Cell cycle arrest allows centrin translation but not basal body formation during spermiogenesis in *Marsilea*

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SUMMARY

Spermiogenesis in the water fern *Marsilea vestita* is a rapid process that requires the de novo formation of basal bodies in a cytoplasmic particle known as a blepharoplast. Spermiogenesis is activated by placing dry spores into water and is dependent upon the translation of new proteins from stored mRNAs with little, if any, new transcription. We looked at the necessity of cell division cycles in the gametophyte as a prerequisite for the activation of centrin translation and for the consequent formation of blepharoplasts. Cell cycle arrest was induced by treatments of gametophytes with hydroxyurea, with olomoucine, or after RNAi, employing dsRNA derived from *Marsilea* cyclin A or cyclin B. In all cases, centrin is translated from stored mRNA at the normal time, approximately 4 hours after imbibition, and it accumulates to maximal levels ~6 hours after imbibition. In spite of the fact that centrin is translated at essentially normal times and accumulates to nearly normal levels, no blepharoplasts form in the gametophytes where division cycles have been disrupted. These results provide a clear demonstration that the new translation of centrin, by itself, is insufficient for blepharoplast formation, the de novo formation of basal bodies, and the assembly of a motile apparatus.

Key words: Mitosis, Centrin, Basal body, Blepharoplast, RNAi, *Marsilea*

INTRODUCTION

The motile male gametes produced by a few members of the algae, the bryophytes and most of the non-flowering vascular plants, are known as spermatozoids, and these gametes are strikingly different from the cells that gave rise to them. The process of spermiogenesis in these organisms involves the de novo formation of basal bodies in cells that lack pre-existing centrioles (Hepler, 1976; Klink and Wolniak, 2001), in a novel particle known as a blepharoplast (Webber, 1897; Webber, 1901). The basal bodies function in the assembly of a motile apparatus, which can be simple or complex, consisting of as few as two, to many thousands of cilia (Mizukami and Gall, 1966; Norstog, 1986; Norstog, 1990). For the past several years, we have been interested in the de novo formation of basal bodies and ciliogenesis in male gametophytes of the water fern, *Marsilea vestita* (Hart and Wolniak, 1998; Hart and Wolniak, 1999; Klink and Wolniak, 2001). In this organism, the spermatozoid is a coiled, free-swimming cell that possesses approximately 140 cilia (Sharp, 1914; Myles and Hepler, 1977).

Spermiogenesis in *M. vestita* is a rapid process that is initiated by placing dry microspores into water (Sharp, 1914; Mizukami and Gall, 1966; Hepler, 1976; Myles and Hepler, 1977; Myles and Hepler, 1982; Hyams et al., 1983; Pennell et al., 1986; Pennell et al., 1988; Hart and Wolniak, 1998; Hart and Wolniak, 1999; Klink and Wolniak, 2000; Klink and Wolniak, 2001). It consists of nine mitotic division cycles followed by the de novo synthesis of basal bodies and the assembly of a complex cytoskeleton with its attached motile apparatus in each of 32 spermatids in the gametophyte. There is little, if any, transcription required for spermiogenesis to reach completion in these gametophytes (Hart and Wolniak, 1998; Klink and Wolniak, 2000; Klink and Wolniak, 2001). Instead, the dry microspore of *M. vestita* contains large quantities of stored proteins and stored mRNA (Hart and Wolniak, 1998). Spermiogenesis in this organism is dependent upon the translation of the stored mRNA (Hart and Wolniak, 1998; Hart and Wolniak, 1999; Wolniak et al., 2000; Klink and Wolniak, 2001), and the coordinated utilization of these newly made proteins with stored proteins for the formation of the blepharoplast, and the subsequent development of the motile apparatus. Ciliogenesis in each spermatid is dependent on the de novo synthesis of basal bodies in the blepharoplast. A blepharoplast appears in each of the spermatogenous cells of the gametophyte about 4 hours after imbibition (Hepler, 1976), coincident with the increase in centrin protein levels (Hart and Wolniak, 1998). Centrin is a small, protein that is present in a variety of structures associated with microtubule nucleation and cytoskeletal organization, including centrosomes, centrioles, and basal bodies (Salisbury, 1995). The blepharoplast functions as the centrosome for the last mitotic division in the spermatocytes of *M. vestita* (Hepler, 1976).

Both anti-β-tubulin and anti-centrin antibodies become localized in blepharoplasts in untreated gametophytes fixed 4-5 hours after imbibition (Klink and Wolniak, 2000; Klink and Wolniak, 2001). β-tubulin is abundant in the cytosol of the microspore as a stored protein, and centrin is translated

from stored mRNA (Hart and Wolniak, 1998). We recently demonstrated that the blepharoplast does not form if centrin translation is blocked by RNAi treatments, which appear to destroy the stored centrin mRNA (Klink and Wolniak, 2001). Thus, centrin translation appears to serve as a rate-limiting step in blepharoplast assembly and as an essential component in the de novo formation of basal bodies. Since the gametophytes lacking centrin (and failing to form blepharoplasts) become arrested at the stage where blepharoplasts serve as centrosomes, it seemed reasonable to ask if centrin translation, and perhaps blepharoplast formation, would occur in the absence of mitotic divisions. In other words, do the cell division cycles create a set of antecedent conditions that allow blepharoplast formation, once centrin is made?

In this manuscript, we show that centrin translation, by itself, is insufficient for blepharoplast formation. We asked whether the early mitotic divisions of the gametophyte are necessary prerequisites for the activation of centrin translation and the consequent appearance of the blepharoplast. We disrupted the mitotic division cycles in the gametophyte in three independent ways: we treated the gametophytes with hydroxyurea, an inhibitor of DNA replication (Anand et al., 1995), with olomoucine, an inhibitor of cyclin-dependent kinase activity (Glab et al., 1994; Meijer, 1996) and by RNAi (Fire et al., 1998; Tabara et al., 1998; Tabara et al., 1999; Grishok et al., 2000; Klink and Wolniak, 2000; Klink and Wolniak, 2001), using dsRNA probes derived from cyclin A and from cyclin B cDNAs that we isolated from a *M. vestita* male gametophyte library. We show total or partial cell cycle arrest with these different treatments. Moreover, we show that centrin translation is largely unaffected by the arrest of cell cycle activity in this gametophyte, but the aggregation of centrin protein into blepharoplasts fails to occur after partial or complete blocks to mitotic cycling. As expected, the absence of blepharoplasts precludes the formation of the motile apparatus. However, in treated gametophytes where some of the divisions have occurred, we observe some events that are reminiscent of normal spermiogenesis, even though there are fewer cells present in the gametophytes.

MATERIALS AND METHODS

Microspore culture and fixation

Dry sporocarps were collected from *M. vestita* sporophytes raised in 10 ponds at the greenhouse facilities at the University of Maryland. Sporocarps were ground with a few bursts in a commercial coffee grinder (Braun, model KSM2; Lynnfield, MA) and sifted through 425 μm and 212 μm wire sieves to separate the microspores from the megaspores and cell debris (Hepler, 1976; Klink and Wolniak, 2001). 10 mg of isolated microspores were immersed in 10 ml of sterile deionized water or Laetsch's medium (Laetsch, 1967) and cultured in a shaking water bath at 20°C (New Brunswick Scientific, Gyrorotary, Model G76; New Brunswick, NJ) maintained at 20°C (Brinkmann Lauda, model RM6; Germany). At designated time points, microspores were fixed with 4% paraformaldehyde, dehydrated, and embedded in polymethacrylate using procedures described earlier (Klink and Wolniak, 2001).

Preparation of mitotic inhibitors

Olomoucine and hydroxyurea (HU) were purchased from Calbiochem (La Jolla, CA). A 100 mM stock solution of olomoucine was made in DMSO (dimethylsulfoxide). A 1 M stock solution of hydroxyurea was made in deionized water.

Immunocytochemistry

Immunogold cytochemistry was employed to localize proteins in the gametophytes because of high autofluorescence from the spore walls. Microspores were sectioned at a thickness of 1-3 μm, placed onto slides, dried, and then transferred to an acetone solution for 30 minutes to dissolve most, if not all of the polymethacrylate from the tissue. The slides were transferred to PBS (pH 7.4) for 10 minutes before further processing.

For immunolabeling, the slides were transferred to a blocking solution (3% Carnation nonfat dried milk in PBS). After one hour incubation at room temperature, slides were briefly washed in PBS and PBST (PBS with 0.1% Tween-20). 1-2 drops of diluted primary anti-centrin monoclonal 20H5 antibodies directed against *Chlamydomonas reinhardtii* (a gift from Jeffery Salisbury, Mayo Clinic, Rochester, MN, was diluted to 1:25 in PBST) were placed on top of the sections. Slides were then incubated at 37°C in a humid chamber for 1 hour. After a brief wash with PBST, the cells were incubated with gold-conjugated goat anti-mouse IgG (Research Diagnostics Inc., Flanders, NJ) at a 1:500 dilution, in a humid chamber for 1 hour at 37°C. The slides were briefly washed in PBST and transferred to distilled water. Silver enhancement of immunogold labeling was performed according to the manufacturer's directions (Ted Pella, Inc., Redding, CA). Labeled cells were observed with reflected light and transmitted DIC with a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, New York), using a 100× Plan Apochromatic Objective Lens. Image stacks with reflected light were obtained through the 1-3 μm thickness of the tissue section and superimposed on a transmitted DIC image to depict antibody distribution within the section of the gametophyte.

Protein extraction, gel electrophoresis and western blots

Microspores were grown in distilled water at a concentration of 1 mg/ml. At designated time points after imbibition (i.e. the time the dry spores were immersed in liquid), spores were collected by centrifugation at 3000 ***g*** for 5 minutes. Spore pellets were ground in 200 μg sample buffer (62.5 mM Tris base pH 6.8, 2% SDS, 0.02% bromophenol blue, 25% urea, 10% glycerol and 5% 2-mercaptoethanol) with a mini-pestle for 5 minutes. Samples were boiled for 5 minutes and supernatants were collected by centrifugation. Protein quantification was carried out according to procedures first described by Bradford (Bradford, 1976).

20-30 μg of proteins were separated on discontinuous 12.5% polyacrylamide-SDS gels (Laemmli, 1970) and transferred to PVDF membranes (Immobilion P, Millipore; Bedford, MA) in 50 mM Tris pH 8.2, 0.5 M glycine, 0.01% SDS and 20% methanol for 1 hour at 60 volts in a BioRad Protein II Mini Gel System. The membranes were then blocked for one hour in PBS containing 5% Carnation non-fat dry milk. Anti-Centrin antibody (the same antibody described above) was diluted 1:300 in PBST and incubated with the membranes for 1-2 hours. Anti-mouse-alkaline phosphatase conjugated secondary antibodies (New England Biolabs, Beverly, MA) were used at a dilution of 1:3000 for 1 hour at room temperature. PBST washes were performed and antibody complexes were detected by enhanced chemiluminescence using ECL reagents (Amersham, Piscataway, NJ). Fluorescence was detected using the STORM 840 PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

The generation of probes for RNAi

Using a library that had been constructed from *M. vestita* male gametophyte mRNAs isolated at all stages of spermiogenesis (Hart and Wolniak, 1999), we successfully isolated and sequenced a large number of cDNAs, including inserts that encode ORFs for cyclin-A (Mvu 9) and cyclin-B (Mvu 98). Mvu 9 and Mvu 98 insert fragments (250 bases) were PCR amplified with pUC/M13 forward and reverse

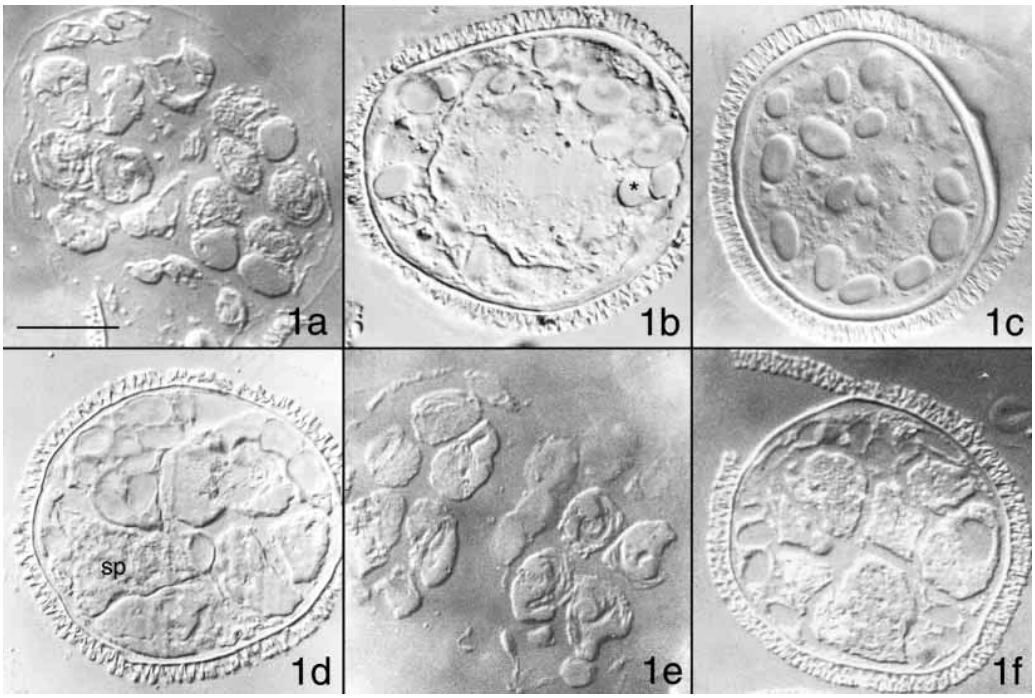

Fig. 1. Mitotic division cycles can be arrested in male gametophytes of *M. vestita*. (a) Untreated control cells. Gametophytes were fixed 8 hours after the time of imbibition. Nine mitotic division cycles have occurred, producing 32 spermatids and 7 sterile cells. (b) Hydroxyurea (1 mM) was present in the medium at the time of imbibition. No division cycles were observed, and the gametophyte is unicellular. (c) Gametophytes were loaded with cyclin B dsRNA, present in the imbibition medium at a concentration of 200 μg/ml. No cell division cycles were observed in the gametophytes after 8 hours.The prominent structures in this gametophyte are plastids; one plastid is labeled with an asterisk. (d) Microspores were placed into an imbibition medium containing 100 μM olomoucine. After 8 hours, a few, anomalous cell divisions were observed in the vast majority of gametophytes. A presumptive spermatogenous cell is labeled (sp). (e) Microspores were placed into an imbibition medium containing 100 μM iso-olomoucine and, after 8 hours, development of the gametophytes was normal. (f) Gametophytes were loaded with cyclin A dsRNA, present in the imbibition medium at a concentration of 200 μg/ml. After 8 hours, a few incomplete cell divisions were observed in the vast majority of gametophytes. All images were obtained with DIC. Bar, 25 μm.

17mers. The PCR mixture consisted of 4 pmole of each pUC/M13 forward and reverse primers, 250 μM dNTPs, 1.5 mM MgCl, 1 U *Taq* polymerase (Promega, Madison, WI) and a large *E. coli* colony carrying the target plasmids in 20 μl of 1× reaction buffer. Mixtures were amplified through 30 cycles at 96°C for 45 seconds, 53°C for 1 minute, 72°C for 30 seconds, and final extension at 72°C for 1 minute. Amplification products were purified with Wizard PCR DNA purification systems (Promega, Madison, WI).

Amplified Mvu 9 and Mvu 98 inserts were transcribed in both directions, in vitro, separately, according to manufacturers instructions (Epicentre Technologies, T3, T7 Ampliscribe® transcription kit; Madison, WI). Double stranded RNAs were then generated by incubating and annealing sense with anti-sense RNA strands. In order to perform RNAi experiments with each of these dsRNA probes, 4 mg of dry microspores were cultured in 1 ml of RNAse free sterile water and 200 μg dsRNA in 2 ml centrifuge tubes, as described (Klink and Wolniak, 2001). Spore cultures were agitated with aeration for 8 hours at 20°C on an Orbitron® agitator (model 260200, Boekel Industries, Feasterville, PA). Then, the gametophytes were harvested and fixed for microscopic observation, or fractionated for protein analyses, as described above.

In situ hybridization

Anti-sense RNA probes were generated using an in vitro transcription kit from Epicentre (Epicentre Technologies, T3 Ampliscribe® transcription kit; Madison, WI). RNA probes were labeled by substituting dUTP with digoxigenin-dUTP (Boehringer Mannheim, Indianapolis, IN). The transcription reaction was precipitated with an equal volume of 5 M ammonium acetate. The mixtures were incubated in ice for 15 minutes and centrifuged at 10000 ***g*** for 15 minutes at 4°C. The pellet was washed in 70% ethanol and resuspended in RNase-free water. Digoxigenin-labeled probes were further quantified by electrophoresis and spectrophotometry.

1-2 μm cell sections were placed on the slides pretreated with 1 mg/ml of poly-D-lysine and dried on an 80°C heat block for 1 hour to increase cell adherence to the slides. Slides were then treated with 100% fresh acetone for 15 minutes and subjected to 1 μg/ml proteinase K digestion for 20 minutes at 37°C. Proteinase activity was inactivated by immersing slides in 0.1 M glycine in PBS, (pH 7.4) for 5 minutes at room temperature. Post-fixation took place in 4% paraformaldehyde to prevent possible diffusion of target nucleic acid. Slides were then immersed in 0.25% acetic anhydride in 0.1 M trithanolamine (pH 8.0) to acetylate tissue and prevent non-specific binding of probe (Steel et al., 1998; Warren, 1998). The sections were hybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 10% dextran sulfate, 2% SDS, 100 μg/ ml single stranded salmon sperm DNA and 1 μg of a DIG-labeled-RNA probe in a moist chamber, overnight at 50°C. The slides were washed and then submerged in 3% BSA in Buffer I (0.1 M Tris, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$) for 10 minutes at room temperature, and then in a 1:1000 dilution of anti-digoxigenin-alkaline phosphatase Fab fragments (Boehringer Mannheim, Indianapolis, IN) for 1 hour at room temperature. The slides were washed in Buffer I and rinsed in Buffer II (0.1 M Tris, pH 9.5, 0.1 M NaCl, 0.5 mM $MgCl_2$). The AP-conjugated antibody was detected with 4.5 μl/ml nitro-blue tetrazolium (NBT)+ 3.5 μl/ml 5 bromo-4 chloro-3 indolyl-phosphate (BCIP) (Boehringer Mannheim) in Buffer II. The color was allowed to develop for 30 minutes to overnight in a dark humid chamber at 4°C.

RESULTS

In order to understand the linkage between cell proliferation and centrin translation during the process of spermiogenesis, we employed three independent procedures to block cell division cycles in male gametophytes of *M. vestita*. In our first set of experiments, we cultured microspores in water or

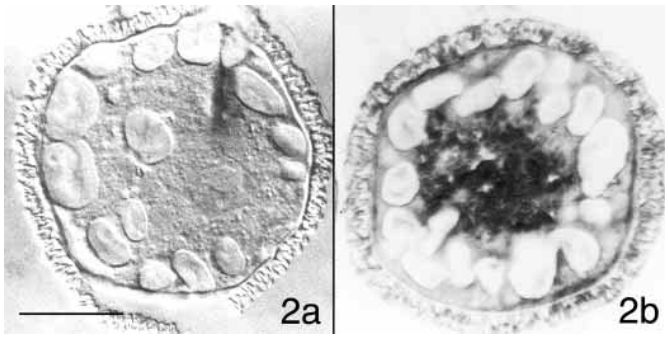

Fig. 2. Cyclin B mRNA becomes undetectable after cells are treated with cyclin B dsRNA at the time of imbibition. (a) Cells treated with cyclin B dsRNA at the time of imbibition. (b) Untreated control cells. DIC images of cells assayed by in situ hybridization shows that the cyclin B mRNA is abundant 30 minutes after imbibition, but that it is undetectable after RNAi treatments. Bar, 25 μm.

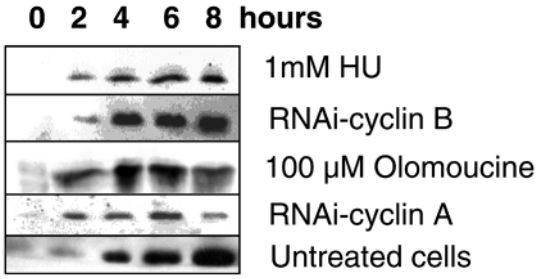

Fig. 3. Centrin translation proceeds even after cell division cycles are arrested. We show anti-centrin immunoblots performed on protein isolates that were obtained on identical cell populations at various intervals after imbibition. Male gametophytes were treated at the time of imbibition with HU, cyclin B dsRNA, olomoucine or cyclin A dsRNA. Irrespective of effects on cell division cycles, all of the gametophytes show a substantial increase in centrin protein abundance 4 hours after imbibition, which is similar to that observed in untreated controls.

aqueous culture medium for 8 hours (Fig. 1a) and we compared these untreated control cells with gametophytes cultured for 8 hours in 1 mM hydroxyurea (HU, Fig. 1b), an inhibitor of DNA replication. We found that the mitotic divisions were completely arrested in gametophytes treated with HU at the time of imbibition (Fig. 1b). These gametophytes remain unicellular for 8 hours with their plastids polarized on one side of the microspores (Fig. 1b).

We then took a different approach to block mitotic divisions in the gametophytes, by employing RNAi strategies (Klink and Wolniak, 2001). We treated cells with dsRNA probes made from a cyclin B cDNA. The cyclin B cDNA used to generate our RNA probes was originally isolated from a *M. vestita* male gametophyte library (Hart and Wolniak, 1999). In the presence of a cyclin B dsRNA, added to the microspores at a concentration of 200 μg/ml (Klink and Wolniak, 2001), mitotic divisions were completely arrested for extended periods; the gametophytes remained unicellular for over 8 hours (Fig. 1c).

The purine derivative, olomoucine, is a potent inhibitor of cyclin dependent kinase (Cdk1); the drug competes with ATP for binding to the enzyme (Glab et al., 1994; Meijer, 1996). When *M. vestita* microspores were imbibed in solutions containing 100 μM olomoucine, the gametophytes typically initiated one or two rounds of cell division, but failed to develop further (Fig. 1d). Most gametophytes did not undergo the standard set of division cycles that produce the normal complement of 39 cells. Moreover, the cell divisions that did occur were often incomplete, producing gametophytes with only partially separated cells. When the divisions were complete, the division patterns deviated significantly from the well-defined pattern that produces regularly shaped spermatogenous surrounded by sterile cells that reside in particular places within the microspore wall (Sharp, 1914; Mizukami and Gall, 1966; Hepler, 1976; Klink and Wolniak, 2001). The production of fewer and irregularly shaped spermatogenous cells (Fig. 1d, sp) is probably the result of the failure of phragmoplast growth or maturation. Mitosis does not appear to have occurred, but we were unable to assess whether DNA replication had taken place in these gametophytes. The inactive derivative, iso-olomoucine, was added to microspores in the same manner and at the same concentration as olomoucine. The gametophytes treated with iso-olomoucine at the time of imbibition (Fig. 1e) were indistinguishable from untreated controls (Fig. 1a). In these control solutions, spermatids reached full maturity and normal spermatozoids were released at 10-11 hours after imbibition, just like untreated control gametophytes (data not shown).

We also treated microspores with RNAi probes derived from a cyclin A that we had isolated from the *M. vestita* cDNA library (Fig. 1f). The treated gametophytes were virtually identical in appearance to microspores imbibed with olomoucine (Fig. 1d). A few rounds of anomalous cytokinesis were common in gametophytes treated with 200 μg/ml cyclin A dsRNA, but it is unclear if DNA replication or mitosis had been arrested by the presence of this probe. When we reduced the concentration of dsRNA-cyclin B from 200 μg/ml (Fig. 1c) to 20 μg/ml (data not shown), we observed that ~30% of the gametophytes exhibited abnormal cell divisions, which resemble the effects observed with 200 μg/ml of RNAi-cyclin A (Fig. 1f).

We suspect that our RNAi treatments affect translation of specific mRNAs by causing the targeted degradation of particular transcripts (Fire et al., 1998). As a control to assess the efficacy of these RNAi treatments, we performed a series of in situ hybridization assays on gametophytes treated with cyclin B dsRNA. We found that exposure to cyclin B dsRNA substantially reduced the detectable level of cyclin B mRNA (Fig. 2a), when compared with untreated control gametophytes (Fig. 2b). We observed a substantial decline in apparent cyclin B mRNA abundance within 30 minutes of imbibition in the dsRNA-treated cells.

The translation of centrin occurs, even after cell division arrest

We have already shown that centrin is translated from stored mRNA in untreated gametophytes, with substantial accumulation of centrin protein becoming obvious 4 hours after imbibition (Hart and Wolniak, 1998). Total proteins from populations of HU-treated gametophytes were extracted at various time points after imbibition and probed with anti-centrin antibody on immunoblots (Fig. 3). Gametophytes arrested prior to the first mitotic division cycle show a substantial increase in the abundance of centrin protein after 4

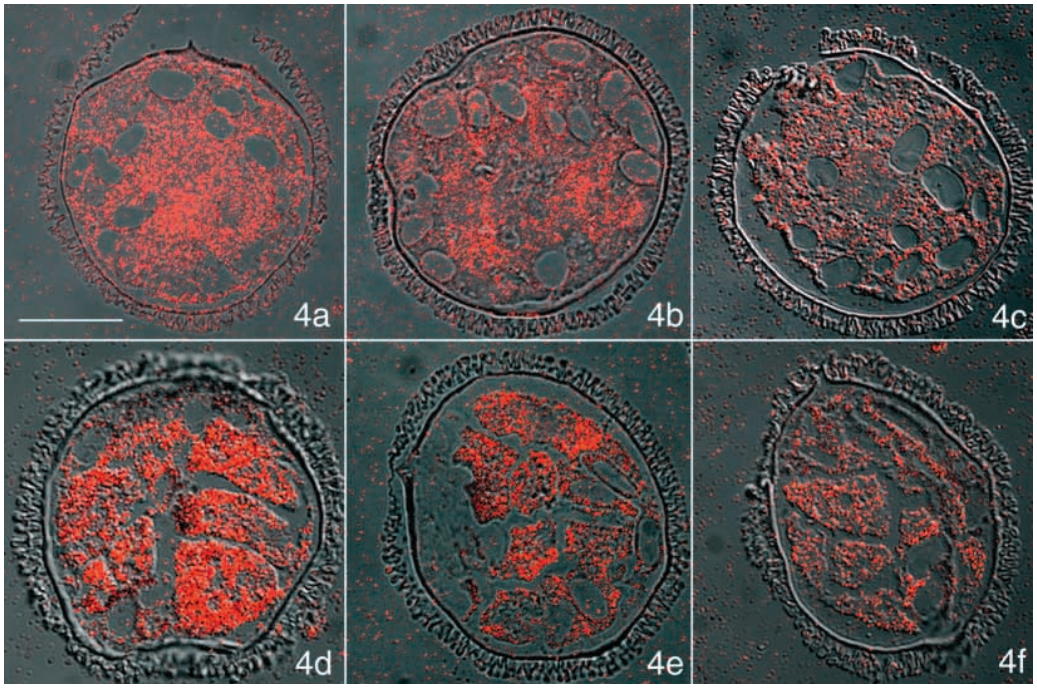

Fig. 4. Immunolabeling with anti-β-tubulin and anti-centrin antibodies shows that blepharoplasts fail to form after the arrest of cell divisions. (a) HU-treated gametophytes, fixed 8 hours after imbibition, and labeled with anti-β-tubulin antibody reveal a random distribution of the antigen in the absence of cell divisions. (b) HU-treated gametophytes, fixed 8 hours after imbibition, and labeled with anti-centrin antibody reveal a random distribution of the antigen in the absence of cell divisions. (c,d) Gametophytes treated with cyclin B dsRNA (c) or treated with olomoucine (d) and labeled with anti-centrin antibody, also show a random distribution of the antigen. (Aggregation of the antigen would be indicative of the formation of blepharoplasts or a motile apparatus – see text.) (e,f) Gametophytes, treated with cyclin A dsRNA and labeled with anti-centrin antibody (e) or anti-β-tubulin antibody (f) show no aggregation of either antigen. (Antigen aggregation would be indicative of the formation of blepharoplasts or a motile apparatus.) Each micrograph is a reflected light confocal image stack superimposed upon a transmitted DIC image as described in Materials and Methods. Bar, 25 μm.

hours, similar to that observed in untreated controls (Hart and Wolniak, 1998). However, the overall abundance of centrin protein in the HU-treated cells appears to be lower than the untreated controls from 4 hours through 10 hours after imbibition (Fig. 3).

Proteins extracted from RNAi-cyclin B treated cells at various time points revealed that centrin translation increased 4 hours after imbibition, even in the absence of mitotic divisions (Fig. 3). Total protein samples from the olomoucine and RNA-cyclin A-treated gametophytes were also isolated at various times after imbibition and immunoblots were probed with anti-centrin antibody (Fig. 3). The patterns of protein accumulation from new translation resembled those observed in HU-treated microspores; centrin protein abundance increases dramatically at approximately 4 hours after imbibition.

Blepharoplast formation is blocked in the absence of mitotic divisions in the gametophyte.

When we looked at gametophytes treated with HU at the time of imbibition, anti-β-tubulin antibody labeling (Fig. 4a) revealed a random distribution of the antigen in the gametophytes that remained unchanged in cells fixed at various intervals after imbibition. We could not detect the formation of blepharoplasts in the HU-arrested gametophytes. In addition, we saw no accumulation of anti-β-tubulin antibody labeling that resembled the assembly of a complicated cytoskeleton, which is involved in spermatid elongation and serves as an anchor for the basal bodies of the motile apparatus in the mature gamete (Wolniak et al., 2000). Anti-centrin antibody labeling revealed that the antigen began to accumulate at what appeared to be a normal time in gametophyte development, ~4 hours after imbibition (Hart and Wolniak, 1998) (Fig. 3), but the antigen remained randomly distributed throughout the cytosol of HU-treated cells, even in cells fixed 8 hours after imbibition (Fig. 4b). Thus, in the presence of HU, we observed no anti-β-tubulin or anti-centrin antibody aggregations into blepharoplasts, and both β-tubulin and centrin distributions remained random throughout the cytosol of the one-cell gametophytes through 8 hours after imbibition. By contrast, with untreated gametophytes fixed at this stage of spermiogenesis, the anti-centrin antibody label would have accumulated at regular intervals along the length of the microtubule ribbon in the anterior portion of the spermatids, depicting the placement of basal bodies (Klink and Wolniak, 2000; Klink and Wolniak, 2001), in close association with an elaborate cytoskeletal array, known as the multilayered structure (MLS) (Carothers, 1975; Myles and Hepler, 1977; Marc and Gunning, 1986; Hoffman and Vaughn, 1995).

Immunolocalizations with anti-β-tubulin and anti-centrin antibodies were also performed on gametophytes that had been treated at the time of imbibition with cyclin B dsRNA (Fig. 4c), with olomoucine (Fig. 4d) or with cyclin A dsRNA (Fig. 4e,f), and fixed 8 hours later. Both β-tubulin and centrin proteins remained randomly dispersed in the cytoplasm of the incompletely divided cells of these gametophytes. In the absence of normal division cycles, blepharoplast formation was not observed in these gametophytes.

Changes in gametophyte permeability after imbibition

Our earlier work (Hart and Wolniak, 1998; Klink and Wolniak,

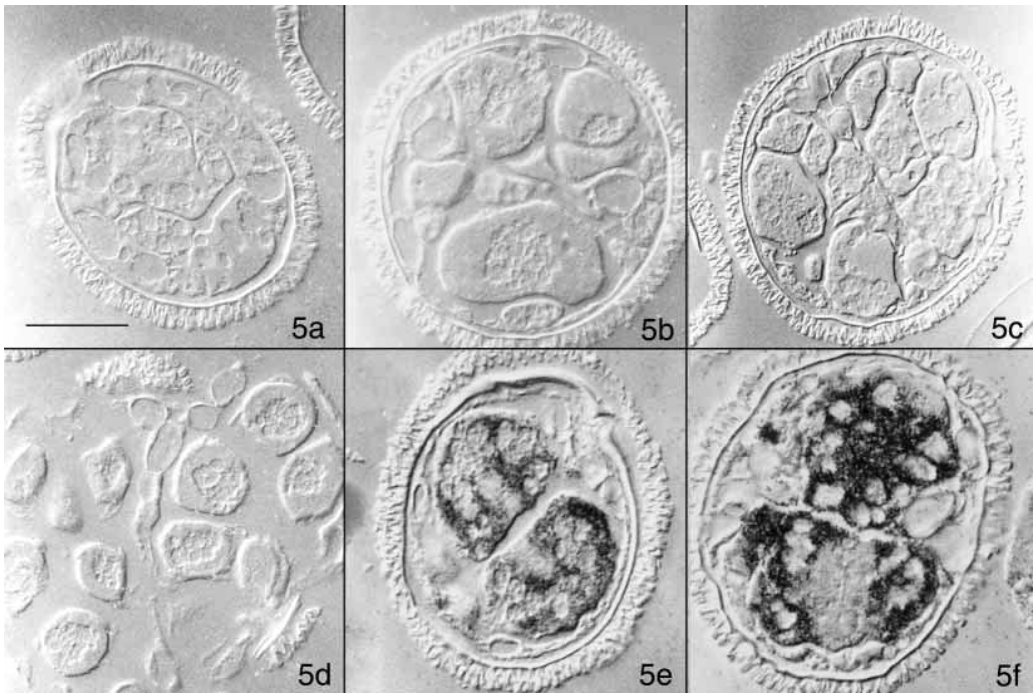

Fig. 5. The addition of 10 mM HU to developing gametophytes of *M. vestita* at various times after imbibition shows that the cells remain permeable to small molecules for ~3 hours. (a) HU was added 60 minutes after imbibition and fixed 8 hours after imbibition; arrest occurred after the second cell division cycle. (b) HU was added 120 minutes after imbibition and fixed 8 hours after imbibition; arrest occurred after the second cell division cycle. (c) HU was added 180 minutes after imbibition and fixed 8 hours after imbibition; arrest occurred during the fourth cell division cycle. (d) HU was added 200 minutes after imbibition and fixed 8 hours after imbibition; there was no mitotic arrest, and spermatids developed normally. (e) Anti-centrin antibody immunolocalization of a gametophyte treated with HU, 120 minutes after imbibition. The antigen distribution is heavily concentrated in the spermatogenous cells at the time of fixation, 8 hours after imbibition. This pattern of distribution does not at all resemble the localization pattern observed in normal spermatids (Klink and Wolniak, 2001) (f) Anti-β-tubulin antibody localization of a gametophyte treated with HU, 120 minutes after imbibition. The antigen abundance in spermatogenous cells is normal at the time of fixation, 8 hours after imbibition, although the distribution within these cells is anomalous (Klink and Wolniak, 2001). All gametophytes were photographed with DIC. Bar, 25 μm.

2001) confirmed other observations (Hyams et al., 1983; Pennell et al., 1986; Pennell et al., 1988), that the cytosolic compartment of the gametophyte is accessible at the time of imbibition to molecules normally not permeant across the plasma membrane. As we focused on the relationship between mitotic divisions and blepharoplast formation, we performed a series of time-course experiments, where 10 mM HU was added to spores at selected times after imbibition, and asked when the gametophytes became impermeant to small molecules. Treated cells were allowed to grow for a total of 8 hours at 20°C before fixation. The growth medium, supplemented with HU at various times within 3 hours of imbibition, halted further cell division at the developmental stage when it was added. When gametophytes that had developed in medium supplemented with HU at various times were fixed at 8 hours and observed with DIC, we observed gametophytes with as few as 2 to as many as 16 spermatogenous cells, depending on the time of HU addition (Fig. 5a-c). Gametophytes treated within 2 hours of imbibition consisted of fewer cells than those treated between 2 and 3 hours after imbibition. The gametophytes became insensitive to HU additions to the medium after three hours (Fig. 5d), presumably because the cells became impermeable to the drug at this stage of development.

Although HU blocked mitosis effectively in the gametophytes, some post-mitotic development of spermatogenous cells still proceeded normally: the (fewer) spermatogenous cells were larger than normal, and they became well-separated from each other (Fig. 5a-c). This pattern of development resembles some aspects of spermatid maturation, but is distinctly anomalous in terms of the number of spermatogenous cells present. Immunolocalizations of these HU-treated gametophytes with anti-centrin antibody (Fig. 5e) showed that centrin protein was made in the spermatogenous cells, by the time of fixation, 8 hours after imbibition. The β-tubulin protein localization pattern was similar to that observed with anti-centrin antibody labeling (Fig. 5f), in that the antigen was heavily concentrated in the spermatogenous cells; however, the antibody distribution within spermatogenous cells was strikingly different from that in normal spermatids, where it is intensely concentrated along one edge of the cell (Klink and Wolniak, 2001). We did not detect any evidence of centrin or tubulin aggregation into blepharoplasts in gametophytes that had been arrested part way through the cell division process. The abnormally large spermatogenous cells failed to become pointed at their anterior ends, a macroscopic sign that the cells had undergone nuclear elongation and had formed a motile apparatus (Myles and Hepler, 1977; Klink and Wolniak, 2001). In the absence of apparent blepharoplasts, it was no surprise that basal bodies and cilia were also undetectable in these arrested gametophytes.

DISCUSSION

In this study, we have used three approaches to uncouple cell divisions from gamete development in the male gametophyte of *M. vestita*. After blocking the mitotic division cycles by treatments with HU or olomoucine, or with RNAi probes made from cyclin cDNAs, we have found that the translation of

stored mRNAs proceeds in the absence of mitosis and cytokinesis, but neither the motile apparatus nor its associated cytoskeleton assemble in the treated gametophytes. The assembly of the motile apparatus is dependent on the de novo formation of basal bodies, in a unique particle known as a blepharoplast (Sharp, 1914; Mizukami and Gall, 1966; Hepler, 1976). Blepharoplast composition is not well known, but the translation of stored centrin mRNA coincides with blepharoplast formation (Hart and Wolniak, 1998), and newly made centrin protein is present in the particle (Klink and Wolniak, 2001). β-tubulin, present as stored protein in the microspores at the time of imbibition (Hart and Wolniak, 1998) also associates with the blepharoplast during basal body formation (Hepler, 1976; Doonan et al., 1986; Klink and Wolniak, 2001).

Our treatments with HU and RNAi(cyclin B) block divisions completely, and prevent the formation of blepharoplasts, although they have no substantial effect on the translation of centrin. In the absence of cell divisions, we conclude that centrin translation by itself, cannot induce blepharoplast formation. With earlier RNAi experiments, we found that that in the absence of centrin translation, cell divisions occurred through eight cycles, but blepharoplasts failed to form (Klink and Wolniak, 2001). Now, in an obverse experimental design, we show that in the absence of normal cell division cycles but in the presence of newly made centrin protein, the formation of blepharoplasts is also blocked. We believe that blepharoplast formation is not only dependent on the presence of existing, stored proteins such as the tubulins, and the synthesis of new proteins, such as centrin, but also on the synthesis of other components, or on the aggregation of existing blepharoplast components into functional particles that give rise to basal bodies. Thus, the partitioning of the gametophyte by successive cell division cycles creates a set of conditions necessary for blepharoplast formation. In male gametophytes of *Ginkgo biloba* and some cycads, only two spermatozoids are formed. Each gamete possesses thousands of cilia, whose basal bodies were produced in large blepharoplasts. With treatments that induce a reduction in the numbers of cell cycles, it would have been striking to produce greatly enlarged blepharoplasts in the gametophytes of *M. vestita*.

Neither the initiation nor the completion of mitotic division cycles appears to serve as a prerequisite for the activation of translation. However, the reorganization of the cytoplasm of the gametophyte that presages the formation of spermatogenous initials is clearly linked with blepharoplast formation, although the blepharoplast appears several hours and several division cycles later. The cell divisions that segregate the spermatogenous initials from the sterile jacket cells are apparently essential for the proper aggregation of centrin and other proteins into organized blepharoplasts. Centrin translation only occurs in the spermatogenous cells, and the stored tubulin proteins become heavily concentrated in the spermatogenous initials before they are fully partitioned from the sterile jacket cells (Klink and Wolniak, 2001). By the time that the seventh and eighth division cycles are completed, the spermatid mother cells contain sufficient centrin for blepharoplast assembly. Blepharoplasts are made synchronously in all of the spermatid mother cells, leading us to suspect that the successive division cycles led to equal partitioning of components necessary for blepharoplast formation.

The cytoskeleton in developing plant spermatids appears to be nucleated by a signature structure, the MLS, which resides at the anterior portion of the gamete, and is tightly associated with a mitochondrion (Hepler, 1976; Hoffman and Vaughn, 1995). A ribbon of crosslinked microtubules extends along the dorsal side of the spermatozoid. The ribbon, known as a spline, is coiled into a spiral and is attached to an elongated nucleus. In the absence of some of the mitotic divisions in the gametophyte of *M. vestita*, we found that the although β-tubulin and centrin proteins were concentrated in spermatogenous cells in the altered gametophytes, the cells did not form discrete aggregates of immunolabeled material that is prominent in untreated cells (Klink and Wolniak, 2001), and indicative of MLS and spline formation. This difference in labeling indicates that the spline is not well organized, and suggests that the MLS may be anomalous, or even absent from the cells.

We have used RNAi strategies to generate phenocopy knockouts of cyclin A and cyclin B. Recently, we showed that RNAi effects are sequence (gene) specific and concentration-dependent in *M. vestita* (Klink and Wolniak, 2001). Although cyclin A and cyclin B are both involved in cell cycle progression, they appear to have different roles in mitosis in the developing male gametophyte. Equal concentrations of RNAi-cyclin A and RNAi-cyclin B result in distinctly different cell morphologies. The enhanced sensitivity to RNAi-cyclin B suggests that cyclin B plays a more critical role in cell cycle progression in this rapidly-developing gametophyte, or that it is more sensitive to RNAi. Other functionally redundant cyclin proteins may compensate for the presence of RNAi-cyclin A. The rapid disappearance of cyclin mRNA after RNAi treatment is consistent with the currently-held notion that dsRNA targets the gene-specific destruction of mRNA in a wide variety of eukaryotic cells (Fire et al., 1998; Tabara et al., 1998; Tabara et al., 1999; Sanchez-Alvorado and Newmark, 1999; Grishok et al., 2000; Boscher and Labouesse, 2000; Klink and Wolniak, 2000).

Our results obtained from supplementing the medium with HU at various times after imbibition indicate that there is a gradual loss of membrane permeability in these developing gametophytes. Our earlier work (Hart and Wolniak, 1998; Hart and Wolniak, 1999; Klink and Wolniak, 2001) shows that the cytosolic space is accessible to macromolecules of considerable size (e.g. 250-400 nt strands of RNA) at the time of imbibition, and our current experiments show that for at least small molecules such as HU, the gametophytes remain permeant for approximately 3 hours. Thereafter, HU is without effect on spermiogenesis, a result consistent with exclusion of the drug from the cytosol (Hyams et al., 1983; Pennell et al., 1986; Pennell et al., 1988). The extended interval of permeability in these gametophytes was somewhat unexpected; we initially thought that the cells would not respond to the presence of the drug at any point more than a few minutes after imbibition. An extended interval of permeability provides the opportunity to perform a variety of new experiments where treatments can be administered during early phases of gametophyte development, up to 3 hours after the dry spores were placed into an aqueous medium. Some of our future experiments will take advantage of the extended interval of enhanced permeability exhibited by these gametophytes.

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