THE ZYGOSPORE WALL OF CHLAMYDOMonas MONOICA (CHLOROPHYCEAE): MORPHOGENESIS AND EVIDENCE FOR THE PRESENCE OF SPOROPOLLENIN

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ABSTRACT

Chlamydomonas monoica Strehlow is being developed as a model for genetic analysis of zygospore morphogenesis, and many relevant mutant strains are available. To provide the basis for interpreting the ultrastructural phenotypes of zygospore mutants, an analysis of wall morphogenesis in wildtype zygospores of C. monoica was undertaken. Following synthesis of a thick, fibrous, primary zygote wall, granular material accumulated between the plasma membrane and the primary zygote wall and aggregated into a repetitive array of electron-opaque fibrous stripes. A new wall layer, the outer layer of the secondary zygospore wall, first appeared as segments with a fibrous outer surface overlying a well-defined band of electron-translucent material. These segments gave rise to an intact sheath adjacent to the plasma membrane. Beneath this sheath, electron-opaque material (forming the inner layer of the secondary zygospore wall) accumulated unevenly and forced the surface sheath to undulate, creating a pattern of peaks and valleys that was exposed to the external environment by rupture and release of the primary zygote wall. The zygospore wall included material resistant to degradation by potassium hydroxide, 2-aminoethanol, and acetylase, but it was destroyed by exposure to trichloroacetic acid. These characteristics, in combination with the autofluorescence of untreated zygospore walls and their failure to stain with phloretin, suggest that sporopollenin may be responsible for many of the resistant properties associated with the mature zygospore of Chlamydomonas.

Key Index words: Chlamydomonas monoica; sexual reproduction; sporopollenin; zygospore; zygote wall

Despite their apparent simplicity, many microorganisms, including unicellular algae, undergo extensive morphological change during the course of their reproductive life cycles. In response to nitrogen limitation, Chlamydomonas vegetative cells differentiate into gametes, which acquire the ability to fuse. In C. monoica, this differentiation is homothallic, with gametes of the opposite mating type arising in clonal populations (van den Ende and VanWinkle-Swift 1994).

A progression of ultrastructural and physiological changes transforms the zygote into a morphologically distinct zygospore. The zygospore is a resting stage adapted to survive a variety of environmental stresses including starvation, desiccation, mechanical abrasion, and freezing. The hallmark of zygospore maturation is the synthesis of an elaborately sculptured and multilayered wall, which must protect the zygospore during dormancy while remaining responsive to the environmental signals that will later trigger germination and disintegration of the spore wall (Levine and Ebersold 1960, Harris 1989).

Ultrastructural studies of Chlamydomonas sexual development have focused on the analysis of gamete activation, gamete interactions, and early stages of cell fusion, primarily with the heterothallic species C. reinhardtii (Friedmann et al. 1968, Cavalier-Smith 1975, Goodenough and Weiss 1975, Martin and Goodenough 1975, Triemer and Brown 1975b, Goodenough et al. 1982, Detmers et al. 1983) and with the distantly related species C. moewusii (Trie mer and Brown 1975a). Although electron microscopy has been applied to the mature zygospore (Brown et al. 1968, Cavalier-Smith 1976, Minami and Goodenough 1978), morphogenesis of the zygospore wall has not been well documented. The zygospore wall bears no ultrastructural resemblance to the walls of vegetative cells, gametes, or young zygotes. In addition to having a thickness as great as 600 nm, the zygospore wall lacks the crystalline appearance of the glycocalyx walls of vegetative cells (Roberts 1974) and includes several distinctive layers.

Earlier studies (VanWinkle-Swift and Bauer 1982, VanWinkle-Swift and Burrascano 1983, VanWinkle-Swift et al. 1987) on zygosporulation in C. monoica have focused on the identification of gene loci expressed specifically in zygotes and required for morphogenesis of the zygote into a zygospore. A classical genetic analysis of spore morphogenesis requires the isolation of appropriate mutant strains, namely, those that are unable to produce a normal zygospore. Because the zygospore is diploid, and because most mutations are recessive, a zygospore mutant phenotype will be seen only if both gametes (i.e. both mating types) carry the mutation. An additional problem in isolating the zygospore mutants is the fact that the mutant gene and its phenotype are not

1 Received 17 October 1996, Accepted 6 March 1997.
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expressed in vegetative cells. Mutants cannot be detected until after gamete fusion has occurred. These problems make it technically unfeasible to obtain the desired mutants from heterothallic species, such as *C. reinhardtii*. In contrast, the ability to obtain zygotes from a single strain has made possible the identification of numerous genes required for zygospore morphogenesis in the homothallic species *C. monoica*. We have mutants carrying zygote-specific recessive lethal mutations that define more than 40 complementation groups (VanWinkle-Swift and Burrascano 1983, VanWinkle-Swift 1989, VanWinkle-Swift et al. in preparation), several of which are specifically defective for synthesis or assembly of the zygospore wall (Parmelee and VanWinkle-Swift 1983, VanWinkle-Swift 1989, VanWinkle-Swift et al. unpubl.). However, the use of such mutants to help define the ultrastructural details of spore morphogenesis requires prior analysis of morphogenesis in wild-type zygo-
spores with an emphasis on the morphogenesis of the zygospore wall.

**MATERIALS AND METHODS**

The wild-type strain (WT15e) of *Chlamydomonas monoica* Streblow was derived from the wild-type strain maintained in the Cambridge Culture Collection of Algae and Protozoa (see VanWinkle-Swift and Burrascano 1983). Vegetative cells were maintained on agar-solidified HS medium (Sueoka 1960) at 20-23°C under continuous cool white fluorescent illumination at 80 µmol·sec⁻¹·m⁻².

To induce gametogenesis, mating, and zygote maturation, vegetative cells were removed from HS plates and were suspended in mating-induction medium, LPN (VanWinkle-Swift and Bauer 1982), at a final cell density of approximately 5 x 10⁷ cells·mL⁻¹. Division in this nitrogen-limiting medium produced gametes within 36-48 h that matured (intracelaral, homothallic mating) and fused to produce quadriflagellate zygotes. Zygote maturation was usually completed within the next 36 h, as revealed by the release of primary zygote walls into the culture medium. Because gametogenesis and mating were not perfectly synchronized, zygotes within the culture differed from one another in terms of age and morphological stage.

After transferring cells to LPN medium, cultures were viewed by phase-contrast microscope periodically. Samples were taken for analysis by transmission electron microscopy (TEM) when young quadriflagellate zygotes first appeared, and periodically thereafter until mature zygospores and discarded primary zygote walls were found in the culture medium. Cells were pelleted by microcentrifugation and were immediately embedded at high cell density in ultralow-temperature freezing agarose. The agar block was then transferred to a 1.1 mix of LPN and 4% glutaraldehyde and fixed overnight at 4°C. After washing in 5 mM phosphate buffer containing 1 mM GAC, the agar-embedded cells were postfixed in 1% osmium tetroxide in phosphate buffer for 4 h on ice or overnight at 4°C. The agar block was washed again in phosphate buffer with CaCl₂ and the agar block dehydrated through 30, 50, 70, 95, and 100% ethanol. The final dehydro step was repeated three times for 15 min each. The dehydrated agar block was transferred to a 7:3 mix of ETOH:Spurr's lowviscosity resin for 2 h and then to a 3:7 ETOH:resin mix overnight at room temperature. The agar block was then transferred periodically to fresh 100% resin several times over the next 2 days, and was finally transferred (to fresh 100% resin in BEEM capsules for curing at 65°C overnight. Thin sections cut with a Sorvall MT 3000 ultramicrotome were picked up on 300-mesh copper grids and were stained in saturated ethanolic uranyl acetate for 1 h followed by staining in alkaline lead citrate 30 min or in Sato's (1968) modified lead stain for 10 min. Sections were viewed with a Zeiss EM 109 transmission electron microscope operated at 50 kV.

Acetolysis of mature zygospores and unmated cells was performed on cells removed from LPN mating induction medium after a 7-day incubation period. The cells were washed in 1.5 mL glacial acetic acid, pelleted by microcentrifugation, and were then resuspended in 1 mL of a 9:1 mix of acetic anhydride and concentrated sulfuric acid (Atkinson et al. 1972). The microcentrifuge tube (with the cap previously pierced) was then suspended in a boiling water bath for 10 min, after which the residual material was pelleted by microcentrifugation for 5 min. All the bottom 100 µL of the supernatant was removed, and the remaining acetolysis solution was diluted by addition of 1 mL of glacial acetic acid. The residual material was resuspended, washed, and repelleted. The majority of the supernatant was then removed, allowing the pellet to be resuspended at a higher concentration for viewing by phase-contrast and ultraviolet fluorescence microscopy using a Zeiss Axiosplan Phase/Fluorescence microscope.

For additional tests, zygospores were purified by layering a 2-mL sample from a 7-day-old LPN culture onto a 2.5-mL cushion of 40% sucrose in a 15-mL centrifuge tube. The cells were centrifuged at 5,000 x g for 15 min. Zygospores remained in the surface layer while unmated cells were pelleted at the base of the cushion. Mature zygospores were removed, washed in distilled water, and tested for resistance to boiling in 10% potassium hydroxide for 1 h (Delwiche et al. 1989), heating at 95°C for 1 h in 2-aminoethanol (Southworth 1974), and incubation at room temperature in chronic acid (2 g CrO₃ in 4 mL 50% glacial acetic acid, Puel et al. 1987). To assay for the presence of lignin in mature zygospore walls, the zygospores were stained with phloroglucinol (1 g in 100 mL 20% HCl. Berkaloff et al. 1983).

**RESULTS**

The life cycle of *Chlamydomonas monoica* including sexual reproduction is shown diagrammatically in Figure 1. When vegetative cells were transferred to a nitrogen-limiting liquid medium, the first appearance of gamete agglutination and pair formation usually occurred between 36-40 h after the transfer. However, because mating was not synchronous, samples taken at 72 h contained young zygotes as well as zygotes with a fully developed zygospore wall. This suggested that morphogenesis of the spore wall was completed within 36 h.

The mature zygospore was recognized by its reticulate surface in phase-contrast light microscopy and...
by transmission and scanning electron microscopy (Figs. 2–4). The zygote formed by gamete fusion initially lacked the distinctive zygospore wall and was most easily recognized at the ultrastructural level by looking for evidence of gamete wall lysis, which is prerequisite to plasma membrane fusion. After the gamete walls were lysed in the region separating the flagellar bases (VanWinkle-Swift et al. 1987, Shi 1995), the walls further relaxed to allow full fusion of the gamete cell bodies but were not degraded further (Figs. 5, 6).

Young zygotes could be recognized by the presence of two nuclei located centrally in the cytoplasm (Fig. 5), with the two chloroplasts occupying much of the remaining cell volume. Starch granules were abundant within the plastids, and large lipid bodies accumulated in the cytoplasm (Figs. 5, 6).

Early in zygote development, the only wall layers visible were the residual gamete walls (Figs. 7, 8). However, Golgi bodies and associated vesicles were very prominent in young zygotes (Fig. 7). The first evidence of new wall synthesis in young zygotes was
the appearance of a fibrous wall layer much thicker than the residual gamete walls and less crystalline in appearance (Figs. 9, 10). This wall layer, the primary zygote wall, underlay the broken gamete walls and encased the entire zygote.

The plasma membrane of young zygotes undulated into a pattern of sharp peaks and valleys (Fig. 11). The region between the plasma membrane and the primary zygote wall expanded and filled with a granular material. This matrix acquired an irregular mottled appearance (Fig. 12) or showed a highly regular pattern of electron-opaque stripes or ripples (Fig. 13). Rough endoplasmic reticulum (ER) and small vesicles continued to be abundant adjacent to the plasma membrane.

The next distinctive zygote wall layer observed after completion of the primary zygote wall included a thin electron-opaque fibrous coat adherent to a more electron-translucent layer of similar thickness but having a more uniform staining pattern. These two layers appeared simultaneously, first as relatively short segments or plaques (Fig. 14) and finally as an intact layer, the trilamellar sheath, adjacent to the plasma membrane and often in apparent contact with it (Fig. 15).

The first evidence of the construction of an inner
Figs. 11–15. Morphogenesis of the outer secondary zygospore wall layer (trilamellar sheath). Fig. 11. Granular matrix fills the space between the primary zygote wall (pzw) and the undulating plasma membrane (arrowhead). Note dense accumulation of ribosomes on nuclear envelope (arrow). Scale bar = 0.5 μm. Fig. 12. Mottled appearance of matrix material beneath the weakly staining primary zygote wall (pzw). Scale bar = 0.4 μm. Fig. 13. Matrix material self-assembles into a regular array of electron-opaque stripes (arrowhead) radiating from the plasma membrane. Scale bar = 0.5 μm. Fig. 14. Wall plaques with fuzzy surface coat (arrowhead). Scale bar = 0.4 μm. Fig. 15. Intact outer secondary wall layer (arrowhead) appressed against the plasma membrane (long arrow) with underlying RER (short arrows). Scale bar = 0.4 μm.
zygote wall layer was the appearance of an electron-transparent space between the plasma membrane and the trilamellar sheath (Figs. 15, 16). Electron-opaque deposits appeared at intervals within this nonstaining region (Figs. 16, 17). This electron-opaque material continued to accumulate with the regions of deposition eventually contacting and coalescing with one another. However, the original sites of accumulation remained as peaks in the innermost layer that, when completed, was uneven in thickness (Fig. 18). The trilamellar sheath was forced into an undulating pattern by irregularities in thickness of the underlying layer. The now completed, secondary zygospore wall included a surface fuzzy coat, an underlying band of more electron-translucent material, and an expansive electron-opaque inner layer that was not uniform in thickness (Fig. 19).

As the zygospore wall layers were assembled and expanded to their final dimensions, the primary zygote wall ruptured and released the mature zygospore (Fig. 20). As a consequence, completion of zygospore maturation was marked by the presence of discarded primary zygote walls with attached residual gamete walls (Fig. 21).

When unmated cultures of vegetative cells were subjected to acetylation, no residue was recovered. Cultures containing mature zygospores and unmated cells, or purified mature zygospores collected on sucrose gradients, when acetylated, yielded a residue of evacuated walls, which displayed autofluorescence under ultraviolet (UV) light (Figs. 22, 23). Transmission electron microscopy of the residue revealed irregular strands of electron-opaque material. An electron-translucent stripe reminiscent of the trilamellar sheath was also detected (Fig. 24).

Zygospore wall fragments (obtained by vortexing purified zygospores with glass beads and washing to remove cytoplasmic contaminants) also showed UV-induced autofluorescence prior to any chemical treatments (Figs. 25, 26). Boiling zygospores in 10% potassium hydroxide, or prolonged heating in 2-aminoethanol had little effect on zygospore wall integrity relative to untreated controls (Figs. 27–29). However, incubation of zygospores in chromic acid...
at room temperature resulted in swelling and extensive dissolution of the wall within 6 h (Fig. 30). No wall residue could be detected after an additional 2-h incubation (not shown).

**DISCUSSION**

We have documented here some of the ultrastructural changes associated with transformation of the dikaryotic zygote into a heavily walled zygospore. Upon completion of gamete fusion, the zygote was protected only by the residual parental gamete walls that had been opened near the flagellar bases to allow plasma membrane contact and fusion. However, soon after plasmogamy, the primary zygote wall was assembled beneath the residual gametic walls, providing additional protection for regions of the plasma membrane exposed by gamete wall lysis.

Although the primary zygote wall of *C. reinhardtii* is a transient wall layer, it may play a critical role in the assembly of the secondary wall layers beneath it. With regard to function, the primary zygote wall may resemble the callose wall of pollen mother cells, a transient wall layer composed of a β-1,3-glucan (Kauss 1985) and involved in pollen grain maturation (reviewed by Bedinger et al. 1994).

As viewed by transmission electron microscopy, the primary zygote wall was much thicker than the residual gametic walls and had a more fibrous appearance. In thin sections, staining of the primary wall was often weak, especially in younger zygotes. When staining was enhanced, the primary wall appeared to be more densely fibrous along its inner surface and less compact on its outer surface (see for example, Fig. 10). Perhaps the primary wall is composed of distinct inner and outer layers unique in both composition and function.

The young zygote contained numerous Golgi bodies and associated vesicles. Rough endoplasmic reticulum was abundant and ribosomes could be seen clustered on the nuclear envelope (Fig. 11). Clearly, development of the zygospore wall requires secretion of extracellular matrix material, presumably processed through the Golgi bodies. In the distant related species, *C. reinhardtii*, it is known that glucoproteins are present in the zygospore wall (Minami and Goodenough 1978, Woessner and Goodenough 1989) and are the primary components of vegetative and gametic cells walls (Roberts 1974, Woessner and Goodenough 1992).

We have attempted to solubilize proteins from the mature zygospore wall without success. Purified wall fragments, fractured with glass beads and boiled for 30 min in sodium dodecyl sulfate (SDS) showed no detectable change in ultrastructure (Donald 1996). The outer sheath and inner wall layer remained intact. The characteristic peaks and valleys were retained even in wall fragments and the surface fuzzy coat seen by TEM (Fig. 19) appeared undamaged.

The morphology of the zygospore wall is distinctly different from that of gametes or of vegetative cells. The massive dimensions of the zygospore wall and the resistant properties of the zygospore are not
only reminiscent of certain other microbial cell types (fungi spores and algal cysts) but may also be analogous to the outer walls of pollen grains of higher plants.

These observations, in combination with the recent report of sporopollenin in zygospor walls of *C. geitleri* (Kalina et al. 1993), a species interfertile with *C. monoica* (Burrascano and VanWinkle-Swift 1984), led us to consider the hypothesis that zygospor wall stability might be a consequence of a primary structure based on nonprotein, highly insoluble components, such as sporopollenin. Sporopollenin, a class of compounds derived from fatty acid and phenylpropanoid precursors, is responsible for the persistence of spores and pollens in the fossil record (reviewed by Wiermann and Gubat 1992, Scott 1994), although proteins are also important components of pollen walls (Knox 1971, Chay et al. 1992). Sporopollenin has also been reported in a wide variety of free-living green algae (Atkinson et al. 1972, Stachelin and Pickett-Heaps 1975, Good

The UV-induced autofluorescence of mature zygospore walls, their failure to stain with the lignin-specific stain, phloroglucinol, the insensitivity of the zygospore wall to dissolution by potassium hydroxide and acetylation, and its sensitivity to chronic acid, support the hypothesis that sporopollenin is a major component (van Gijzel 1971, Southworth 1974, Brunner and Honegger 1985, Delwiche et al. 1989, reviewed by Wiermann and Gubatz 1992).

The ultrastructure of the acetylsis-resistant wall material does not clearly establish which wall layers are resistant. According to Kalina et al. (1993), only the outer wall layer of C. geitleri is resistant. We believe that the electron-translucent band observed in our acetylsis-resistant material (Fig. 24) is derived from this outer layer. However, the considerable amount of highly electron-opaque material also associated with the acetylsis-resistant residue suggests to us that the inner wall layer may also contain sporopollenin. To further address this question, we are presently using acetylsis to analyze a number of zygospore wall mutants with alterations in specific wall layers.

The majority of descriptions of sporopollenin walls in unicellular algae emphasize the presence of a trilamellar sheath seen in electron micrographs as an electron-translucent central layer sandwiched between two electron-opaque boundary layers. The dimensions of the sheath appear to be similar in diverse algae, that is, approximately 20 nm in cross-sectional diameter. In Chlorella (Atkinson et al. 1972), Prototheca (Puel et al. 1987), and the phycobionts of lichens (Honegger and Brunner 1981), inner polysaccharide-containing wall layers are present beneath the trilamellar sheath. In general, the polysaccharide layers account for the majority of the wall volume in these algae, and only the trilamellar sheath contains sporopollenin. The outer wall layer of the C. monoca zygospore may be equivalent to the trilamellar sheath.

In the alga Phytofertis, both the inner and outer wall layers are apparently constructed from tightly appressed trilamellar layers (Good and Chapman 1978). The cross-sectional diameter of the sporopollenin layer(s) in Phytofertis more nearly approaches that of the C. monoca secondary zygospore wall and that of plant pollen exines, being in the micrometer rather than nanometer range. One could view the entire secondary zygospore wall as a trilamellar sheath with a more extensive deposition of electron-opaque material on the inner surface, and a less expansive fuzzy coat on the outer surface of a lipid-like central layer.

Relatively few studies are available describing the developmental synthesis and assembly of algal sporopollenin walls. However, cell wall development in Chlorella (Atkinson et al. 1972) resembles zygospore wall development in Chlamydomonas. During the formation of Chlorella autospores, which are enclosed within the parental mother cell wall, a granular material first appears in the extracellular space surrounding the autospores. Trilamellar “plaques” then appear close to the plasma membrane. The plaques contain an electron-translucent central layer and a fuzzy electron-opaque coating on the outer surface, which is more pronounced than the electron-opaque layer on the inner surface of the plaques. The plaques gradually increase in length and eventually fuse with the ends of the plaques joining in “perfect register”. The granular material in the extracellular space decreases in amount and a fibrillar material is then deposited between the plasma membrane and the trilamellar sheath. The assembly of the trilamellar sheath from progenitor plaques or lamellae has also been described for pollen exine development (Rowley and Southworth 1967, Dickinson and Heslop-Harrison 1968).

Pollen exine formation in angiosperms also resembles the developmental pattern observed in C. monoca (e.g. Fernandez and Rodriguez-Garcia 1988, review by Scott 1994, Gubaryeva 1995). First, the plasma membrane takes on a wavy undulating appearance, then fibrous material appears in the space between the plasma membrane and the callose wall, next electron-opaque spots with the appearance of homogenous droplets that may be the sporopollenin precursors appear within the fibrous region, the droplets fuse to produce the outer layer of the exine, and finally the membranous lamellae appear near the plasma membrane, accumulate electron-opaque deposits, and move up against the outer layer, producing the lamellate inner exine layer. In contrast to the above sequence—which depicts a very different origin for the inner and outer exine layers of pollen grains—an alternative model (Dickinson and Heslop-Harrison 1968) proposes that all exine layers originate as lamellae near the plasma membrane.

Various pollens and spores may differ not only in the details of wall development but also in their solubility in hot 2-aminoethanol (Southworth 1974). Whereas the exines of fresh pollen of most angiosperms and gymnosperms are soluble in 2-aminoethanol, older pollen is often more resistant, and the spore walls of bryophytes and pteridophytes are insoluble. In this regard, the Chlamydomonas zygospore wall more closely resembles the spore walls of the more primitive plants.

The primary advantage of C. monoca for analysis of zygospore wall morphogenesis, structure, and function lies in the ease with which zygospore mu-
tant can be obtained from this species by using homothallism to reveal recessive mutations in a diploid zygote. Using the baseline observations reported here for wild-type zygospores, future work will be directed at the use of mutant strains to arrest wall development.

Such mutants offer the opportunity to combine ultrastructural and genetic approaches for ordering the sequence of events comprising zygospore wall morphogenesis and for investigating the synthesis, secretion, and assembly of a uniquely complex cell wall remarkably resistant to environmental extremes yet responsive to extracellular signals.

K.V.W.S. gratefully acknowledges support for the early stages of this work from National Science Foundation Grant DMB-9106008 and continuing support from Northern Arizona University's Organized Research Intramural grant program. W.L.R. was supported by a Murdock Summer Research Award and an Enrichment Grant from the University of Puget Sound. We thank both the University of Puget Sound and Northern Arizona University for providing electron microscope facilities. Janine Parmelee, Wilma Cruz, and Liang Shi provided technical assistance during preliminary investigations leading up to this work. K.V.W.S. thanks John Cross for first encouraging these studies and Lawrence Fritz and Marilee Sellers for helpful suggestions, technical assistance, and their continuing interest in this project.


