Daughter Cell Assembly in the Protozoan Parasite *Toxoplasma gondii*

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The phylum Apicomplexa includes thousands of species of obligate intracellular parasites, many of which are significant human and/or animal pathogens. Parasites in this phylum replicate by assembling daughters within the mother, using a cytoskeletal and membranous scaffolding termed the inner membrane complex. Most apicomplexan parasites, including *Plasmodium* sp. (which cause malaria), package many daughters within a single mother during mitosis, whereas *Toxoplasma gondii* typically packages only two. The comparatively simple pattern of *T. gondii* cell division, combined with its molecular genetic and cell biological accessibility, makes this an ideal system to study parasite cell division. A recombinant fusion between the fluorescent protein reporter YFP and the inner membrane complex protein IMC1 has been exploited to examine daughter scaffold formation in *T. gondii*. Time-lapse video microscopy permits the entire cell cycle of these parasites to be visualized in vivo. In addition to replication via endodyogeny (packaging two parasites at a time), *T. gondii* is also capable of forming multiple daughters, suggesting fundamental similarities between cell division in *T. gondii* and other apicomplexan parasites.

INTRODUCTION

Toxoplasma gondii is a ubiquitous protozoan parasite, chronically infecting 10–90% of human populations worldwide. Sexual differentiation occurs only in the cat, but asexual *T. gondii* parasites can invade, proliferate, and encyst in virtually any nucleated cell (Frenkel, 1973; Bonhomme *et al.*, 1992; Smith, 1995; Dubey, 1998; Dubey *et al.*, 1998). Primary infection during pregnancy poses a risk of abortion or severe birth defects. Reactivation of dormant parasite tissue cysts (bradyzoites) gives rise to rapidly replicating tachyzoites, which may be fatal in immunocompromised individuals. Pathogenesis in both congenital infection and immunosuppressed patients is directly attributable to parasite proliferation (Frenkel, 1973; Dubey, 1998). Understanding the replication process of this parasite is therefore essential for the development of improved treatment but little is known about cell cycle control in these parasites.

Like other members of the phylum Apicomplexa, *T. gondii* is an obligate intracellular parasite. Haploid

tachyzoites invade into host cells, establishing a parasitophorous vacuole whose membrane is derived from the host plasma membrane (Joiner *et al.*, 1994; Suss-Toby *et al.*, 1996; Mordue *et al.*, 1999; Figure 1A). Two parasites are typically produced in each mitotic cell cycle (~7–10 h), and replication proceeds synchronously, resulting in geometric expansion of clonal progeny until the host cell is lysed, ~48 h postinfection.

In contrast to replication by binary fission (as observed in most animal, plant, and bacterial cells), parasite replication proceeds via assembly of daughters within the mother (Figure 1B). Because asexual replication of *T. gondii* tachyzoites typically produces two parasites per mitotic cell cycle, this process is often termed “endodyogeny” (Sheffield and Melton, 1968). In contrast, most Apicomplexan parasites (including even certain stages of the *T. gondii* life cycle) undergo schizogony or endopolygony, producing multiple daughters from a single polyploid mother. The diverse replication patterns observed in Apicomplexan parasites have long intrigued biologists, and both endodyogeny and schizogony have been extensively characterized by electron microscopy (Snigirevskaya, 1969; Sheffield, 1970; Aikawa, 1971; Hammond, 1973; Azab and Beverley, 1974; Ferguson *et al.*, 1974; Chobotar *et al.*, 1975; Dubremetz and Elsner, 1979; Dubey and Carpenter, 1991; Bannister *et al.*, 2000).

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Figure 1. *T. gondii* tachyzoites establish a specialized parasitophorous vacuole inside of the host cell, within which they replicate synchronously to produce 2 parasites, 4 parasites, 8 parasites, etc. (A) Phase-contrast image showing a parasitophorous vacuole (dotted line, large arrow) containing eight *T. gondii* tachyzoites (small arrow). HN, host cell nucleus; bar: 5 μ m. (B) Diagram of *T. gondii* showing the formation of daughter inner membrane complexes within the mother during cell division and the partitioning of several organelles. Note that the mother's inner membrane complex is still present at this time.

A scaffold for daughter parasite assembly is provided by the inner membrane complex, a patchwork of flattened membrane vesicles—presumably derived from the Golgi apparatus—that is associated with the subpellicular microtubules and additional cytoskeletal elements (Nichols and Chiappino, 1987; Tilney and Tilney, 1996; Morrisette *et al.*, 1997). Organelles are partitioned between these membrane/cytoskeleton assemblies during cell division (Figure 1B; Ogino and Yoneda, 1966; Sheffield and Melton, 1968; Snirevskaya, 1969; Aikawa, 1971; Hammond, 1973; Bannister *et al.*, 2000; Striepen *et al.*, 2000). Eventually, all of the mother's organelles either degenerate or are partitioned between the daughters, which acquire their plasma membrane by budding from the mother, leaving behind only a small residual body.

The length of G1, S, and M phases has been estimated by FACS sorting of synchronized BrdU-labeled transgenic parasites expressing thymidine kinase. Unfortunately, the small size and featureless appearance of Apicomplexan parasites at the light microscopic level has complicated attempts to directly observe dynamic aspects of their replication in living cells (Radke and White, 1998; Radke *et al.*, 2001). Many important questions remain unanswered: How is DNA replication coordinated with cytokinesis? How are the replication and segregation of various intracellular organelles coordinated? How is the mother's inner membrane complex dismantled while the daughter complex is being assembled? What is the relationship between endodyogeny and schizogony? What checkpoints regulate the parasite cell cycle? To investigate questions such as these, we have combined IMC1 (Mann and Beckers, 2001), a subunit of the membrane skeleton of the inner membrane complex or subpellicular network, with various fluorescent reporter proteins (Chalfie *et al.*, 1994; Pepperkok *et al.*, 1999). IMC1-YFP has been exploited to directly observe daughter assembly in real time

using time-lapse video microscopy, yielding insights into the biology of mitotic replication in Apicomplexan parasites.

MATERIALS AND METHODS

Toxoplasma gondii tachyzoites (strain RH) were cultivated in human foreskin fibroblast (HFF) cells as previously described (Roos *et al.*, 1994).

Plasmid *ptubIMC1-YFP/sagCAT* was engineered by amplifying the IMC1 gene from a cDNA plasmid (Mann and Beckers, 2001) using primers 5'-GTTagtctATGTTTAAGGACTGCGCCGATCCTTGCA-3', and 5'-TGGcctaggGCACTGGCATCGGCACACACCACACC-3', digestion with *Bgl*II and *Avr* II, and ligation in place of β TUB in *ptub- β TUB-YFP/sagCAT* (Striepen *et al.*, 1998). The resulting plasmid is based on Bluescript pKS+ (Stratagene, La Jolla, CA), and contains an α -tubulin promoter (Nagel and Boothroyd, 1988) separated from the IMC1 coding sequence by a *Bgl*II site, an in-frame *Avr* II site separating IMC1 coding sequence from YFP, a 3' untranslated region derived from the *T. gondii* DHFR-TS gene (Roos, 1993), and a *Not*I site separating these sequences from a chloramphenicol acetyl transferase selectable marker under the control of 5' and 3' sequences derived from the *T. gondii* P30 gene (Kim *et al.*, 1993). Parasites ($n = 10^7$) were transfected with 50 μ g plasmid DNA and inoculated into host cells as previously described (Roos *et al.*, 1994). To produce stable transgenics, chloramphenicol was added 24 h later to a final concentration of 6 μ g/ml, and drug-resistant clones were isolated by limiting dilution after several rounds of selection.

For immunofluorescence microscopy, confluent HFF cultures on glass coverslips were fixed in 3% paraformaldehyde ~18–24 h after infection with parasites and permeabilized in 0.25% Triton X-100. The following antibody reagents were used: rabbit anti-IMC1 polyclonal antibody (Mann and Beckers, 2001), mouse anti-IMC1 mAb (kindly provided by Dr. G. E. Ward, University of Vermont), monoclonal anti-ROP1 (kindly provided by Dr. J. C. Boothroyd; Ossorio *et al.*, 1992), polyclonal anti-ACP (kindly provided by Drs G. I. Macfadden and R. F. Waller; Waller *et al.*, 1998), monoclonal anti-centrin (kindly provided by Dr. J. L. Salisbury; Paoletti *et al.*, 1996). All antibodies were diluted 1:1000 in 2% BSA and detected using either FITC-conjugated goat anti-rabbit antibody (F-0511; Sigma Chemical Co., St. Louis, MO), FITC-conjugated goat anti-mouse antibody (F-4018; Sigma), or Alexa-594-conjugated goat anti-mouse antibody (A-11032; Molecular Probes, Eugene, OR). After antibody labeling, coverslips were incubated in 2.8 μ M DAPI (Molecular Probes) for 5 min, followed by several brief washes in PBS. Coverslips were mounted with Fluormount G (Southern Biotechnology Associates, Birmingham, AL).

Images were captured using Openlab software (Improvision, Coventry, United Kingdom) on a Zeiss Axiovert (Thornwood, NY) equipped with appropriate barrier/emission filters (Chroma Technology, Brattleboro, VT) and a 1280 $\times$ 1024 pixel, 12-bit, ORCA interline transfer chip CCD camera (Hamamatsu, Bridgewater, NJ). The brightest pixels in an image were typically in the range of <3000 (CCD wells were saturated at 4096 [= 2^{12}]). Some figures were contrast enhanced for display purposes, but all quantitative measurements were carried out using unprocessed data. Images were also collected with a Zeiss LSM510 confocal microscope, taking care to avoid image saturation by adjusting the exposure time and laser power.

For time-lapse video microscopy studies of YFP-expressing parasites, host cell monolayers were cultivated in Δ T chambers (Biopetech, Inc., Butler, PA). HEPES (10 mM, pH 7.0) was added to the medium immediately before imaging at 37°C using a Biopetech heated microscope stage and objective lens heater. Quantitation of IMC1-YFP fluorescence was carried out using Openlab software by summing all the pixel intensities of an entire vacuole and deducting background fluorescence (estimated by the fluorescence in a region with no discernable structure adjacent to the vacuole).

For quantitation of DAPI fluorescence, pixel intensities within a rectangular region completely enclosing the nucleus were summed to give a raw total fluorescence, from which net DAPI fluorescence was calculated by subtracting background (measured on an adjacent area outside the nucleus). The reference for 2n DNA was the average integrated DNA fluorescence of parasites containing two already segregated nuclei within or close to the test vacuole (*T. gondii* tachyzoites are haploid; Pfefferkorn and Pfefferkorn, 1977). SE of the mean for this reference population was 0.02n. DNA content was measured in 284 parasites containing 2 daughters, 90 parasites containing 3 daughters, and 35 parasites containing 4 daughters. Additional details are provided in the text and relevant figure legends.

FRAP (fluorescence recovery after photobleaching) was performed on a Zeiss confocal microscope. A 30 mW Ar/Kr laser (488-nm line, 70% of maximum tube current, 1.8- μ s dwell time per 0.06- μ m pixel) was used for Figure 4A; a 25 mW Ar laser (514-nm line, 70% of maximum tube current, 0.88- μ s dwell time per 0.06- μ m pixel) was used for Figure 4B. Recovery after photobleaching was observed by scanning the sample with 1–2% of the bleaching power at various time intervals. In preliminary measurements we determined the number of scans at high laser power required to produce a 50% decrease in fluorescence (one “photobleach half-life”), and FRAP analysis was carried out after bleaching with 4 “photobleach half-lives” (typically 20 scans). Photobleaching did not affect viability, because bleached parasites entered into and completed cell division at approximately the same rate as controls.

RESULTS

IMC1-YFP Is Properly Targeted to the Inner Membrane Complex, Permitting Visualization of Daughter Scaffold Formation during T. gondii Replication

Previous studies on intramembranous particles located within the inner membrane complex of *T. gondii* parasites led to the prediction that an unknown cytoskeletal filament meshwork must be involved in coupling subpellicular microtubules with the flattened vesicles of the inner membrane complex (Morrisette *et al.*, 1997). IMC1 is a subunit of the subpellicular network, the apparent membrane skeleton associated with the cytoplasmic face of the inner membrane complex. It associates with the inner membrane complexes of both mother and daughters (Mann and Beckers, 2001).

During mitotic cell division in *T. gondii* (endodyogeny), daughter inner membrane complexes develop within the mother while the mother's inner membrane complex is still present, as diagrammed in Figure 1B (Ogino and Yoneda, 1966; Sheffield and Melton, 1968). Antibody staining of proteins associated with the inner membrane complex permits visualization of both the mother and the two developing daughters (Figure 2A). To observe inner membrane complex assembly in living parasites, we constructed a recombinant fusion between IMC1 and either yellow- or cyan-fluorescent protein reporters (YFP or CFP, respectively), as described in MATERIALS AND METHODS. Stable IMC1-YFP and IMC1-CFP transgenic parasites were produced by transfection and selection as previously described (Striepen *et al.*, 1998; Striepen *et al.*, 2000). Both the mother and daughters were clearly visible during mitotic division (Figure 2B), exhibiting a similar pattern to that observed by IMC1 antibody staining (compare with Figure 2A). These fluorescent protein reporters therefore appear to be appropriately incorporated into the cytoskeleton, providing a useful marker for visualizing the inner membrane complex.

Figure 2. IMC1 labeling patterns of wild-type and transgenic parasites. (A) Wild-type parasites (RH) stained with IMC1 antibody during early (left) and late (right) stages of cell division. (B) Living IMC1-YFP transgenic parasites during early (left) and late (right) stages of cell division. Maternal inner membrane complexes are indicated by white arrowheads; daughter inner membrane complexes by white arrows. Note the bright concentration of fluorescence visible in IMC1-YFP transgenics (black arrowheads). Bar: 5 μ m.

Interestingly, daughters exhibited much stronger labeling than the mother in IMC1-YFP transgenics, especially during the early stages of cell division when the daughter inner membrane complexes are small (Figure 2B, left). This effect is also observed—although to a smaller degree—when parasites are stained with anti-IMC1 antibodies (compare left-hand panels in Figures 2A and 2B). Bright, punctuate concentrations of IMC1-YFP were observed in 10–15% of the parasites (black arrowheads in Figure 2B) at various stages of cell division.

Time-lapse Video Microscopy of IMC1-YFP Transgenics

The ability to visualize both mother and daughter inner membrane complexes in living parasites permits analysis of daughter scaffold assembly *in vivo*, as shown in Figure 3A. Selected images from a 12-h time-lapse series show that IMC1-YFP labels the daughter scaffolding from its earliest stages. Elevated concentrations of IMC1-YFP first appear in close proximity to the nucleus ($t = 0$ and 10 h in Figure 3A) before any of the changes in gross nuclear shape characteristic of mitosis are apparent (see Figure 5A). Continued growth of the daughter inner membrane complexes within

Figure 3. Time-lapse images of IMC1-YFP transgenic parasites throughout the cell division cycle. (A) Time-lapse video microscopy of IMC1-YFP transgenic parasites through two mitotic divisions over the course of 12 h. Pictures were taken at 0.5-h intervals, but only time points from 0 to 2 h and 10 to 12 h are shown, because little change was observed from 2 to 10 h; bar: 5 μ m. (B) Fluorescence intensity of three parasitophorous vacuoles in a single field, corrected for background and photobleaching and plotted as a function of time. These three vacuoles were at different points of cell division at the beginning of the experiment; to facilitate comparison, curves have been shifted horizontally to align the second peak.

the mother produces a pair of domes into which the nucleus is drawn, in an elongated or a horseshoe-shaped form (see Figure 5B). The time between first appearance of the daughter complex and full emergence of daughter is typically 1.5–2 h (Figure 3A). Fluorescence intensity rises continuously throughout this period (cf. 9.5–11 h time points in Figure 3B). Total fluorescence declines markedly after daughter scaffold assembly, and IMC1-YFP intensity and distribution remain approximately uniform for 7–8 h between cell divisions (Figure 3B).

Assembly and Maintenance of the IMC1 Network

FRAP experiments have been conducted to study how IMC1-YFP is incorporated into the parasite scaffold (Figure 4). In Figure 4A, one of the two daughters in each dividing parasite was bleached. The fluorescence of bleached daughters recovered to ~80% of that of the unbleached daughters after 40 min. Recovery was uniform over the entire daughter scaffold. When an entire parasite was bleached at the onset of cell division (Figure 4B), daughter scaffolds subsequently formed with approximately the same intensity as those in unbleached parasites in the same vacuole, indicating that the IMC1-YFP incorporated into daughter scaffolds is newly synthesized, rather than being salvaged from maternal scaffolds or assembled from a preexisting cytoplasmic pool.

These results are consistent with time-lapse experiments during cell division (Figure 3B), showing a dramatic increase in total IMC1-YFP fluorescence. Because the fluorescence of the mother remains constant throughout cell division (Figure 3A), IMC1-YFP incorporated into the daughters' scaffold must account for this increase. The mother's IMC1 network is clearly less dynamic than the daughters'. Fluorescence recovery was significantly slower in the mother when part (arrow heads in Figure 4A) or all (Figure 4B) of the mother parasite was bleached.

Markers of the *T. gondii* Cell Cycle

T. gondii has a haploid genome in its asexual cycle, (i.e., DNA content is 1n) and both Feulgen staining (Cornelissen *et al.*, 1984) and FACS-sorting of BrdU-labeled TK⁺ transgenic parasites (Radke and White, 1998) have been used to assay DNA content. Without a marker for daughter scaffold assembly, however, it has not been possible to examine the timing of DNA replication relative to daughter scaffold formation. By measuring the DNA content of parasites in different stages of cell division (as judged by the morphology of daughter scaffold assembly), we have been able to integrate DNA replication into the overall time-course of daughter scaffold formation.

Figure 4. FRAP analysis of YFP-IMC1 incorporation and exchange. (A) One daughter (dashed circle and ellipse) in each of the two parasites in this vacuole was bleached, along with a small portion of the maternal wall (arrowheads). After 20 min, YFP fluorescence recovered uniformly in the daughters, but a defect remains visible in the maternal wall. (B) An entire mother containing two daughters at an early stage of development was bleached (dashed ellipse). After 20 min, the daughters recovered fluorescence and appear almost as bright as unbleached daughters in adjacent parasites. Bars: 5 μ m.

Centriolar replication (Striepen *et al.*, 2000) provides the earliest morphologically observable event associated with mitotic division in *T. gondii*, as has been described in other systems (Robbins *et al.*, 1968; Sluder and Rieder, 1985; Sluder, 1989; Salisbury, 1995; Paoletti *et al.*, 1996; Marshall and Rosenbaum, 1999). The two halves of each centriole could not be distinguished during interphase, but two distinct centrin-positive dots become visible near the nucleus before the initiation of daughter inner membrane complex assembly (Figure 5C). The average DNA content measured in 11 parasites with visibly separated centrioles—but which had not yet formed recognizable daughter inner membrane complex—was $1.2n \pm 0.15n$, indicating that centriolar replication initiates close to the onset of S-phase ($2n$ DNA content was defined by measurement of total DAPI fluorescence within adjacent vacuoles that contain two completely separated nuclei that had not yet budded out of the mother). Daughter inner membrane complex scaffolding first becomes apparent at a DNA content of $1.8n \pm 0.1n$ (sample size = 29). At this stage, the duplicated centrioles have moved apart and are located just under the developing inner membrane complexes.

T. gondii Is Capable of Assembling Multiple Daughters

As noted above, *T. gondii* tachyzoites typically divide by endodyogeny, forming two daughters within the mother. The vast majority of parasitophorous vacuoles contain 2^n

parasites (2, 4, 8, etc), where n indicates the number of parasite divisions since invasion of the host cell (Fichera *et al.*, 1995). Aberrant numbers of parasites have usually been attributed to the inability to resolve all parasites within a vacuole or to slightly asynchronous division.

The ability to visualize the inner membrane complex scaffolding using the IMC1-YFP marker, as shown above, permits careful examination of replication in many parasites. Surprisingly, these studies revealed a significant number of cases where more than two daughters formed within a single mother, as shown in Figure 6. This phenomenon is not attributable to IMC1-YFP overexpression, because multiple daughters were observed at comparable frequencies in both IMC1-YFP transgenics and in wild-type parasites fixed and stained for IMC1 (Figure 7A). The frequency of multiple daughter formation was typically in the range of 0.5–0.7%, although—for unknown reasons—certain cultures exhibited higher frequencies (up to 5–10%). In both RH (type I) and P-strain parasites (type II; Howe and Sibley, 1995; Howe *et al.*, 1997), multiple daughters were observed.

Interestingly, although the formation of more than two daughters within a single mother is rare within the population as a whole, whenever this phenomenon is observed it is common to find several such parasites within the same parasitophorous vacuole (cf. Figure 6, and below). This observation suggests that genetic factors or local conditions may increase the tendency to form multiple daughters.

Figure 5. Markers of the *T. gondii* cell cycle. Fixed IMC1-YFP transgenic parasites were stained with DAPI to visualize nuclear DNA (blue), and with anticentrin antibody to reveal the centrioles (red). IMC1-YFP (green) labels the inner membrane complexes. (A) Daughter scaffold formation (arrows) is initiated close to the nucleus, which remains roughly spherical at this early stage of cell division. (B) As the daughter inner membrane complexes grow in size, the dividing nucleus extends to form a horseshoe or elongated shape, depending on the orientation of the two daughters. HN, host cell nucleus. (C) Anticentrin staining reveals centriolar replication before the establishment of daughter inner membrane complexes or nuclear division. Quantitation of DAPI staining indicates that DNA replication has initiated shortly before this stage (see text). Bars: 5 μ m.

Figure 6. Multiple cases of multiple daughter formation within mothers in a single parasitophorous vacuole. Five IMC1-YFP transgenic parasites in this parasitophorous vacuole are in the process of producing three daughters each (arrows), while 12 are producing two daughters each. The presence of 17 mothers in this vacuole suggests that one mother produced three daughters in the previous replicative cycle. Bar: 5 μ m.

Viability of Multiple Daughters Assembled from a Single Mother

Can three (or more) of the multiple daughters formed within a single mother be viable, or is the apparent development of multiple daughters simply attributable to aberrant daughter scaffold assembly? Each of the daughters appears to acquire a full complement of subcellular organelles, as shown in Figures 7–9. Figure 7 illustrates nuclear partitioning between three daughters (parasite at upper left in panel A, and parasites A, B, and D in panel B). Each daughter also acquires an apicoplast (Figure 7B; Striepen *et al.*, 2000; He *et al.*, 2001), a mitochondrion (Figure 8A; Melo *et al.*, 2000), and rhoptries (Figure 8B)—specialized secretory organelles thought to be essential for host cell invasion (Nichols *et al.*, 1983; Ossorio *et al.*, 1992; Carruthers and Sibley, 1997; Dubremetz *et al.*, 1998). Each developing daughter parasite contains a centriole pair (Figure 8C), which is thought to be critical for assembly of these highly polarized cells (Striepen *et al.*, 2000).

The ability to examine daughter scaffold assembly in parallel with organellar morphology provides important clues about how organelle division proceeds. For example, the partitioning of a single apicoplast between three daughters precedes nuclear division and is usually characterized by three projections extending from a single base, with one branch protruding into each one of the daughters (Figure 7B). This pattern is reminiscent of apicoplast division during schizogony in *Plasmodium* (Waller *et al.*, 2000) and in *T. gondii* parasites that have been treated with dinitroanilines to inhibit microtubules polymerization (Striepen *et al.*, 2000). Dividing mitochondria also extend from a common base (Figure 8A), with multiple branches extending to surround the daughter scaffolding. Mitochondria enter into the daughters only after nuclear segregation, just before cytokinesis (M. Nishi and D. S. Roos, unpublished observations).

Quantitative analysis of DNA levels based on DAPI staining (and comparison with interphase nuclei in the same

Figure 7. Nuclear and apicoplast segregation in wild-type and IMC1-YFP transgenics forming multiple daughters or nuclei. (A) Top panels: wild-type parasite stained with anti-IMC1 antibody (green) and DAPI (blue). (Note: only two of four mothers in this vacuole are shown.) The parasite in the top left corner is in the process of forming three daughters. Nuclear division produces three symmetric lobes (arrows), one for each daughter. Quantitation reveals 4n DNA content in this nucleus (see text). Lower panels: IMC1-YFP transgenic parasites (green) stained with DAPI (blue). Both parasites in this vacuole are finishing one round of nuclear division without forming daughter scaffolds (most obvious in the lower parasite), similar to what happens in schizogony. (B) IMC1-YFP transgenic parasites (green) stained with anti-ACP (a nuclear encoded apicoplast protein [Waller *et al.*, 1998]; red) and DAPI (blue), highlighting the apicoplasts and nuclei, respectively. Four parasites are present in this vacuole (circles at lower left), and three of these (A, B, and D) are in the process of forming three daughters each. Daughter formation proceeds more rapidly when only two daughters are assembled; parasites C1 and C2 are already emerging from the mother. Note the dividing apicoplast intermediate with three symmetric branches in parasite A (arrowhead) and a nucleus in parasite B containing three lobes of 1n DNA content (arrows) and a vestigial body of 1n DNA content (see text for further description). DNA content was established by reference to the integrated DAPI fluorescence in parasites in the same or adjacent vacuole that had just finished their nuclear division. Bars: 5 μ m.

Figure 8. Mitochondria, rhoptries, and centrioles in IMC1-YFP transgenics forming multiple daughters. Green indicates IMC1-YFP fluorescence, and blue indicates DAPI-stained DNA. Circles indicate parasites producing multiple daughters. (A) Circles indicate one parasite within this vacuole in the process of forming three daughters and another in the process of forming four daughters. Each one of the three or four daughters is associated with one nuclear lobe (or one nucleus) and one branch of the dividing mitochondrion (visualized using Hsp60-RFP; red). At this stage, one parasite contains distinct nuclei of 1n and 2n; note that daughters associating with different nuclei (antiparallel red arrows) share the same dividing mitochondrion (white arrowhead). (B) Staining with anti-ROP1 reveals that each daughter contains rhoptries. (C) One of the two parasites in this vacuole is in the process of forming three daughters (circle, red arrows). Its nucleus has divided into two nuclei (1n + 2n DNA content); the latter is in the process of further division to form a total of three daughters. Each daughter is associated with one nucleus or nuclear lobe and one centriole pair. Bars: 2 μm.

Figure 9. Relative DNA content among populations of parasites forming 2, 3, or 4 daughters. (A) Integrated fluorescence intensity was used to establish DNA content in individual parasite nuclei. Calibration was based on DAPI staining of neighboring parasites that had recently completed DNA replication (daughter formation well underway, but before complete emergence from the mother; see text). SE of the mean for this reference population was 0.02n. Sample sizes were as follows: 2 daughters (black), 284; 3 daughters (gray), 90; and 4 daughters (white), 35. (B) Morphological patterns exhibited by parasites that had recently completed DNA replication in only one nucleus (center panel), or (ii) replication in only one nucleus (center panel), or (ii) replication in only one nucleus (center panel) followed by packaging of only three daughters with 1n DNA content left behind as a residual body (right panel). (C) Morphological patterns exhibited by parasites in the process of forming four daughters are also consistent with this model, forming either two nuclei which each divide to form two daughters (center panel), or forming four daughters simultaneously from a 4n nucleus (left panel). Insets illustrate the IMC1-YFP labeling in the corresponding parasites (masked to hide other parasites in the same vacuole to facilitate interpretation). Bars: 2 μ m (insets reduced by threefold).

sample) reveals that each daughter receives a full complement of DNA (1n), even when three or more daughters are formed within a single mother. Total DNA content does not necessarily correspond to the number of daughters produced; however, the upper right-hand parasite in panel 7B appears to have undergone two complete cycles of DNA replication (producing 4n DNA content) but assembles only three daughters. Figure 9 shows DNA content distribution among parasites having 2, 3, and 4 daughters. The vast majority of *T. gondii* parasites replicate by classical endodyogeny and contain $2n \pm 0.02n$ DNA content (black columns). Parasites producing three daughters may contain either 3n or 4n DNA, as revealed by the skewing of the DNA content histogram toward $>3n$ range (Figure 9B); when 4n DNA was observed in parasites packing three daughters, however, one genome equivalent sometimes appeared to be

excluded from the daughters (Figure 7B; also see DISCUSSION).

Various morphologies were observed in developing daughters, including simultaneous separation of 3 or 4 nuclei (Figure 9, B and C, left-hand panels), or initial separation into $1n + 2n$ (9B, center), $2n + 2n$ (9C, center), or $1n + 3n$ (Figure 9C, right). The number of centriole pairs correlates well with the number of nuclei and daughters produced (cf. Figure 8C). When multiple daughters were produced from a single mother, assembly of the daughter scaffolding was usually delayed very slightly relative to siblings dividing by endodyogeny within the same vacuole (cf. Figure 7, A and B).

Direct assessment of daughter parasite viability is technically challenging, because it is difficult to follow an individual parasite after escape from the host cell, particularly among the many other parasites emerging from neighboring

Figure 10. Time-lapse video microscopy of multiple daughter formation in IMC1-YFP transgenic parasites. Replication proceeds in parallel for the three parasites that form two daughters each, and the single parasite that forms four daughters (lower right, arrows). Bar: 5 μ m.

cells at the same time. Time-lapse imaging clearly shows that “triplets” (our unpublished results) and “quadruplets” (Figure 10) can traverse the cell cycle as effectively as “twins” (i.e., progeny derived from endodyogeny), however, suggesting that these parasites are viable.

DISCUSSION

Visualizing *T. gondii* Replication in Living Cells

In this study, we report the engineering of IMC1-YFP as a marker for visualizing the *T. gondii* inner membrane complex in living parasites. For the most part, IMC1-YFP colocalizes with native IMC1 (Figure 2), but subtle differences can be identified. Daughter scaffolds are labeled more brightly than the mother in both wild-type and transgenic parasites, but the distinction appears more pronounced in IMC1-YFP transgenics. This difference could be attributable to C-terminal processing of mature IMC1 (T. Mann and C. J. M. Beckers, in preparation), or to preferential incorporation of IMC1-YFP into daughter scaffolds. Cell-cycle-specific differences are unlikely to be attributable to expression from a heterologous promoter, as such differences have not been previously noted when using the *T. gondii* α -tubulin promoter (Kim *et al.*, 1993; Striepen *et al.*, 1998; Striepen *et al.*, 2000). Bright particles of IMC1-YFP sometimes appear within the cytoplasm, possibly because of overexpression under control of the tubulin promoter.

Stable IMC1-YFP transgenics replicate at the same rate as wild-type parasites, and because daughters are assembled within the mother—on a scaffold consisting of the inner membrane complex and cytoskeletal elements (Figure 1), IMC1-YFP provides an ideal marker for studying parasite replication. Combining time-lapse microscopy of IMC1-YFP-labeled parasites (Figure 3) with previous morphological studies on fixed specimens (Sheffield and Melton, 1968; Radke and White, 1998; Radke *et al.*, 2001; Striepen *et al.*, 2000) makes it possible to draw up a schedule for cell cycle events in *T. gondii*. Duplication and separation of the centrioles and Golgi apparatus (Sheffield and Melton, 1968;

Striepen *et al.*, 2000) are the earliest morphologically recognizable events during cell division, and quantitative analysis reveals that DNA replication initiates at approximately the same time (Figure 5). Formation of the inner membrane complex scaffold is first detected slightly before the completion of DNA replication ($\sim 1.8n$) and is completed over the subsequent ~ 1.5 h. These data correspond closely with analyses of the cell cycle based on thymidine incorporation into synchronized TK⁺ transgenics (Radke *et al.*, 2001), which proposed a G2+M phase of ~ 1 h.

Division of the nucleus is characterized by nuclear elongation with or without folding into a horseshoe shape (depending on the relative orientation of the developing daughters) and takes place only after DNA replication is complete ($2.0n$). Scaffold formation is quite far advanced by the time that nuclear division becomes visible. Other organelles also replicate at characteristic times within the division cycle: apicoplast segregation (Striepen *et al.*, 2000) precedes nuclear division while mitochondrial segregation takes place after nuclear division, as the daughter inner membrane complex formation is nearing completion (M. Nishi and D. S. Roos, unpublished results). New micronemes and rhoptries are formed *de novo* in each daughter, during the early stages of scaffold assembly (Sheffield and Melton, 1968).

Dynamics of the IMC1 Network

FRAP analysis of subpellicular microtubules shows that new subunits are incorporated only at the growing ends of the daughter scaffolds (manuscript submitted). In contrast, newly synthesized IMC1-YFP seems to be incorporated throughout the entire daughter scaffold, as shown by the uniform recovery of the daughter scaffolds after photobleaching, independent of bleaching of the mother parasite. The fluorescence increase of unbleached daughters is lower than that of bleached daughters 20 min after photobleaching, indicating that IMC1-YFP within the network is removed in parallel with the addition of new subunits. Thus, the IMC1 network appears to be constantly remodeled with

the growth of daughter parasites. In contrast, the mother's IMC1 scaffold is much less dynamic, as indicated by the low recovery rate after photobleaching.

Endodyogeny vs. Schizogony: Coordination of DNA Replication with Daughter Parasite Assembly

The bright labeling of daughter inner membrane complexes in IMC1-YFP transgenics permits observation of daughter assembly from the very earliest stages (Figure 3). *T. gondii* tachyzoites have generally been thought to replicate exclusively via endodyogeny, in which two parasites are assembled within the mother, although some EM images do show multiple daughters (Sheffield, 1970; Azab and Beverley, 1974; Ferguson *et al.*, 1974; Dubey and Carpenter, 1991; Dubey *et al.*, 1998). In contrast, other Apicomplexan parasites typically form polyploid nuclei, leading to the simultaneous assembly of multiple daughters via schizogony (nuclear division occurs before daughter assembly) or endopolygeny (daughter assembly precedes nuclear division; Snigirevskaya, 1969; Aikawa, 1971; Hammond, 1973; Brockelman *et al.*, 1985; Dubey *et al.*, 1998; Bannister *et al.*, 2000; Speer, 2001). Our studies have yielded the unexpected observation of multiple daughters in a small but significant fraction of *T. gondii* tachyzoites (Figure 6). We have observed examples of both schizogony (e.g., center panels in Figures 9, B and C), and endopolygeny (e.g., right-hand panel in Figure 9B and left-hand panel in Figure 9C) in *T. gondii* with multiple daughters. Even when multiple daughters are formed within a single mother (up to 8 daughters have been observed), each daughter appears to acquire a full haploid genome-equivalent and a full complement of organelles (Figures 6 and 7). These parasites proceed normally through the entire cell cycle (Figure 10) and are presumed to be fully viable, although technical limitations have thus far precluded isolation of tachyzoites producing multiple daughters and subsequent inoculation into new host cells.

In sum, our observations suggest that there is no fundamental distinction between these various modes of nuclear division (endodyogeny, endopolygeny, and schizogony).

It is relatively easy to understand how it might be possible to produce parasites containing 4n DNA content, if the parasite nucleus proceeds through two cycles of DNA replication before daughter scaffold assembly (cytokinesis). But how is it possible to produce 3n DNA? Presumably, the haploid nucleus first replicates to produce 2n DNA content. DNA may then be partitioned into two nuclei, without cytokinesis (parasites containing two nuclei without any obvious daughter inner membrane complex formation are occasionally observed; Figure 7A, lower panels). If only one nucleus were then to replicate its DNA again before assembling the daughter scaffolding, the net result would be two nuclei, with 1n and 2n DNA content. Such patterns are commonly observed, as shown in Figures 8, A and C. This suggests that in multinucleated parasites, individual nuclei could be autonomous with respect to DNA replication. Other Apicomplexan parasites have occasionally been noted with nuclei in numbers that deviate from powers of two, possibly for the same reason (Vikerman, 1967; Bannister *et al.*, 2000). Rigorous testing of this model will require time-lapse imaging of parasite replication using quantitative markers of DNA content that do not require sample fixation.

Within the parasites that form multiple daughters, the sizes of daughters (indicated by the IMC scaffold) are very similar, even when the nuclei with which these daughters are associated have replicated to differing extents (e.g., 1n + 2n nuclei, Figure 8, A and C). This suggests that scaffold formation is always synchronous (in contrast to nuclear division). Inner membrane complex formation is probably subject to checkpoint control and is suppressed during DNA replication. Organelle duplication appears to be coordinated with scaffold formation, including cases where some DNA is excluded from the developing daughter scaffolds (e.g., Figure 7B). This is consistent with the observation that organelle duplication is coupled to scaffold formation rather than DNA replication in the presence of DNA replication inhibitors (Shaw *et al.*, 2001). To date, the number of centriole pairs detected by centrin staining has always coincided with the number of daughters. Experiments are underway to increase the frequency of parasites having multiple daughters so that we can carry out more extensive analysis of centriole distribution in parasites having different nuclear morphologies.

It is still not clear what triggers *T. gondii* to make multiple daughters. Because centriole replication is one of the first events observed during *T. gondii* cell division and we have observed perfect coincidence between the number of daughters and the number of centriole pairs, it seems likely that the decision point precedes centriole separation. It is intriguing that this initial decision seems not to be completely binding on all subsequent events, because we observed occasional disparities between the DNA copy number and the number of daughter scaffolds (e.g., parasites with 4n DNA, but three daughters; Figure 8, A and B). How to ensure that each daughter gets exactly one copy of all essential organelles is for the present a mystery.

Polyploidy resulting from endomitosis (multiple rounds of DNA replication occur without cytokinesis) has been observed in a wide variety of plant and animal cells (e.g., megakaryocytes; Paulus, 1968; Nagata *et al.*, 1997; Zimmer and Ravid, 2000). The DNA replication of most Apicomplexan parasites is similar to endomitosis, but these parasites go on to complete cytokinesis, producing as many as hundreds of daughters per cell cycle in some *Eimeria* species. How these parasites are able to coordinate DNA replication, cytoskeletal assembly, nuclear division, organelle segregation, and cytokinesis is extremely interesting. We found that *T. gondii* is quite versatile in its ability to organize terms and coordinate its daughter scaffold assembly and nuclear division, producing various nuclear division patterns observed in diverse Apicomplexan species. These observations suggest that multiple daughter formation in *T. gondii* is not a "mistake," but represents a trait that lies within the range of normal phenotypic variation. It is even possible that this parasite might assemble multiple daughters as a matter of routine under certain circumstances (or might have done so in the recent evolutionary past). Regardless, *T. gondii* provides an attractive model for studying cell division in the Apicomplexa. In pursuit of a deeper understanding of the coordination of this process, future experiments will require labeling of the nucleus and centrioles in living cells, and direct cloning of parasites will be necessary to examine heritability of the multiple daughter phenotype. Our observations also raise the possibility that it might be possible to

identify mutants altered in their pattern of DNA replication, nuclear division, and the coordination of these two processes.

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