identified, and its expression and localization during the synchronized vegetative cell cycle have not yet been determined.

Results

Isolation of sporangin cDNA

In order to begin the characterization of the molecular details of the hatching enzyme sporangin, the enzyme was purified by anion exchange and gel filtration chromatography from the hatching medium of synchronized cultures, by an assay that monitors digestion of the sporangial cell walls using glutaraldehyde-fixed sporangia as a substrate (Matsuda et al. 1995, also see Materials and Methods). The purified enzyme was a single 125 kDa polypeptide on SDS–PAGE (see Matsuda et al. 1995). Amino acid sequencing of the sporangin N-terminus was attempted, but in spite of three trials we failed to obtain the sequence, suggesting blockage by N-terminal amino acid modification. During the course of this sequencing analysis, however, we noticed that the purified single polypeptide of 125 kDa produced a small amount of 76 kDa polypeptide when intact sporangin was stored or incubated under low salt conditions (Fig. 1). It is known that serine proteases identified in higher plants such as Cucumis melo L. (melon) have a limited autolysis activity that produces smaller fragments of the enzymes (Yamagata et al. 1994). Therefore, the band of 76 kDa was probably derived from degradation of the purified 125 kDa polypeptides, presumably by autolysis of the enzyme. Immunoblot analysis using the anti-125 kDa antibody detected two forms of degradation products: a 76 and a 62 kDa form (Fig. 1).

Amino acid sequence of the cDNA encoding sporangin,

The hatching enzyme (sporangin) released into the culture medium acts only on the sporangial cell walls and not on those of single cells (Schlösser 1981, Matsuda et al. 1995). Hatching of the newly formed daughter cells from the sporangial cell walls occurs just before the beginning of the next cell cycle. The hatching enzyme sporangin released into the culture medium acts only on the sporangial cell walls and not on those of single cells (Schlösser 1981, Matsuda et al. 1995). However, the molecular details of sporangin and its expression and cellular localization during the synchronized vegetative cell cycle have not yet been determined.

We present here the complete nucleotide and deduced amino acid sequence of the cDNA encoding sporangin, revealing that the Chlamydomonas hatching enzyme is, like the Volvox VheA (Fukuda et al. 2006), a subtilisin-like serine protease domain. The 143 kDa proenzyme has a transmembrane segment and is synthesized long before the hatching stage and stored throughout the life cycle.

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Sporangin is a member of subtilase family

An NCBI conserved domain search revealed that sporangin unequivocally contains a domain structure of the subtilase family of serine protease (Siezen and Leunissen 1997) in the first half of the polypeptide (positions 190–480), where the Asp219/His296/Ser484 catalytic triad residues and the Asn401 substrate-binding residue are found. Sporangin seemed to contain pro- and mature regions in its entire sequence (Fig. 2A). The putative proregion (positions 1–182) was defined by a cleavage site that has a typical conserved sequence: a pair of threonine residues (positions 183–184), which are conserved at the putative N-terminus of the mature peptide of subtilase-like serine proteases in higher plants such as Arabidopsis, Alnus glutinosa, C. melo and Lilium longiflorum (Kobayashi et al. 1994, Yamagata et al. 1998). We are somewhat puzzled by the observations that the N-terminus of the mature VheA of V. carteri does not contain a pair of threonine residues (Fukada et al. 2006) and that the sequence of the N-terminus of the mature sporangin could not be obtained (see above). Therefore, we do not know at present what is the exact cleavage site between the pro- and mature polypeptides of sporangin.

A secondary structure prediction performed by the TMAP (Persson and Argos 1994) and TMHMM (Sonnhhammer et al. 1998) programs suggested that a transmembrane segment is present at positions 33–65 within the putative proregion (Fig. 2A and Supplementary Fig. S1). The mature region was divided into two domains (Fig. 2A): a catalytic subtilase domain (positions 183–547), presumably corresponding to the 62 kDa fragment derived from the 125 kDa glycoprotein, and a C-terminal domain (positions 548–1117), corresponding to the 76 kDa fragment (Fig. 1). There were 14 putative N-glycosylation sites (Asn-X-Ser/Thr) in both the subtilase domain and the C-terminal domain (Supplementary Fig. S1).

Structural similarity between sporangin and VheA

Fig. 2A shows that two hatching enzymes derived from Chlamydomonas (sporangin) and Volvox (VheA) (Fukada et al. 2006) resemble each other as they have: (i) similar lengths of pro- and mature polypeptides; (ii) involvement of a transmembrane segment in the propolyptide region; and (iii) similar lengths of a subtilase domain and a functionally unknown C-terminal domain in the mature polypeptide region. Sequence identity between sporangin and VheA proteins is 53% in the prodomain, 64% in the subtilase domain and 45% in the C-terminal domain (Fig. 2A).

A search of the JGI Chlamydomonas genome database (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) revealed that the gene for sporangin encompasses 8.2 kb (scaffold 1, 5000563–5009677; Protein ID, 190128) and consists of a total of 24 exons, which range in size from 75 to 387 bp (Fig. 2B). Southern blot analysis showed that a sporangin cDNA probe gave a single band with the Chlamydomonas genomic DNA digested with BamHI, SacI and NotI, respectively (Fig. 2C), suggesting that the sporangin gene is a single-copy gene.

The VheA gene in the JGI Volvox genome database (http://genome.jgi-psf.org/Volca1/Volca1.home.html) consists of 21 exons within a 15 kb region (scaffold 86, 391952–407157; Protein ID, 127481). The nucleotide sequences of the exons in the sporangin gene had 44–69% homology with those of the VheA gene (Fig. 2B), while the non-coding regions upstream of the gene and untranslated regions of the primary transcript showed low homology (26–45%) between the two genes. Three pairs of exons adjacent to each other in sporangin are one exon in the VheA gene structure (Fig. 2B: exon 7 and 8 in the sporangin gene to exon 7 in the VheA gene, exon 15 and 16 to exon 14, and exon 20 and 21 to exon 18), indicating that the structure of exons has diverged during the evolution of the unicellular Chlamydomonas and the multicellular volvocine lineage from their common ancestor. Since the VheA gene is also a single-copy gene in the Volvox genome (Fukada et al. 2006), the VheA and sporangin genes appear to be an orthologous pair.

Transcriptional profiles of the sporangin gene in the vegetative cell cycle

The accumulation of sporangin mRNA during the synchronized vegetative cell cycle was determined by RNA gel blot analysis. Synchronized cells were placed in constant light; total RNA was isolated from cells sampled every 2 h throughout the 18 h time course, which represented one mitotic cell cycle (Fig. 3A), and was subjected to RNA blot analysis with a fragment of the sporangin cDNA as a probe. The L27a ribosomal protein gene was used as a loading control since the gene is expressed constitutively in every stage of the mitotic cell cycle (Abe et al. 2004). As shown in Fig. 3B, no sporangin mRNA was detected in the G1 phase, which for vegetative growth is from 2 to 12 h. The accumulation of sporangin mRNA was detectable by 14 h when the cells shifted into S/M phase and the cells had started mitosis and cytokinesis.
to form new daughter cells. After the hatching of sporangia, the mRNA still remained, but rapidly disappeared as the cells entered into the next G1 phase of vegetative growth (Fig. 3B). The data suggest that expression of the sporangin protein during the cell cycle is regulated transcriptionally, with the message being up-regulated when the cell starts mitotic cell division in the S/M phase.

**Synthesis and release of sporangin during the mitotic cell cycle**

To examine the molecular form of sporangin in the sporangial cells, immunoblot analysis was performed using mature sporangia just before hatching, liberated daughter cells after hatching and the cell-free culture medium collected before and after hatching (Fig. 4A). The anti-Spo antibody detected...
a single band of 127 kDa in cell lysates of sporangia, but not in the culture medium. After hatching, the 127 kDa band disappeared in cell lysates of daughter cells, and a 125 kDa band was detected in the culture medium. Similar results were obtained when the anti-125 kDa antibody was used for the immunoblot analysis (data not shown). We also examined the enzyme activity using the cell homogenates and culture medium, showing that the sporangin activity was

**Fig. 3** Expression of sporangin during the synchronized cell cycle. (A) Time course of synchronous growth under continuous light. Synchronously grown cells were harvested at the beginning of the light period (L-0 cells), resuspended in M medium and incubated for 20 h in the light. At the times indicated, the numbers of cells (filled circles with a solid line) and the percentages of dividing cells (filled squares with a dotted line) were determined. (B) Expression of the sporangin gene (Spo). Cell samples were taken at the indicated time points, and Northern blot analysis was performed. The L27a gene was used as a loading control. (C) Accumulation of sporangin polypeptides. Equal volumes of cell suspensions (0.1 ml containing 1.5×10⁵ cells) were taken at the indicated time points and subjected to Western blot analysis by using anti-Spo antibody. Anti-transketolase antibody (Anti-Tra) was used as a loading control.

**Fig. 4** Accumulation and release of sporangin before and after hatching of sporangial cells. (A) Immunoblot analysis of cells and culture medium derived from sporangia before and after hatching. Mature sporangia recovered at 16 h in Fig. 3A (lane C before hatching, 1.5×10⁵ sporangia), hatched daughter cells recovered at 18 h in Fig. 3A (lane C after hatching, 1.2×10⁶ daughter cells) and culture medium after removing the cells (lane M before and after hatching, 0.1 ml) were subjected to Western blot analysis by using the anti-Spo (left panel) and anti-Prospo (right panel) antibodies. The arrows indicate the prosporangin (127 kDa) found in the mature sporangia before hatching and the mature sporangin (125 kDa) found in the culture medium after hatching. The enzyme activities in cell homogenates or culture medium are indicated in units per 10⁹ cells. (B) The peptide fragment (residues 92–166) derived from the proregion was fused with GST and overexpressed in *E. coli*. The fusion protein was loaded on the SDS–polyacrylamide gel, and stained with Coomassie brilliant blue (CBB) or subjected to immunoblot analysis with anti-Prospo antibody.
detected only in the culture medium after daughter cells hatched (Fig. 4A). These results suggest that sporangin is stored in an inactive 127 kDa proenzyme in sporangia and released as an active 125 kDa enzyme into the culture medium concurrently with the digestion of sporangial cell wall.

The difference in the molecular mass between the deduced pro- and mature enzyme was only about 2 kDa on SDS–PAGE, the value being much smaller than expected from the length of the predicted prodomain of 182 amino acid residues at the time of synthesis (Fig. 2A). In Volvox VheA, a 143 kDa proenzyme, which is anticipated to involve the entire prodomain with the transmembrane segment, has been detected in spheroids before hatching (Fukuda et al. 2006). To examine whether the 127 kDa proenzyme had the entire prodomain, we prepared an anti-propeptide antibody (anti-Prospo) that should recognize the proregion. A part of the prodomain (residues 106–134 that are downstream of the transmembrane segment) was used to make a fusion protein with glutathione S-transferase (GST) and raise polyclonal antibodies. The anti-Prospo antibody, however, recognized neither the 127 kDa proenzyme present in sporangia nor any larger polypeptides that might represent the full-length, unprocessed sporangin (Fig. 4A, right panel). To confirm the antigenicity of the deduced proregion of the sporangin against the anti-Prospo, part of the propolypeptide (residues 92–166) was synthesized, fused in-frame with GST, expressed in Escherichia coli, purified by GST affinity column chromatography, and then processed with thrombin. Immunoblot analysis using the peptide antigen showed that the anti-Prospo antibody recognized both GST and a 9.4 kDa propolypeptide fragment containing residues 92–166 (Fig. 4B). We anticipated, therefore, that the 127 kDa proenzyme might lose the N-terminus of the propolypeptide including the transmembrane segment.

Fig. 3C shows that the 127 kDa proenzyme became detectable from 14 h when the cells shifted into the S/M phase, the levels peaked when the cells formed sporangia maximally at 16 h, and then were greatly reduced concurrently with hatching of daughter cells at 18 h.

Flagellation is important for hatching

We were interested to know how prosporangin is mechanically transported to its site of action on the sporangial cell walls to release the daughter cells at the time of hatching without damaging daughter cell walls. A physiological experiment to examine the correlation between flagellation and hatching of sporangia is shown in Fig. 5. The mature sporangia were subjected to pH shock (Witman et al. 1972) in order to detach the flagella of daughter cells from their cell bodies. The pH-shocked sporangia were then incubated in culture medium to allow regeneration of the flagella, sampled at designated time intervals, fixed with glutaraldehyde and assayed for both the percentage of hatched sporangia and the length of the regenerated flagella on the daughter cells. For the measurements of flagella lengths, gametolyisin in its crude form was added to the glutaraldehyde-fixed sporangia in order to digest the sporangial cell walls; thereby the daughter cells hatched artificially (see Materials and Methods).

Daughter cells of untreated, mature sporangia had flagella of almost full length (∼8 μm) and hatched within 10 min of incubation. In contrast, the pH-shocked daughter cells completely lost their flagella, and could not hatch until 50 min of incubation. Then, hatching occurred gradually, when the daughter cells had regenerated the flagella to 6–7 μm (Fig. 5, upper panel). To examine whether the pH shock treatment itself causes the delay of hatching, the mature sporangia...
were first treated with neomycin, an antibiotic known to inhibit flagella excision by pH shock in *Chlamydomonas* (Quarmby et al. 1992), and then subjected to the pH shock treatment. The results (Fig. 5, lower panel) showed that these neomycin–pH-shocked cells hatched normally with no detachment of the flagella, suggesting that the pH shock per se has no direct effect on the hatching of sporangia.

**Sporangin is released through flagella during hatching**

Since the flagellation of daughter cells plays an important role in the hatching process, we speculated that prosporangin might be localized to the flagella. Therefore, anti-Spo antibody was used for immunofluorescence localization of sporangin at the time of hatching. The images of synchronized sporangia showed that fluorescence was highly enriched in the several ‘rope-shaped’ regions between the daughter cells in sporangia (Fig. 6A, B). To confirm that these rope-shaped regions correspond to the flagella of the daughter cells, flagella from both sporangia and hatched daughter cells were isolated, and immunofluorescence stained for prosporangin. Strong fluorescence could be observed over the entire length of flagella derived from sporangia (Fig. 6C), while only a few dots of fluorescence were observed on the flagella derived from the hatched daughter cells (Fig. 6D), indicating that prosporangin is localized to the flagella of the daughter cells before hatching.

Immunoblot analysis showed that the 127 kDa prosporangin band found in whole sporangial cells was derived mostly from their flagella detached from cell bodies (Fig. 6E). This band, however, was absent from the isolated flagella derived from the daughter cells after hatching (Fig. 6F).

**Discussion**

**Hatching enzyme of Chlamydomonas and Volvox**

We show in the present study that the *Chlamydomonas* hatching enzyme, sporangin, is a subtilisin-like serine protease, which is similar to the *Volvox* hatching enzyme, VheA, identified previously (Fukuda et al. 2006). Comparative analysis of the genes encoding the two hatching enzymes shows that the coding regions are highly conserved although the gene structures are slightly changed (Fig. 2A, B). The data suggest that sporangin and VheA are orthologous and the two genes have diverged from the common *Chlamydomonas*-like unicellular ancestor. This is consistent with the fact that the structure and function of the hatching enzyme of the two species are conserved: sporangin is released by daughter *Chlamydomonas* cells for the proteolysis of sporangial cell walls to hatch, and VheA degrades the cell sheet of parental somatic cells to allow juvenile *Volvox* spheroids to be released. Furthermore, both enzymes are responsible for proteolytic digestion of the ECM, enclosing the cells of the next generation.

**The expression of sporangin is phase specifically regulated in the vegetative cell cycle**

We found that transcription of the sporangin gene was specifically induced in the S/M phase of the vegetative cell cycle (Fig. 3B), suggesting that its expression is under the control of timing for hatching. Recently, the cell cycle regulators in *C. reinhardtii* such as cyclin-dependent kinases (CDKs) and their binding proteins were identified by genome annotation (Bisova et al. 2005). The cell cycle regulator may interact indirectly or directly with cis-elements of the sporangin gene promoter to regulate expression during the S/M phase of the mitotic cell cycle.

Protein blot analysis (Fig. 3C) shows that 127 kDa prosporangin is stored specifically in daughter cells after mitotic cell divisions. Upon hatching, this inactive enzyme is activated and released into the culture medium as 125 kDa sporangin (Fig. 4A). The 127 kDa prosporangin appeared to lose a large part of the prodomain (182 amino acids) including the putative transmembrane segment (Fig. 2A) since anti-Prosporo antibody that should recognize the middle part of the prodomain did not react with the stored proenzyme (Fig. 4B).

The present findings are in contrast to the expression profile of VheA: the proenzyme is synthesized long before the hatching stage and accumulated as a 143 kDa polypeptide with the entire prodomain (Fukuda et al. 2006). We speculate, therefore, that the *Chlamydomonas* sporangin might be synthesized as a proenzyme with the entire prodomain, like 143 kDa VheA, but rapidly processed into the 127 kDa storage form. In the case of *Chlamydomonas* gametolysin, the 65 kDa proenzyme, which is stored in the periplasm of gametes, contains only a 25 amino acid propeptide at the N-terminus of the mature peptide, thus losing a large part of the entire 155 amino acid prodomain (Matsuda and Kubo 2004, Matsuda et al. unpublished).

**Prosporangin is localized to the flagella as an export apparatus for hatching**

Immunofluorescence images and immunoblot data (Fig. 6) show that prosporangin is localized to the flagella of daughter cells after mitotic cell divisions. In addition, we found the presence of two segments of sporangin protein in the *Chlamydomonas* flagellar proteome (*ChlamyFP*) database (Pazour et al. 2005, http://labs.umassmed.edu/chlamyfp/index.php). The database contains two peptide sequences of subtilisin-like serine proteases (ID, C_180170), which correspond to the internal sequence of mature sporangin (Supplementary Fig. S1, positions 328–343 and 791–823). These data are further evidence that sporangin is localized...
and accumulated on the flagella of the daughter cells prior to hatching during the mitotic cell cycle.

The importance of flagella for hatching is also suggested by the phenotype of flagella-less mutants. It has been observed that daughter cells of flagella-less mutants have a property that cells are slow to be released from the sporangial cell wall after mitosis, and clumps of eight, 16, 32 and 64 cells are commonly observed (Goodenough and St. Clair 1975, Harris 1989). This phenotype is known to be a property of flagella-less strains of *Chlamydomonas*, including strains with a defective basal body and a defective flagellar structure or elongation (McVittie 1972, Goodenough and St. Clair 1975).

**Fig. 6** Localization of sporangin in sporangial cells. (A) Phase contrast image of mature sporangia (B) Immunofluorescence image of mature sporangia. (C) Immunofluorescence image of flagella isolated from mature sporangia (D) Immunofluorescence image of flagella isolated from daughter cells after hatching in vivo. In these experiments, anti-Spo antibody was used for the immunofluorescence localization of prosporangin. Bar = 10 µm. (E) Immunoblot analysis of mature sporangia. Samples of whole cells, cell bodies and detached flagella were prepared from the mature sporangia (2×10^6 sporangia) and they were reacted with anti-Spo. (F) Immunoblot analysis of flagella isolated from the mature sporangia and daughter cells after hatching. Samples of flagella (2.5 µg per lane) were reacted with anti-Spo (upper panel). The tubulin protein was detected by Coomassie brilliant blue staining (lower panel).
is exported in concert with the construction of flagella as flagella after completion of cell division: one is that sporangin for how sporangin would be exported to the daughter cells’ fraction (Pazour et al. 2005), there are two possibilities two polypeptides from sporangin were found in the axoneme and the flagellar proteome database showed that both of could distinguish between these two models.

In the vegetative cell cycle of *C. reinhardtii*, flagella of the cells gradually regress at the end of G1 phase, and the development of new flagella occurs only after the completion of mitotic cell divisions and synthesis of cell walls of daughter cells (Cavalier-Smith 1974). Since sporangin is specifically expressed in the S/M phase of the cycle (Fig. 3), synthesis and export of this enzyme might be regulated synchronously with the construction of the new flagella of the daughter cells. Electron microscopic observation also showed that during the S/M phase newly formed daughter cells have flagella, some of which are shorter than usual, suggesting that the flagellum is assembled sequentially beginning at the base and growing by adding materials to the tip (Cavalier-Smith 1974, Rosenbaum and Witman 2002). Since the pH shock experiment showed that the hatching did not occur until new daughter flagella had grown to a certain length (6–7 µm) within the sporangial cell wall (Fig. 5, upper panel), and the flagellar proteome database showed that both of two polypeptides from sporangin were found in the axoneme fraction (Pazour et al. 2005), there are two possibilities for how sporangin would be exported to the daughter cells’ flagella after completion of cell division: one is that sporangin is exported in concert with the construction of flagella as one of the materials of this organelle, and the other is that when development of flagella is completed or they have reached a certain length, sporangin then begins to be exported from the cell body to the flagella. In either case, sporangin is specifically synthesized when cell division is totally finished and flagella can reform, and this prevents sporangin getting access to the inside of the wall of the daughter cells. Future investigation with detailed observation of both flagellar development and location of sporangin could distinguish between these two models.

**Materials and Methods**

**Preparation of sporangin**

The SAG 11-32b (*mt*+) strain of *C. reinhardtii* was cultured synchronously in minimal (M) salt medium under a cycle of 12 h light and 12 h dark as described (Matsuda et al. 1995). To obtain mature sporangin released into the medium, the synchronized vegetative cells (2–3×10⁶ cells ml⁻¹) were harvested by centrifugation (5,000×g, 1 min) at 5 h into the dark period, when the population of cells was >90% mature sporangia. The synchronized sporangia were suspended in 10 mM Tris-acetate, pH 7.5, at a density of 2×10⁷ cells ml⁻¹ and then illuminated for 15–30 min without aeration to allow the release of the daughter cells. After completion of the liberation of daughter cells (confirmed by phase-contrast microscopy), a cell-free supernatant was prepared by centrifugation (15,000×g, 20 min) at 4°C and used as the crude solution of sporangin. For further purification, the solution of crude enzyme was purified according to the method described previously (Matsuda et al. 1995). The crude sporangin solution was mixed with 200 ml of DEAE-cellulose (DE-12; Whatman, Maidstone, USA) that had been equilibrated with 10 mM Tris-acetate buffer, pH 7.5. The mixture was stirred for 1 h at 4°C, collected by and through a filter in a funnel, and then washed with the same buffer. The filtrates in which most of the sporangin was recovered were combined and concentrated in a collodion bag to about 0.2 ml, and fractionated by gel filtration using an HPLC system (Tosoh, Tokyo, Japan) equipped with a TSK-Gel G3000SWXL column (0.78 cm i.d. ×30 cm). Elution was performed with 20 mM Tris-acetate buffer without salt at a flow rate of 0.5 ml min⁻¹ at room temperature. The fractions containing sporangin activity were pooled, concentrated and supplemented with glycerol to 50% (v/v), and stored at −80°C. The activity of the purified enzyme was assayed, and the protein contents were determined by the Folin method (Lowry et al. 1951).

**Assay of sporangin activity**

Enzymatic activity of sporangin was measured by the method described previously (Tamaki et al. 1981). Sporangia were fixed in 0.125% glutaraldehyde and were used as substrates in this assay. The reaction mixtures contained 10 mM Tris–HCl (pH 9.0), 0.5 mg ml⁻¹ bovine serum albumin (BSA), 1×10⁶ cells ml⁻¹ fixed sporangia and 0–50 µl of enzyme solution in a total volume of 125 µl. The reaction was started by the addition of the enzyme and was stopped after 30 min at 35°C by the addition of EDTA-2Na to a final concentration of 20 mM. After the mixture was stirred vigorously for 15 s using a vortex mixer, the number of sporangia was counted in a hemocytometer using a phase contrast microscope. One unit of enzyme is defined as the amount that liberates daughter cells from 50% of sporangia. The cell homogenates for the determination of the enzyme activity were prepared by passing the cells through a French pressure cell as described previously (Matsuda et al. 1987).

**Amino acid sequencing**

Purified sporangin and the 76 kDa autolysis product of sporangin were subjected to SDS–PAGE, electrotransferred to Hybond-P membranes (GE Healthcare, Buckinghamshire, England) and visualized with silver staining or Coomassie brilliant blue R-250 staining. The N-terminal amino acid sequence of the blotted sporangin was determined by the
automated Edman degradation method using a peptide sequencer (model 477A/120A; Applied Biosystems).

**Isolation of cDNA encoding sporangin**

To identify the EST clones encoding sporangin, the N-terminal amino acid sequence of the 76kDa fragment was used to search the *Chlamydomonas* EST database by TBLASTN programs (http://est.kazusa.or.jp/en/plant/chlamy/EST/blast.html). One EST clone, MX089e06, was identified as the longest clone containing the sporangin gene. MX089e06 was subcloned into pBluescriptII SK−/KS− (Stratagene, La Jolla, CA, USA) and its sequence was determined using the Thermo Sequence Cycle Sequencing Kit (GE Healthcare) with T3 and T7 primers (Nisshinbo, Tokyo, Japan) using a DNA auto-sequencer LIC-4200L (Li-Cor, Lincoln, NE, USA) (Kubo et al. 2001).

To obtain the full-length cDNA sequence of the sporangin gene, 5′ RACE was carried out using the Marathon cDNA amplification Kit (Clontech, Mountain View, USA), according to the manufacturer's instructions. RACE-PCR was performed between universal primer, and VLE-R1 primer (5′-CTTGGCGTACCCGTAGTCGATGG-3′) and VLE-R2 primer (5′-GTCATCCACCACCTTGAGAACCATCTG-3′) as 5′ RACE reverse primers. The template was poly(A)+ RNA isolated previously (Kubo et al. 2001, Abe et al. 2004). Amplified fragments were ligated into pT7blue (Novagen, San Diego, CA, USA) and transformed into *E. coli* JM109. The inserted fragments were sequenced using the T7 primer as described previously (Kubo et al. 2001).

**DNA gel blot analysis**

Cells were lysed in a buffer containing 3% SDS, 250 mM NaCl, 25 mM EDTA and 60 mM Tris–HCl (pH 8.0) at 42°C with gentle shaking, and genomic DNA was isolated by using CsCl and ultracentrifugation. DNA blot analysis was carried out as described (Kubo et al. 2001). The blotted membrane was hybridized with a probe, the *EcoRI–BamHI* fragment (1.5 kb) isolated from MX089e06.

**RNA gel blot analysis**

RNA gel blot analyses were performed as described previously (Kubo et al. 2001, Abe et al. 2004). The *EcoRI–BamHI* fragment (1.5 kb) isolated from MX089e06 was used as a probe. Aliquots of 15 µg of total RNA were isolated from each sample during the time course of the synchronous vegetative cell cycle and loaded into each lane of a 1% RNA gel. The *L27a* gene encoding a *Chlamydomonas* 60S ribosomal protein (accession No. AV640287) was used as a standard or control because it is constantly expressed during the vegetative cell cycle, gametogenesis and early zygote formation (Abe et al. 2004, Kubo et al. 2008).

**Antibodies to sporangin and Western blot analysis**

The segment of Kazusa EST clone MX68e09 encoding the catalytic region (residues 197–290) of mature sporangin was used to make a fusion protein with GST. An *EcoRI–HindIII* DNA fragment (279 bp) was ligated into the pGEX4T-1 vector (GE Healthcare). The resulting fusion protein was expressed in *E. coli* JM109, and recovered from lysates as reported previously (Smith and Johnson 1988). The purified fusion protein was injected into rabbits to raise polyclonal antibody. The IgG fraction was purified using a protein G affinity column (GE Healthcare) as specified by the manufacturer, and named anti-Spo. In some experiments, polyclonal antibody was raised against the purified 125kDa sporangin (anti-125kDa), although this antibody reacted weakly with multiple bands in Western blots due to shared carbohydrate determinants (Adair 1985).

Protein samples were separated on 8% SDS–polyacrylamide gels as described previously (Matsuda et al. 1995). Cells, flagella and proteins in cell-free culture medium were freeze-dried, resuspended in 1× SDS loading buffer [2% SDS, 10% glycerol, 0.01% bromophenol blue, 50 mM Tris–HCl, pH 6.8, and 100 mM dithiothreitol (DTT)] and boiled for 5 min. The protein concentrations were always checked on test gels stained with Coomassie blue R-250 before use in Western blots. The proteins in the gel were transferred to a Hybond-P membrane (GE Healthcare) in a semi-dry blotting apparatus with transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). For Western analysis, blots were incubated in blocking solution [0.1% Tween-20 and 5% skim milk in Tris-buffered saline (TBS)] for 2 h, followed by primary antibodies (1:5,000–1:10,000 dilution) for 1 h, three changes of TBS–TWEEN buffer, and secondary antibodies for 1 h at room temperature. Alkaline phosphatase-conjugated goat anti-rabbit IgG (GE Healthcare) was used as the secondary antibody. The detection procedure was carried out with the ECL immunoblotting detection kit (GE Healthcare) as specified by the manufacturer.

**Flagella regeneration experiment**

To monitor the regeneration process of flagella and the ratio of hatching, sporangial cells were harvested and resuspended gently in buffer (10 mM Tris–HCl, 2 mM MgCl₂, pH 7.5) to a final density of 2×10⁶ cells ml⁻¹, then subjected to pH shock (Witman et al. 1972) for deflagellation. The pH-shocked sporangia were transferred into M medium and shaken gently at room temperature to regenerate the flagella of daughter cells inside the sporangial cell walls. Every 10 min, cells were sampled, fixed by 0.125% glutaraldehyde (Tamaki et al. 1981), and counted to calculate the ratio of hatching. The glutaraldehyde-fixed sporangia were also treated with gametolysin (2 U ml⁻¹) to digest the sporangial cell walls and liberate the daughter cells (Tamaki et al. 1981).
Flagella lengths of the liberated daughter cells were measured for >30 cells by using a micrometer. To inhibit the detachment of flagella from cell bodies by pH shock, neomycin was added at a final concentration of 100 μg ml⁻¹ (Quarmby et al. 1992).

**Isolation of flagella**

Sporangial cells were harvested and re-suspended gently in buffer (10 mM Tris–HCl, 2 mM MgCl₂, pH 7.5) containing 7% sucrose to a final density of 2×10⁷ cells ml⁻¹. To isolate the flagella, 15 and 30% sucrose layers in 70 ml centrifuged at 20,000×g for 30 min in a swinging bucket-type rotor. The 15% layer was removed and centrifuged at 15,000×g for 30 min to sediment the detached flagella. The isolated flagella were re-suspended in the buffer solution containing 7% sucrose and stored at –80°C. In the case of daughter cells after hatching in vivo, the flagella were detached from the cell bodies by the pH shock method and then isolated as above.

**Immunofluorescence microscopy**

Immunofluorescence staining was carried out as described previously (Cole et al. 1998). Sporangial cells, hatched daughter cells and the detached flagella from both cells were used for immunostaining. Multiwell slide glasses were coated with 1% polyethyleneimine for 10 min at room temperature, rinsed with distilled H₂O, and then air dried for 60–90 min. 1% polyethyleneimine for 10 min at room temperature, for immunostaining. Multiwell slide glasses were coated with 1% polyethyleneimine for 10 min at room temperature, rinsed with distilled H₂O, and then air dried for 60–90 min. The cells were blocked with 3% bovine serum albumin (BSA) for 10 min, and washed with PBST for 10 min three times. The dried cells or flagella on slide glass were washed in PBST for 10 min three times to wash off the excessive cells, then permeabilized with 0.5% NP-40 in PBS for 10 min, and washed with PBST for 10 min three times. The cells were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature and washed in PBS for 5 min three times. The cells or flagella were incubated with anti-Spo as the primary antibody diluted 1:10 with PBS. Aliquots of 20 μl of anti-Spo solution were dropped on samples and incubated for 90 min at room temperature in a humid chamber. The slide glass was washed in PBST for 5 min three times, then in PBS for 5 min three times in a Coplin jar. The secondary antibody, 2 μg ml⁻¹ of Alexa488-conjugated mouse anti-rabbit IgG (Molecular Probes, Carlsbad, CA, USA), was dropped on the samples and incubated for 60 min at room temperature. After incubation, the slide glass was washed in the same way as the primary antibody. After mounting medium [2.5% 1,4-diazabicyclo[2,2,2]octane (DABCO), 50% glycerol and 50% PBS] was placed onto the multiwell, a coverslip was sealed to the slide glass using nail polish.

Stained cells were observed using an Olympus IX-70 inverted immunofluorescence microscope. Images were collected using a DP 70 digital camera, DP control and DP manager software (Olympus, Tokyo, Japan), and were exported to Photoshop 7.0 (Adobe Systems, San Jose, CA, USA).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


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