The Plastid of *Toxoplasma gondii* Is Divided by Association with the Centrosomes

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Abstract. Apicomplexan parasites harbor a single nonphotosynthetic plastid, the apicoplast, which is essential for parasite survival. Exploiting *Toxoplasma gondii* as an accessible system for cell biological analysis and molecular genetic manipulation, we have studied how these parasites ensure that the plastid and its 35-kb circular genome are faithfully segregated during cell division. Parasite organelles were labeled by recombinant expression of fluorescent proteins targeted to the plastid and the nucleus, and time-lapse video microscopy was used to image labeled organelles throughout the cell cycle. Apicoplast division is tightly associated with nuclear and cell division and is characterized by an elongated, dumbbell-shaped intermediate. The plastid genome is divided early in this process, associating with the ends of the elongated organelle. A centrin-specific antibody demonstrates that the ends of dividing apicoplast are closely linked to the centrosomes. Treatment with dinitroaniline herbicides (which disrupt microtubule organization) leads to the formation of multiple spindles and large reticulate plastids studded with centrosomes. The mitotic spindle and the pellicle of the forming daughter cells appear to generate the force required for apicoplast division in *Toxoplasma gondii.* These observations are discussed in the context of autonomous and FtsZ-dependent division of plastids in plants and algae.

Key words: chloroplast division • organelle segregation • cell division • mitotic spindle • *Toxoplasma gondii*

Introduction

The Apicomplexa constitute a large and diverse protozoan phylum of obligate intracellular parasites responsible for many diseases of humans and livestock, including malaria, toxoplasmosis, cryptosporidiosis, and coccidiosis. The apical complex that gives this phylum its name consists of an assemblage of cytoskeletal elements (Nichols and Chiappino, 1987; Morrissette et al., 1997) and secretory organelles (Carruthers and Sibley, 1997). These structures appear to be synthesized de novo during each cell cycle and are thought to be involved in host cell invasion and establishment of an intracellular parasitophorous vacuole. Most apicomplexans are only capable of replicating within the parasitophorous vacuole, until they emerge to infect a new host cell (Roos et al., 1999a).

Various members of the Apicomplexa have recently been found to harbor another distinctive organelle just apical to the nucleus, the "apicoplast" (McFadden et al., 1996; Köhler et al., 1997; Lang-Unnasch et al., 1998; Zhu et al., 2000). Unlike organelles of the apical complex, the apicoplast contains its own genome, a 35-kb circular DNA that has been sequenced from both *Plasmodium falciparum* and *Toxoplasma gondii* (Wilson et al., 1996; Köhler et al., 1997). The apicoplast is essential for parasite survival (Fichera and Roos, 1997; McConkey et al., 1997), but the critical function(s) of this organelle are not yet known.

Although well-established phylogenies based on morphological and molecular characters group the Apicomplexa with ciliates and dinoflagellates in the superphylum Alveolata (Gajadhar et al., 1991; Cavalier-Smith, 1993), phylogenetic analyses of apicoplast ribosomal genes, elongation factor A (*tufA*), and organization of the organellar genome reveal a plastid origin (Wilson et al., 1996; Köhler et al., 1997; McFadden et al., 1997; Denny et al., 1998). The algal origins of the apicoplast are attributable to its evolution by secondary endosymbiosis (Palmer and Delwiche, 1996): an ancient ancestor of the apicomplexa engulfed a eukaryotic alga and retained the algal plastid (Roos et al., 1999b). This model is consistent with the presence of multiple membranes surrounding the apicoplast (Köhler et al.,

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1997; Hopkins et al., 1999; McFadden and Roos, 1999)—remnants of the endosomal membrane, the algal cell membrane, and the two plastid membranes. Origin of the apicoplast by lateral genetic transfer is also consistent with the targeting of nuclear-encoded proteins to the organelle via the general secretory pathway (Schwartzbach et al., 1998; Roos et al., 1999b; DeRocher et al., 2000; Waller et al., 2000), a mechanism very different from chloroplast targeting in algae and plants (Schatz and Dobberstein, 1996).

In this study, we have exploited the ease of genetic manipulation in *T. gondii* and the availability of in vivo fluorescent markers to study division of the apicoplast and the segregation of this essential organelle and its DNA between daughter cells. Our results indicate that replication of the apicoplast genome and division of this organelle is significantly different from plastid division in plants and algae: apicoplast division is not autonomous (as observed in plants), but is instead inextricably coupled to cell division. Apicomplexan parasites replicate by assembling daughter cells on a complex scaffolding of cytoskeletal elements and flattened membrane vesicles (the "inner membrane complex;" Morrissette et al., 1997). Daughter cell assembly occurs either after every cycle of DNA replication, producing two daughters at a time (endodyogeny; Scholtyseck and Piekarski, 1965; Ogino and Yoneda, 1966; Sheffield and Melton, 1968), or after multiple rounds of DNA replication, producing multiple daughters (schizogony; Frenkel et al., 1970; Müller, 1975; Roos et al., 1999a; Speer and Dubey, 1999; Waller et al., 2000). These parasites appear to have solved the problem of replication and faithful segregation of a single organelle into two or more daughter parasites by linking apicoplast replication to the machinery already in place for nuclear division: the centrosome/mitotic spindle.

Materials and Methods

Host Cells and Parasites

RH strain *T. gondii* tachyzoites and transgenic lines derived from this strain were maintained by serial passage in primary human foreskin fibroblast cultures (HFF),[1] grown at 5% CO_2 in bicarbonate-buffered Dulbecco's modified Eagle's medium (GIBCO BRL), supplemented with 10% heat inactivated newborn bovine serum (Hyclone) and penicillin/streptomycin/gentamycin (Roos et al., 1994). The culture medium was replaced with modified Eagle's medium containing 1% dialyzed fetal bovine serum (GIBCO BRL) before parasite infection. Dinitroaniline herbicides (synthesized and provided by Dr. J.W. Benbow, Lehigh University, Bethlehem, PA) were stored as 10 mM stocks in DMSO and used at a final concentration of 1 μM.

Plasmid Construction and Parasite Transfection

All fluorescent protein expression plasmids used in this study are related to plasmid *p*tubP30-GFP/sag chloramphenicol acetyl transferase (CAT) (Striepen et al., 1998). In this construct (based on Bluescript plasmid pKS+; Stratagene), a BglII site (compatible with BamHI) separates the 5′ untranslated region of α-tubulin (Nagel and Boothroyd, 1988) from the P30 signal sequence (Burg et al., 1988), an AvrII site separates P30 sequences from the green fluorescent protein (GFP) coding sequence, a PstI site (compatible with NsiI) separates GFP from the 3′ untranslated region of DHFR-TS (Roos, 1993), and a NotI site separates this domain from pKS+ and the selectable marker sagCATsag (Kim et al., 1993). Plasmids encoding the yellow fluorescent protein (YFP, a derivative of *Aequoria victoria* GFP; Miller et al., 1999), cyan fluorescent protein (CFP, a derivative of *Aequoria victoria* GFP; Miller et al., 1999), and red fluorescent protein (RFP; from *Discosoma* sp.; Matz et al., 1999) were obtained from CLONTECH Laboratories, Inc., and used as templates to amplify the respective coding regions.

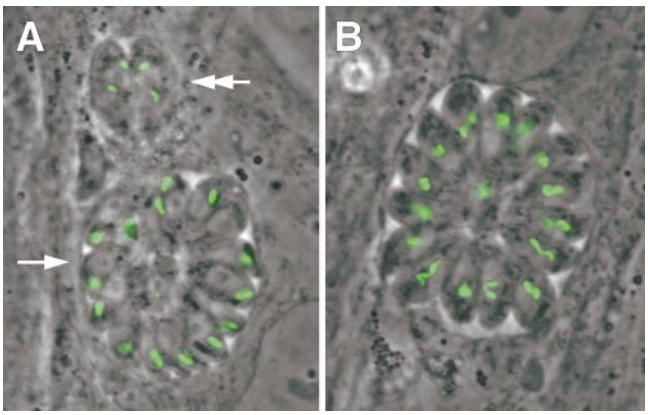

Figure 1. Ovoid, U-shaped, and dumbbell-shaped apicoplasts. Parasites were transfected with construct *p*tubACP-GFP before infection of coverslip cultures and imaged 24 h after electroporation. A single oval apicoplast is observed just apical to the nucleus in most parasites (A, single arrow; see also Köhler et al., 1997; Waller et al., 1998). A small fraction of vacuoles contain two plastids (A, double arrow) or large, dumbbell-, or U-shaped large (B). Note that plastid morphology is uniform for all parasites in a given vacuole, and that parasites containing two plastids or elongated organelles are larger than parasites harboring a single ovoid apicoplast.

Reporters targeted to the apicoplast were engineered as in-frame COOH-terminal fluorescent protein fusions, using PCR primers to introduce a 5′ AvrII site immediately upstream of the ATG initiation and a 3′ NsiI site immediately downstream of the stop codon (CFP/YFP sense primer 5′-acgtCCTAGGatggtgagcaagggcgaggagc-3′, antisense primer 5′-cagtATGCATtacttgtacagctcgtccatgccg-3′; RFP sense primer: 5′-agctCCTAGGatggtgcgctcctccaagaacg-3′, antisense primer 5′-gactATGCATctacaggaacaggtggtggcgg-3′). Amplification products were cloned as AvrII/PstI fragments in place of GFP in vector *p*dhfrCAT-GFP (Striepen et al., 1998), excised together with dhfr 3′ UTR using AvrII/NotI, and introduced in place of GFP in apicoplast targeting vectors tubulin acyl carrier protein (ACP)-GFP/sagCAT (Waller et al., 1998) or tubFNR-YFP/sagCAT (Vollmer et al., 2000). A parasite line stably expressing plasmid *p*graPCNA-GFP (exploiting the GRA1 promoter) was provided by Drs. M. Guerini and M.W. White (Montana State University, Bozeman, MT; Guerini et al., 2000).

β-Tubulin proteins were engineered to contain a COOH-terminal fluorescent domain using primers 5′-actgAGATCTaaaatgagagaaatcgtccacgttc-3′ and 5′-cagtCCTAGGcgcgccttcctctgcaccc-3′, and ligating the resulting β-tubulin PCR product in place of P30 sequences in the plasmids described above (BglII/AvrII). Tubulin proteins with a fluorescent NH_2-terminal domain were constructed by amplifying α- or β-tubulin sequences (Nagel and Boothroyd, 1988) from cDNA clones (α-tubulin sense primer 5′-atcgCCTAGGatgagagaggttatcagcatcc-3′, antisense primer 5′-gcatCTGCAGttagtactcgtcaccatagccc-3′; β-tubulin sense primer 5′-actgCCTAGGatgagagaatcgtccacgttcag-3′, antisense primer 5′-gcatCTGCAGttagtactcgtcaccatagccc-3′), introducing these AvrII/PstI fragments in place of GFP in plasmid *p*tubP30GFP, and replacing P30 sequences with YFP (sense primer 5′-acgtcAGATCTaaaatggtgagcaagggcgaggagc-3′, antisense primer 5′-cagtCCTAGGcttgtacagctcgtccatgccg-3′).

Parasite transfection was performed by electroporation, as previously described (Roos et al., 1994). Briefly, 10^7 purified parasites were resuspended in cytomix [(mM) 120 KCl, 0.15 $CaCl_2$, 10 K_2HPO_4/KH_2PO_4, pH 7.6, 25 Hepes, pH 7.6, 2 EDTA, 5 $MgCl_2$, 2 ATP, and 5 glutathione] containing 50 μg sterilized plasmid DNA, and electroporated in a 2-mm gap cuvette with a 1.5 kEV pulse at a resistance setting of 24 Ω using a BTX 600 electroporation system. Electroporated parasites were used to infect

[1]*Abbreviations used in this paper:* ACP, acyl carrier protein; CAT, chloramphenicol acetyl transferase; CFP, cyan fluorescent protein; FNR, ferredoxin-NADP reductase; GFP, green fluorescent protein; HFF, human foreskin fibroblast; PCNA, proliferating cell nuclear antigen 1; RFP, red fluorescent protein; YFP, yellow fluorescent protein.

HFF cells grown on coverslips (for microscopy) or in T25 flasks (for drug selection). Stable transformants were selected in 20 μM chloramphenicol (Kim et al., 1993); the relatively low level of resistance provided by CAT in *T. gondii* parasites selects for multi-copy plasmid integration (Striepen et al., 1998), enhancing expression levels.

Light Microscopy

For microscopy, HFF cells were grown to confluence on sterilized coverslips in six-well plates. Cultures were infected with 5×10^5 parasites and examined 16–36 h after infection. For imaging of native fluorescent proteins, coverslips were mounted in PBS or medium without further treatment. GFP-expressing parasites were imaged using an Axiovert microscope (Carl Zeiss, Inc.) and a FITC filter set (450–480-nm excitation/515–565-nm emission). RFP was detected using a Texas red filter set (530–585-nm excitation/615-long pass emission). To record registration-free images of YFP/CFP expression, we used a single emission filter (505–555 nm; Chroma) and dichroic mirror for both fluors, introducing specific filters for CFP (399–429 nm) or YFP (480–495 nm) into the excitation path on a slider. Images were collected using an interline chip cooled CCD camera (Orca 9545; Hamamatsu).

For time-lapse video microscopy, cells were grown in 3ΔT dishes in which the culture surface consists of a #1 glass coverslip (Bioptechs). Monolayers were infected with transgenic parasites and cultured for 24 h before imaging. During imaging, the standard infection medium was supplemented with Hepes, pH 7.6 (GIBCO BRL) to a final concentration of 10 mM, permitting culture at ambient [CO_2]. The temperature of the dish and the 100× oil immersion lens was maintained at 37°C for the duration of the experiment using stage and objective heaters (Bioptechs). Electronic shutters (Ludl) were introduced into both light paths, permitting illumination only during imaging, to reduce photobleaching. Shutter and camera control, image contrast, and image overlays were performed using Openlab software (Improvision) and Photoshop (Adobe Systems, Inc.).

For immunofluorescence, parasite-infected cells were fixed in 3% paraformaldehyde and permeabilized with 0.25% Triton X-100 in PBS. Anti–GFP antibodies (Clontech) were used at 1:500 (rabbit polyclonal) or 1:250 (mouse monoclonal), and detected using appropriate FITC-conjugated anti–immunoglobulin (1:200; Sigma-Aldrich). Anti–centrin antibody 26-14.1 (provided by Dr. J.L. Salisbury, Mayo Clinic, Rochester, MN) was used at 1:200 to label centrosomes, and anti–NET1 mAb 45.15 (provided by Dr. G.E. Ward, University of Vermont, Burlington, VT) was used at 1:1,000 to label the *T. gondii* inner membrane complex. Appropriate rhodamine-conjugated anti–immunoglobulins (1:200; Cappel) were used in double-labeling assays. Cells were mounted on slides using Gelmount (Fisher Scientific).

To stain plastid DNA, cells were fixed, permeabilized, incubated 10 min in 1 μg/ml DAPI (Molecular Probes) in PBS, washed to remove unbound dye, and mounted. Stained preparations were viewed on an Axiovert or a DMIRBE microscope (Leica) using Openlab software or an Olympus BX50 confocal system running Fluoview software (Olympus). To remove background haze and reveal greater detail in parasites double-labeled with DAPI and anti-GFP, serial 0.25-μm optical sections were collected and the resulting image stacks were deconvolved using a multiple neighbor algorithm. Deconvolved images were then used to reconstruct images depicting the entire cell using Openlab software.

Electron Microscopy

Infected cells were fixed directly in the culture dish by replacing the culture medium with a freshly prepared solution of 1% glutaraldehyde (8% stock; Electron Microscope Sciences) and 1% OsO_4 in 50 mM phosphate buffer at pH 6.3. Samples were fixed 45 min on ice, rinsed in distilled water to remove excess phosphate, stained overnight in 0.5% uranyl acetate, dehydrated, and embedded in Epon directly in the culture dish. Thin sections were cut using a diamond knife, mounted on uncoated grids, and stained with uranyl acetate and lead citrate before examination with a Phillips 200 electron microscope.

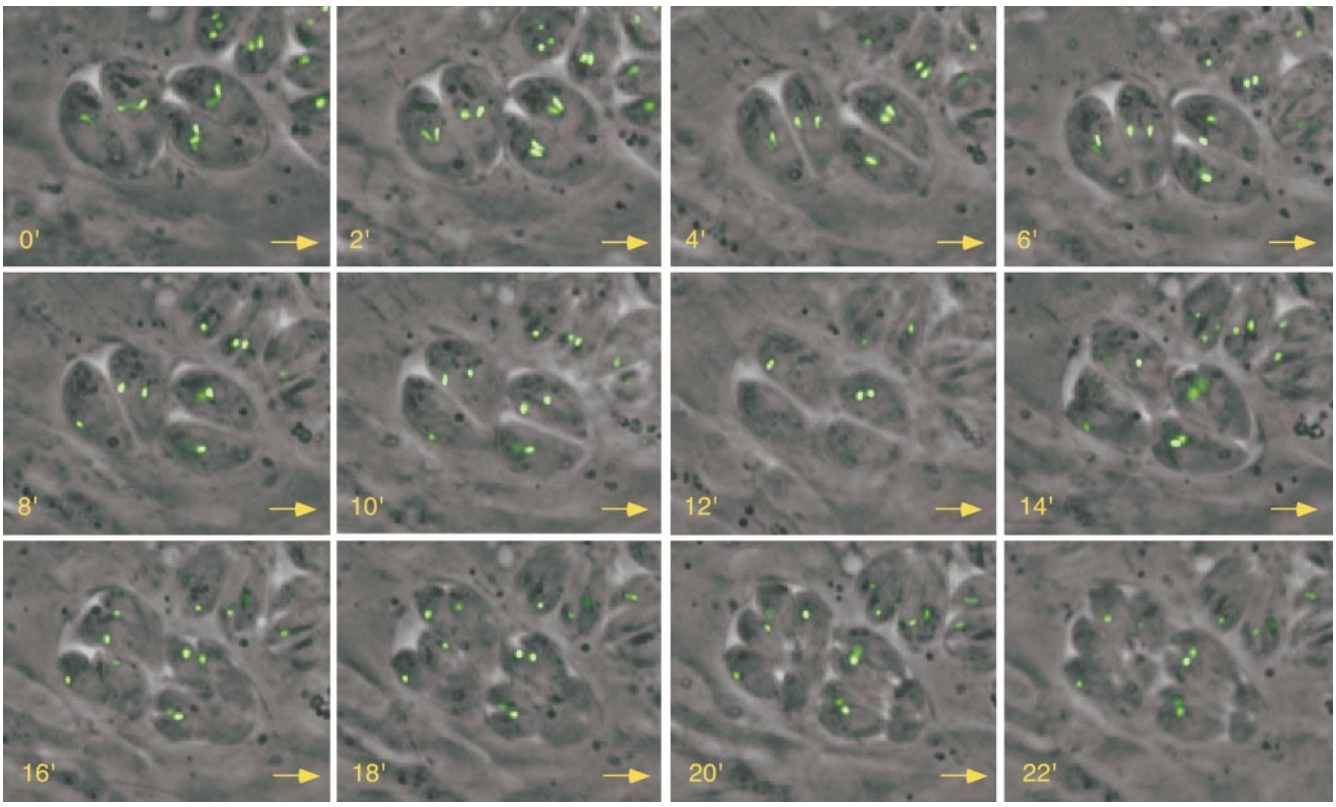

Figure 2. Plastid division precedes cell division and involves dumbbell- and U-shaped intermediates. Infected cells were time-lapse imaged as detailed in Materials and Methods. Phase-contrast and fluorescence images were collected every 2 min and the resulting images from each time point were merged. $t = 0$ shows a vacuole with four large parasites harboring dumbbell-shaped apicoplasts (green). At $t = 2$ min, the ends of the apicoplast tilt sharply towards the apical end to form a U-shaped organelle as the organelle is cut through the middle ($t = 4$ min). With increasing time (6–12 min), the two daughter organelles move apart and towards the apical end of the daughter parasites. Shortly after plastid division (14–20 min), the daughter parasites emerge from their mother, indicating the completion of endodyogeny (Sheffield and Melton, 1968).

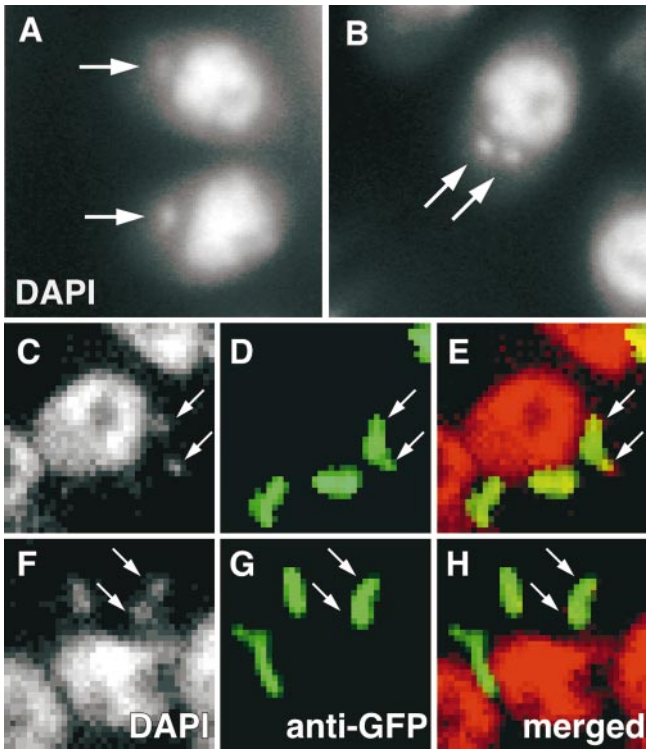

Figure 3. Segregation of the apicoplast genome. Cultures infected with ACP-GFP transgenic parasites were fixed and permeabilized, and stained with DAPI only (A and B) or anti–GFP rabbit serum (green) before DAPI staining (C–H). C–H were generated from a stack of optical sections, which were deconvolved using a multineighbor algorithm, rendered in three dimensions, and superimposed. While most parasites exhibit a single restricted spot of DAPI-stained apicoplast DNA (A), a subset shows two DAPI-stained spots of equal intensity within the organelle (B). Double labeling for DNA (C and F) and the lumenal marker ACP-GFP (D and G) reveals that the organellar DNA localizes to each end of the dumbbell-shaped apicoplast.

Online Supplemental Material

A quicktime movie of Fig. 2 is available at http://www.jcb.org/cgi/content/full/151/7/1423/DC1. Infected cells were time lapse imaged as detailed in Fig. 2 (below). Images of GFP-labeled plastids were taken by fluorescence microscopy (green) and merged with phase-contrast images depicting the outline of the parasite and host cell at the same time point. Additional movie and image files are available at http://webs.cb.uga.edu/~striepen.

Results

Apicoplast Replication Is Linked to Cell Division

We have previously shown that fusion of GFP to a nuclear-encoded apicoplast protein (the acyl carrier protein of a type II fatty acyl synthase) permits direct visualization of the apicoplast in living parasites (Waller et al., 1998). Most parasite cells contain a single plastid (Fig. 1 A, arrow), confirming earlier work where the plastid genome was detected by in situ hybridization (Köhler et al., 1997). At low frequency, however, we encountered relatively large parasites harboring two apicoplasts (Fig. 1 A, double-headed arrow). We also noted that the shape of the apicoplast can be quite variable: in addition to the ellipsoid organelle described in electron microscopic studies, dumbbell and U-shaped plastids were also observed (Fig. 1 B). These distorted apicoplasts can be more than three times longer than the ellipsoid form (up to 3 μm from end to end), and they appear synchronously in every parasite within a given parasitophorous vacuole, suggesting a link to the ~8-h cell cycle (which is synchronous for all *T. gondii* parasites within the vacuole; Fichera et al., 1995).

To evaluate whether the U-shaped and dumbbell forms of the apicoplast might be intermediates in plastid division, we performed time-lapse video imaging on a clonal line stably expressing ACP-GFP (see Materials and Methods for further details). Fig. 2 shows a representative series of images, additional images and movies are available at http://www.jcb.org/cgi/content/full/151/7/1423/DC1. The four parasites in the center of this image at $t = 0$ are all siblings within a single vacuole, and each harbors an elongated apicoplast (contrast with the ovoid apicoplasts in the parasites within a separate vacuole; Fig. 2, top right). 2 min into this time series, all four apicoplasts are bent in half, with their ends pointing towards the apical end of the parasite. 2-min later (4' image), these apicoplasts have clearly divided into two daughter organelles within a single mother cell. 10 min after plastid division (14' image), the parasites initiate cytokinesis, resulting in the formation of eight daughter parasites (clearly visible by 22'). Plastid division as well as cytokinesis was synchronized in all four parasites of the vacuole. Division of the apicoplast consistently took place just before the emergence of the two daughter parasites, arguing that plastid replication might be linked to parasite cell division. Furthermore, it appears that the ends of the organelle are not free but pulled by some cellular structure resulting in the division of the organelle.

The Plastid Genome Is Segregated Early and Associates with the Organelle Ends

The apicoplast genome is a 35 kb DNA circle (Wilson et al., 1996; see also http://www.sas.upenn.edu/~jkissing/toxomap.html), present at approximately six copies per cell in *T. gondii* tachyzoites (Fichera and Roos, 1997). As shown in Fig. 3 A, this genome (in addition to the nuclear DNA) can be visualized in fixed and permeabilized cells by staining with intercalating dyes such as DAPI or Hoechst 33258 (Köhler et al., 1997). Staining of apicoplast DNA reveals a spot smaller than the organelle itself, however, suggesting concentration of the apicoplast genome as a intra-organellar nucleoid (Fig. 3 A). This finding is reminiscent of multicopy chloroplast genomes, which associate with as yet uncharacterized proteins to form multiple nucleoids (Kuroiwa et al., 1998; Cannon et al., 1999). Some parasites exhibit two closely associated apicoplast nucleoids of equal intensity (Fig. 3 B), and double labeling of the plastid genome (with DAPI) and it's lumen (with anti–GFP antibody) reveals that the two closely associated DAPI-labeled spots are invariably associated with elongated apicoplasts, and always localize to the ends of the organelle (Fig. 3, C–H).

The Plastid Divides Concurrently with the Nucleus

To more precisely characterize the timing and context of plastid division, we labeled both the nucleus and plastid in living parasites, taking advantage of a proliferating cell nuclear antigen 1 (PCNA)–GFP fusion protein to label the *T. gondii* nucleus (Guerini et al., 2000). For these experi-

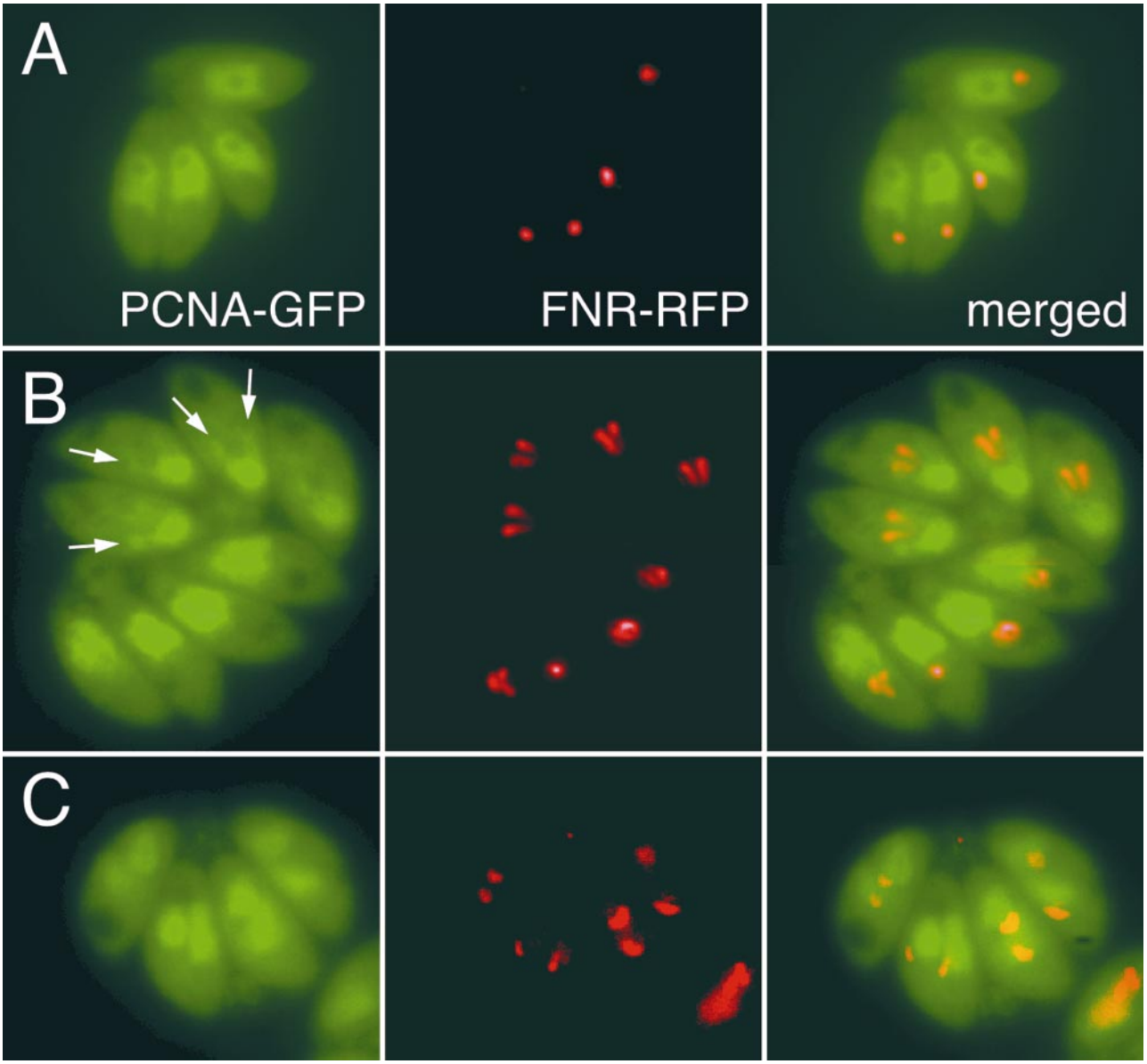

Figure 4. Apicoplast and nuclear division are coordinated. Parasites stably expressing PCNA-GFP (nucleus, green) were transiently transfected with FNR-RFP to permit plastid visualization (red), inoculated into HFF cells, and imaged 24 h after transfection. (A) Parasites with interphase nuclei (90% of all parasites) show a clear nucleolus, and a single ellipsoid plastid. (B) U-shaped plastids (still connected or already divided into two daughter plastids) are coincident with the onset of nuclear division. The nucleus has moved to the basal end of the parasite and two lobes that will establish the daughter nuclei (arrows) are visible. This process is driven by the intranuclear spindle (Sheffield and Melton, 1968). (C) After completion of nuclear division, but before emergence of daughter cells from the mother, each of the daughter nuclei is specifically associated with a single plastid (see Fig. 7 for an electron microscopic image during the same phase of endodyogeny). Note that the top and bottom halves of the FNR-RFP image in B have been adjusted for contrast individually to prevent signal saturation.

ments, the plastid was labeled using a RFP reporter (a native fluorescent molecule that is entirely distinct from GFP; Matz et al., 1999) fused to Ferredoxin/NADPH Reductase (FNR; Vollmer et al., 2000). The FNR cDNA sequence predicts a bipartite apicoplast-targeting domain (Roos et al., 1999b), and FNR-RFP shows identical labeling to ACP-GFP in double transfection experiments (not shown). A stable transgenic parasite line expressing PCNA-GFP was transiently transfected using plasmid *p*tubFNR-RFP/sagCAT. Living parasites were imaged using GFP and RFP filter sets, and the images were collected, colored, and merged.

As shown in Fig. 4 A, 90% of all parasites in the transfected population exhibit a single central nucleus with a well-defined nucleolus, as is typical for G1 and S-phase *T. gondii* tachyzoites (M.W. White, unpublished observation). These parasites invariably contain a single ellipsoid plastid just apical of the nucleus. Nuclear division in *T. gondii*, which takes place without breakdown of the nuclear envelope, is preceded by migration of the nucleus moves to the basal end (Fig. 4 B, bottom half). Next, the nucleus forms two lobes that extend towards the apical end (Fig. 4 B, top half). Ultimately (over the course of ~20 min), two fully separated daughter nuclei appear (Fig. 4 C;

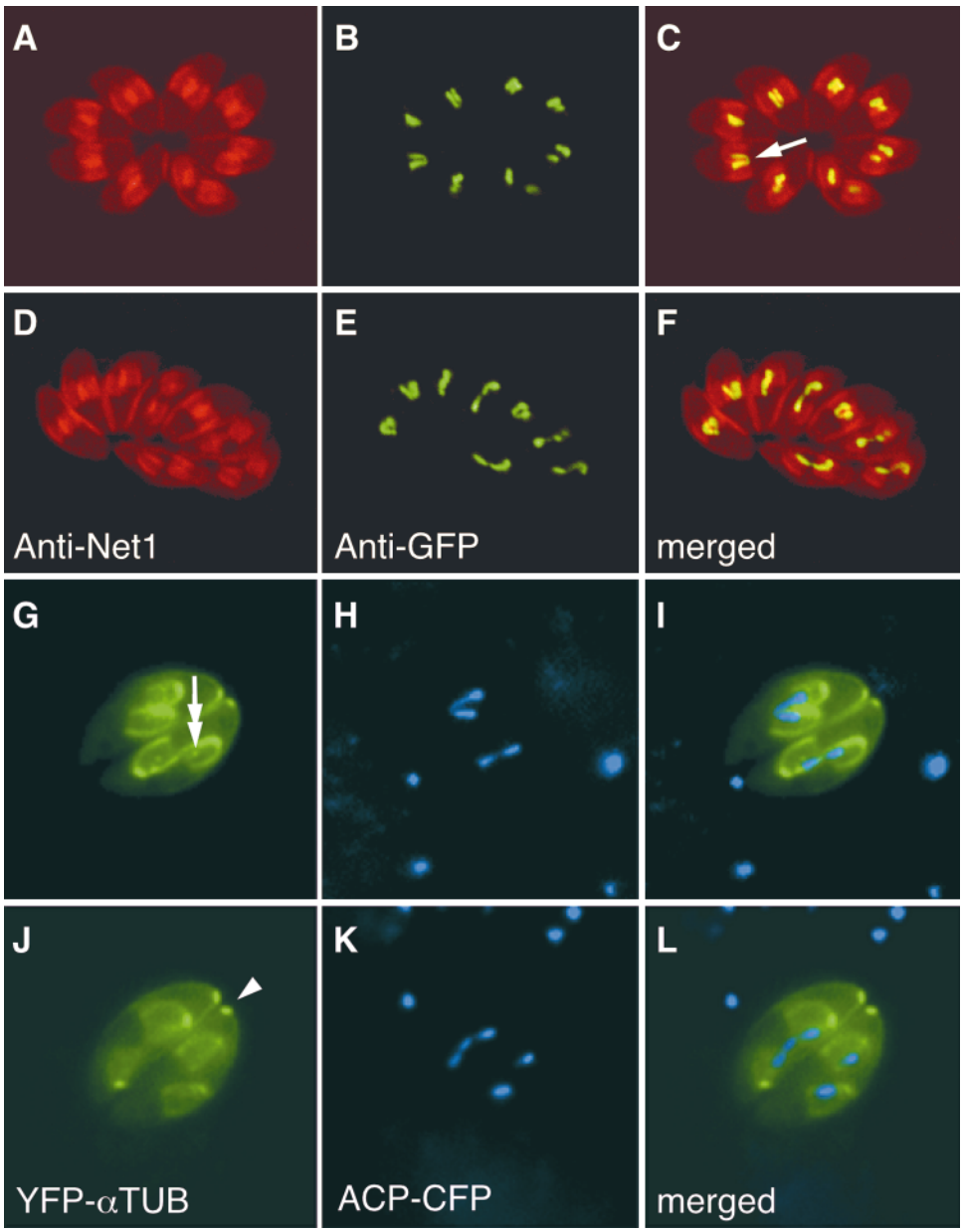

Figure 5. Insertion of the apicoplast into developing daughter parasites. HFF cells grown on coverslips were infected with stable transgenic parasite lines expressing either ACP-GFP (A–F, green) or ACP-CFP (G–L, cyan). The inner membrane complex of developing daughter parasites was visualized 24 h after infection either by staining of fixed and permeabilized parasites with monoclonal anti–NET1 and rhodamine-conjugated rabbit anti–mouse immunoglobulin (A–F, red), or in living parasites transiently transfected with *p*tubYFP–α-tubulin (G–L, green); see Materials and Methods for further details. The apicoplast is elongated to form a U- or dumbbell-shaped organelle in dividing parasites; bending or constriction of the organelle coincides with the basal ends of the growing inner membrane complex (compare arrow in C). The apical end of the plastid is closely associated with the inner face of the developing daughter pellicle, and in some images can be seen to terminate near the intranuclear concentration of tubulin thought to represent the spindle (double-headed arrow in G). The distance between the apical end of daughter parasites (marked by a concentration of tubulin attributable to the conoid and/or apical polar rings; arrowhead) and the apicoplast remains constant throughout growth of the daughter parasites.

movies and time-lapse images of this process are available at http://webs.cb.uga.edu/~striepen/pcnatl2min.html). Division of the apicoplast occurs in close synchrony with nuclear division in these double-labeled parasites: U- and dumbbell-shaped plastids form slightly before the appearance of lobulated nuclei, and daughter apicoplasts separate before division of the nucleus (Fig. 4 B). The apicoplast remains closely associated with the apical end of the nucleus throughout this process (Fig. 4, B and C).

Insertion of the Dividing Apicoplast into Developing Daughter Parasites and Association with the Inner Membrane Complex

As noted above, apicomplexan parasites replicate by assembling daughter parasites upon an inner membrane complex within the mother (Scholtyseck and Piekarski, 1965; Ogino and Yoneda, 1966; Sheffield and Melton, 1968). *T. gondii* tachyzoites divide by endodyogeny, in which two daughters are formed within a single mother. This is a simpler, but fundamentally identical, version of schizogony, in which multiple daughters are assembled simultaneously (e.g., in the formation of *Plasmodium* merozoites, *Eimeria* sporozoites, and feline intestinal epithelial forms of *Toxoplasma*; Frenkel et al., 1970; Müller, 1975; Roos et al., 1999a; Speer and Dubey, 1999; Waller et al., 2000). We have used several probes to study the relationship between apicoplast division and the developing daughter parasites.

T. gondii parasites expressing ACP-GFP were fixed, permeabilized, and incubated with anti–GFP antibody to label the plastid (Fig. 5, B and E, green) and a monoclonal antibody 45.15 specific for NET1 (also known as IMC1), a putative intermediate filament protein associated with the inner membrane complex (Fig. 5, A and D, red). The ends of dividing plastids are always inserted into the forming daughters and appear to be attached to one side of the structure (Fig. 5, C and F). These observations confirm that the characteristic folding of the apicoplast observed

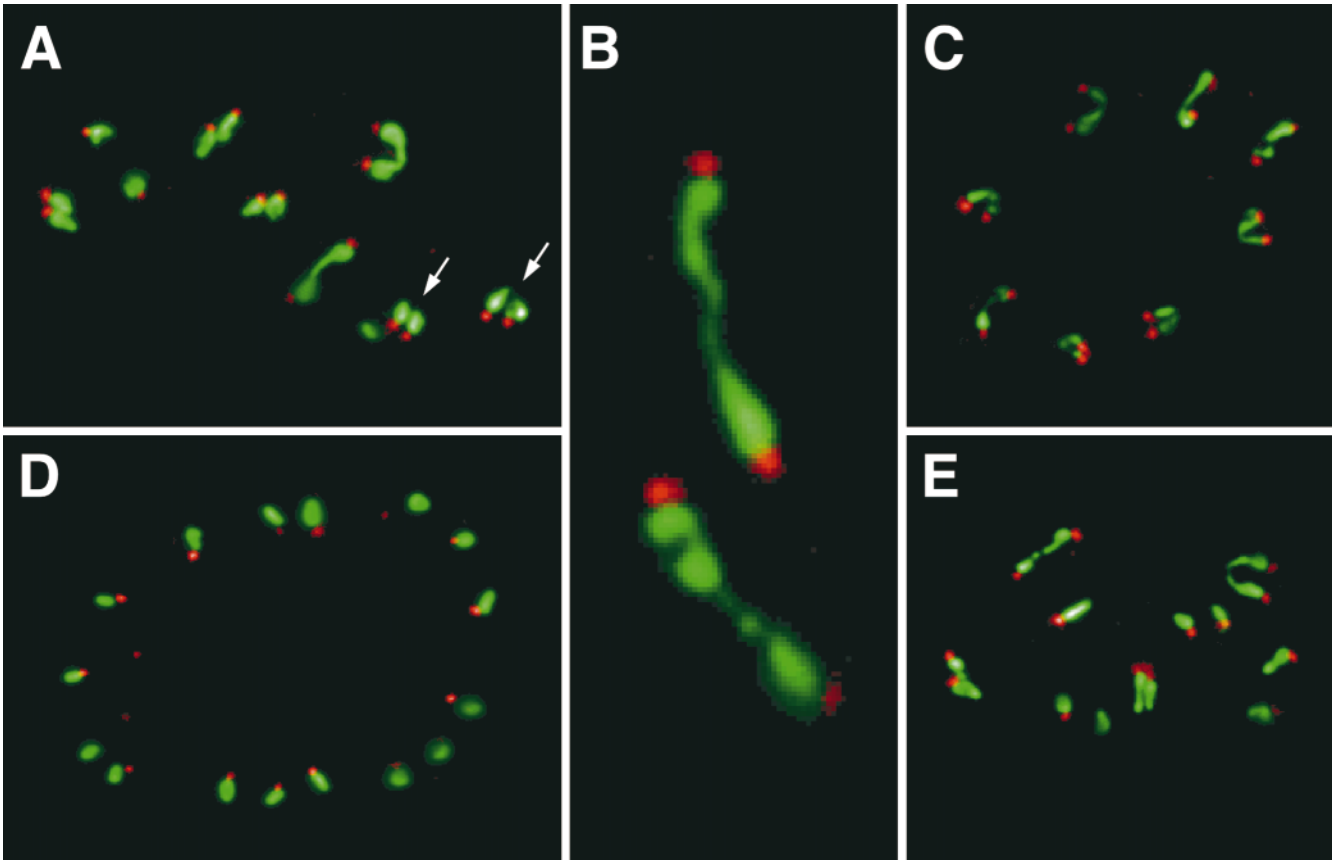

Figure 6. The apicoplast ends are associated with the centrosomes. Transgenic parasites expressing ACP-GFP were fixed, permeabilized, and stained with a monoclonal anti–GFP, followed by a suitable FITC conjugate (green, plastid), and anticentrin antibody 26-14 followed by a rhodamine-conjugated secondary antibody (red, centrosome). The ends of the plastid in dividing apicoplasts are consistently associated with the parasite's centrosomes. This association is maintained even after apicoplast division (arrows) and in nondividing (S-phase) parasites (D). Note: A–D each depict a single vacuole, containing 2, 8, or 16 parasites; E shows parasites in several independent parasitophorous vacuoles, accounting for the heterogeneity of apicoplast architecture. In rosettes such as that shown in D, parasites are oriented with their apical ends pointing outwards (compare Fig. 1).

during replication (Figs. 1 and 2) coincides with bending around the edge of the forming daughter pellicle.

Similar studies were carried out in living parasites by fusing fluorescent proteins to the *T. gondii* α- and β-tubulin genes (Nagel and Boothroyd, 1988), as microtubules form part of both the parasite's pellicle and the mitotic spindle (Nichols and Chiappino, 1987; Morrissette et al., 1997). Both NH_2- and COOH-terminal fusions of YFP with β-tubulin showed general cytoplasmic staining without incorporation into any specific structure, presumably due to steric limitations imposed by the incorporation of a bulky GFP tag into microtubules (data not shown). In contrast, YFP–α-tubulin labeled the subpellicular microtubules of both the mother and developing daughter parasites (Fig. 5, G and J). We also observed a spot of intense staining at the very apical end of the parasite (Fig. 5 J, arrowhead), probably attributable to either the conoid or the apical polar rings (Nichols and Chiappino, 1987; Morrissette et al., 1997). An additional focus of intense labeling in developing daughter parasites was observed in the nuclear region (Fig. 5 G, double arrow). Double labeling with centrosome- and microtubule-specific antibodies (not shown) reveal that this label represents ends of the intranuclear mitotic spindle.

For in vivo double labeling, a parasite line stably expressing ACP-CFP was transiently transfected with a *p*tubYFP–α-tubulin. Overlays of α-tubulin and plastid markers (Fig. 5, I and L) show that the ends of the (U- or dumbbell-shaped) dividing apicoplast are inserted into the developing daughter parasites, and once again indicate that the ends of the plastid are always in contact with the daughter's pellicle, immediately adjacent to the intranuclear spindle. Using the bright apical dot (conoid?) as a marker for the extreme apex of developing parasites, the distance between the point of plastid attachment and the apical complex is ~1 μm (SD ± 0.7 μm; $n = 100$). Time-lapse movies (not shown) confirm the spatial relationship between the apical complex, the intranuclear spindle, and the plastid attachment site on the inner membrane complex throughout endodyogeny.

Ends of the Dividing Apicoplast Are Linked to the Centrosome

To further explore the hypothesis that the ends of the dividing plastid are associated with the mitotic cytoskeleton, ACP-GFP transgenic parasites were stained with polyclonal anticentrin antiserum 26-14.1 (Shaw et al., 2000). Centrins are well-characterized calcium binding proteins found throughout the cytoplasm, but concentrated in the matrix surrounding centrioles (Salisbury, 1995). Parasites express-

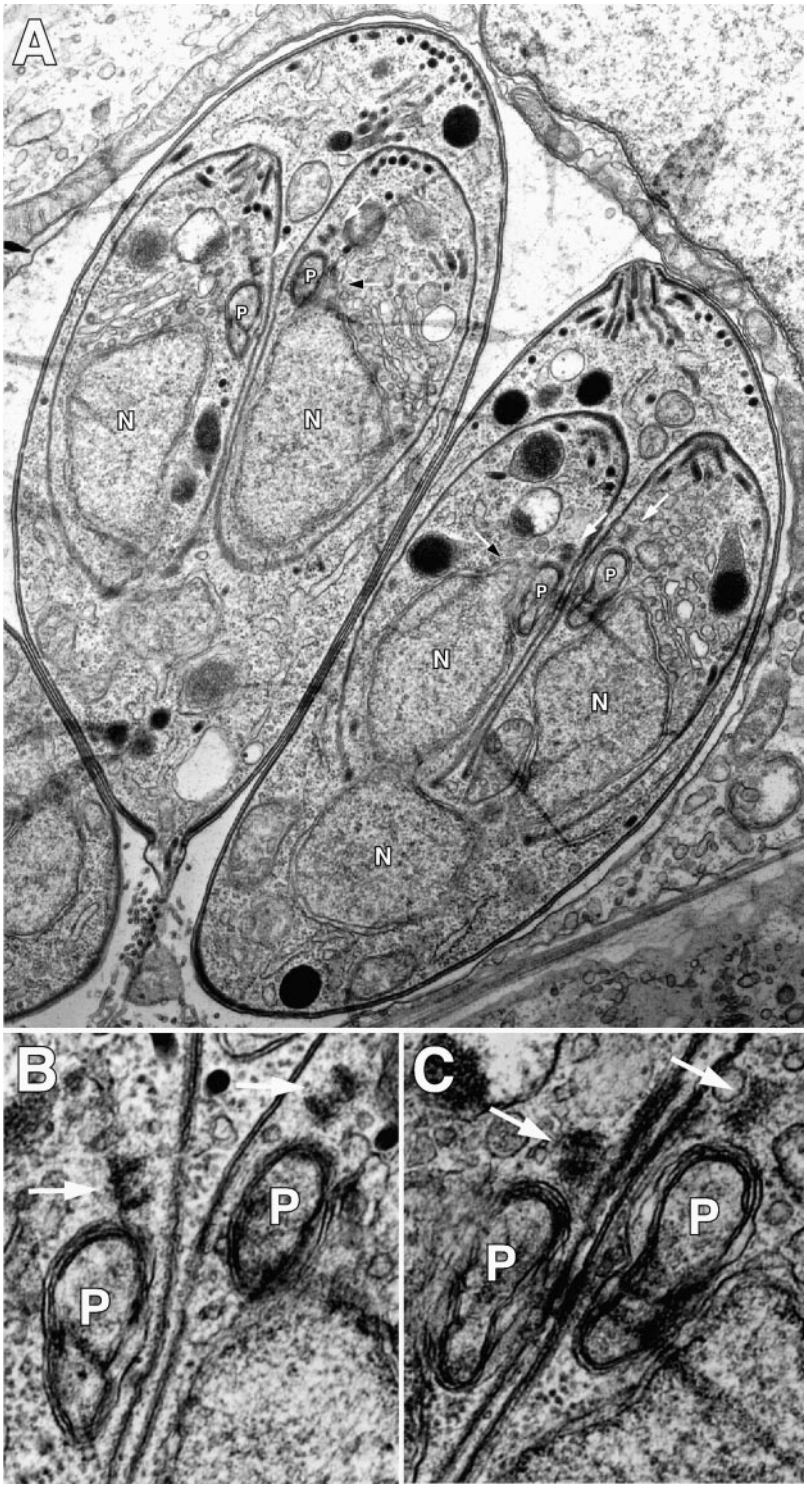

Figure 7. Organization of the centrioles, apicoplast, intranuclear spindle, and inner membrane complex during endodyogeny. Cultures infected with wild-type *T. gondii* tachyzoites (RH strain) were fixed with 1% glutaraldehyde and processed for transmission electron microscopy as described under Materials and Methods. (A) Two dividing parasites within a single parasitophorous vacuole are visible in this micrograph, each containing two developing daughters. The apicoplast (P) is identified by the presence of four delimiting membranes (shown at higher magnification in B and C. Note that the centrioles (white arrows) are located at the apical end of each plastid, confirming the antibody staining in Fig. 6. In two daughters, this section runs through the "centrocones" (Sheffield and Melton, 1968), where the intranuclear spindle exits the nucleus (A, black arrows). This structure is laterally adjacent to the apicoplast, as suggested by the α-tubulin–YFP labeling in Fig. 5 I. Intracellular location of the apicoplast in dividing parasites thus appears to be highly constrained: adjacent to both the inner membrane complex and the centrocone, and surmounted by the centrosome.

ing ACP-GFP were fixed, permeabilized and doubly labeled for GFP (Fig. 6, green) and centrin (red). The anticentrin antiserum always labeled just beyond the ends of the dividing apicoplast, whether dumbbell shaped (Fig. 6 B), U shaped (C), or even after plastid fission (A, arrows). Indeed, images of nondividing parasites (Fig. 6 D) suggest that a close connection between the (nondividing, ovoid) apicoplast and centrosome persists throughout the parasite cell cycle. Intracellular parasites within a single parasitophorous vacuole typically orient with their apical ends pointed outwards; the fact that the ring of green dots in such images lies outside the ring of red dots indicates a more apical localization of the apicoplast during G1 and S phases.

Electron microscopic observation confirms the apicoplast/spindle/centrosome/inner membrane complex association, as shown in Fig. 7. Apicoplasts (P) are readily identified by their multiple membranes (Fig. 7, B and C) in each of the four daughter parasites being assembled within these two *T. gondii* tachyzoites. In each case, the apicoplast is closely apposed to the inner membrane complex, and im-

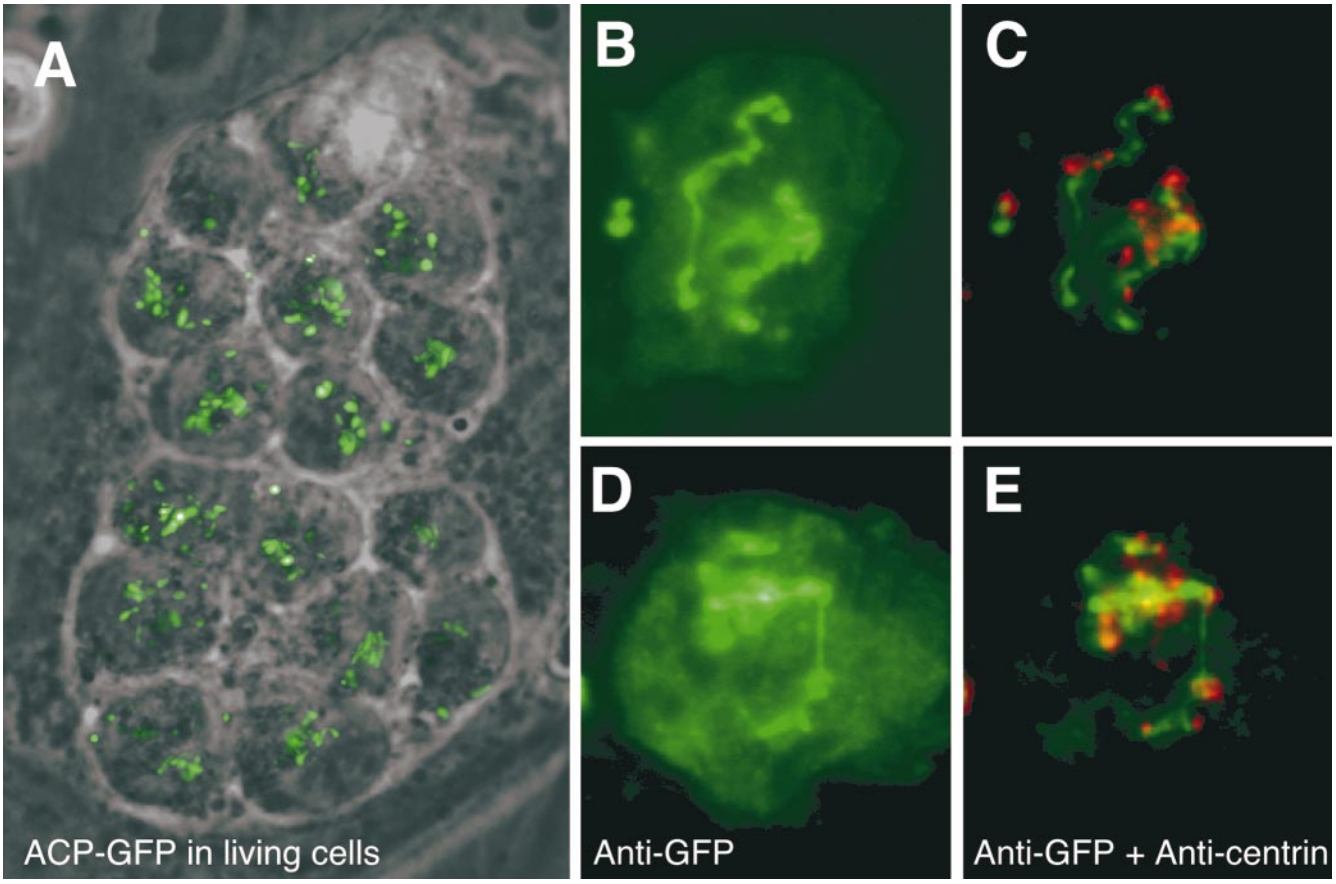

Figure 8. Dinitroaniline treatment blocks cytokinesis, producing parasites with multiple or reticulate plastids. ACP-GFP expressing *T. gondii* tachyzoites were treated with 1 μM oryzalin (A) or ethalfluralin (B–E). After 20 h, parasites were either imaged live (A) or after fixation, permeabilization, and staining for ACP-GFP (B and D, green) and centrin (C and E, red), as described in Fig. 6. Prolonged drug treatment transforms parasites into amorphous blobs as previously described (Stokkermans et al., 1996; Morrissette and Roos, 1998). Oryzalin treatment leads to the production of multiple plastids of normal size, despite the absence of fully completed nuclear division and cytokinesis (A, note that spindles are still formed under these conditions; Shaw et al., 2000). Ethalfluralin treatment (B–E) produces a large reticulate plastid morphology reminiscent of the apicoplast in dividing *Plasmodium* schizonts (Waller et al., 2000). Double labeling with anticentrin antibody showed that the plastids retain their association with centrioles.

mediately posterior to a centriole (white arrows). The intranuclear spindle microtubules are visible in two of the daughter parasites (black arrows), forming conical projections from the nucleus directly adjacent to the apicoplast.

Formation of Multiple Spindle Poles Affects Plastid Division

Microtubule organization in *T. gondii* is sensitive to dinitroaniline herbicides (Stokkermans et al., 1996), which specifically target α-tubulin (Anthony et al., 1999; D.S. Roos, unpublished observation). Treatment of parasites with these drugs does not completely abolish microtubule formation, but affects different microtubule subsets differentially (a remarkable observation given that *T. gondii* contains only a single gene each for α-, β- and γ-tubulin; Nagel and Boothroyd, 1988). Oryzalin treatment completely blocks assembly of subpellicular microtubules, preventing inner membrane complex development and hence daughter parasite assembly; ethalfluralin exhibits a similar but less dramatic effect (Stokkermans et al., 1996; Morrissette and Roos, 1998). The formation of intranuclear spindles seems not abolished by dinitroanilines, however (Shaw et al., 2000). After prolonged treatment, parasites resemble "artificial schizonts," with a swollen lobed nucleus incapable of completing division, and multiple centrosomes and spindles present (Morrissette and Roos, 1998; Shaw et al., 2000).

To test the effect of dinitroanilines on apicoplast division, cultures were inoculated with *T. gondii* tachyzoites stably expressing ACP-GFP, incubated 24 h (by which point most vacuoles contained four or eight parasites), treated with 1 μM oryzalin or ethalfluralin, and examined 20 h later. In the presence of dinitroanilines, parasites form large amorphous bodies, as shown in Fig. 8 A (compare with Fig. 1). Many parasites contain multiple apicoplasts (Fig. 6 A) or large reticulate plastids (Fig. 6, B and D), strikingly similar to the pattern observed in *P. falciparum* schizonts (Waller et al., 2000). Double labeling with anticentrin antibody reveals the presence of multiple centrosomes, closely associated with the reticulate apicoplast (Fig. 6, C and E).

Discussion

The apicomplexan plastid is a remarkable organelle, acquired by secondary endosymbiosis from an algal ancestor and maintained throughout the aeons separating *Plasmodium* from *Toxoplasma* with little genetic change. Al-

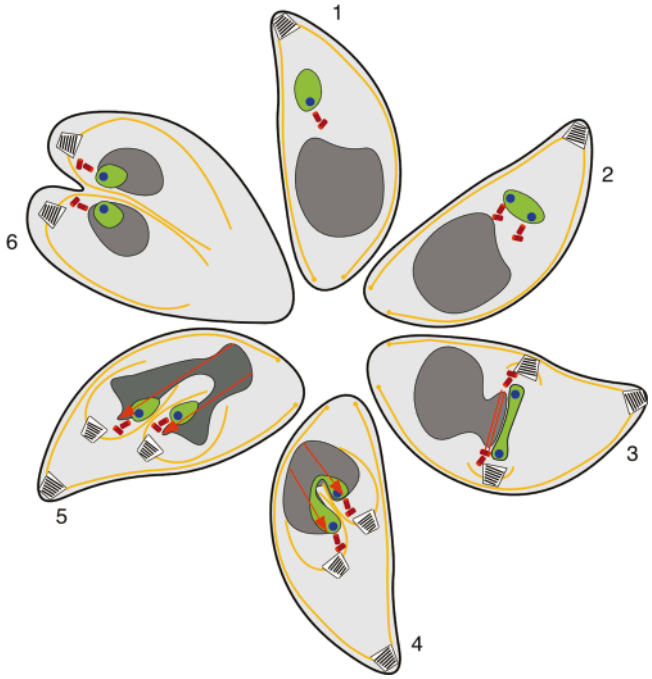

Figure 9. Model of plastid division in *Toxoplasma gondii.* (1) During interphase, the ellipsoid plastid is located apical to the nucleus, it contains multiple copies of its genome concentrated in a nucleoid, and it is associated with the centriole. (2) The plastid DNA is replicated and divided into two equal portions, associated with the recently divided centrioles at the ends of the organelle. (3) The intranuclear spindle forms and the dumbbell-shaped plastid migrates to lie on top of the nucleus. Note that the ends of the apicoplast are closely associated with the centrioles and the pellicle (inner-membrane complex) of the newly forming daughter. (4) As the daughter buds grow and the spindle extends, the plastid is pulled into a U shape, with its ends associated with the centrioles and the middle cut by the growing daughter pellicles. (5) The plastid divides in two, with each half remaining associated with one centriole and migrating into the budding daughter parasite together with the dividing nucleus. (6) After nuclear fission, the two daughter nuclei rebound towards the middle of the daughter parasites; each daughter is left with one centriole, one apicoplast, and one nucleus, which now appear to be less tightly associated with the pellicle. Endodyogeny is completed when the now fully assembled daughters bud out of the mother, picking up their plasma membrane en route. See text for further discussion.

though the apicoplast has lost all photosynthetic function, it is nevertheless an essential organelle (Fichera and Roos, 1997; McConkey et al., 1997). Parasites lacking plastid function die upon entry into the next host cell (Fichera et al., 1995; Fichera and Roos, 1997; C.Y. He, unpublished observation). Several groups are seeking to identify the critical metabolic function(s) provided by the apicoplast, and current evidence indicates a possible role in the synthesis of both fatty acids and isoprenoids (Waller et al., 1998; Jomaa et al., 1999; McFadden and Roos, 1999). Both *T. gondii* and *P. falciparum* harbor a single apicoplast (Fig. 1; Köhler et al., 1997; Waller et al., 2000). How is this essential organelle efficiently replicated and segregated during cell division?

Perhaps the best-studied example of plastid replication occurs in the mesophyll cells of *Arabidopsis thaliana.* Each cell contains numerous chloroplasts, which divide autonomously many times during the growth phase of a mesophyll cell (Pyke, 1999). Plastid replication is independent of mitosis in this system. In contrast, simultaneous visualization of plastid division, nuclear division, daughter cell formation, and cytokinesis (Figs. 2, 4, and 5) demonstrate that these processes in *T. gondii* are intimately coupled (Fig. 9 summarizes our current model of apicoplast replication).

Apicoplast division is not only linked to mitosis in *T. gondii*, but even appears to use the mitotic machinery. The ends of the dividing apicoplast are invariably associated with the centrosomes (Fig. 6). These findings are supported by electron microscopic evidence (Fig. 7; Ogino and Yoneda, 1966; van der Zypen and Piekarski, 1967; Sheffield and Melton, 1968; Speer and Dubey, 1999). Centrosome association could explain how precise plastid distribution is ensured even in large schizonts, where hundreds of progeny must be assembled at once. It is tempting to speculate that the entire daughter cell is built around the centriole.

Chloroplast division in plants and algae is thought to require a fission ring (Kuroiwa et al., 1998; Pyke, 1999) containing the tubulin homologue FtsZ known to be associated with bacterial cell division. The *A. thaliana* genome encodes at least two genes exhibiting strong similarity to FtsZ, and one of these genes carries a putative plastid leader sequence (Lutkenhaus and Addinall, 1997; Osteryoung et al., 1998). One model suggests that a combination of stromal and cytoplasmic FtsZ rings are required for chloroplast division (Kuroiwa et al., 1998; Osteryoung et al., 1998). An algal mitochondrial FtsZ has also been identified, suggesting a conserved mechanism of bacterial origin for plastid and mitochondrial division in algae (Beech et al., 2000). To date, however, we have not been able to identify a candidate FtsZ gene from the *P. falciparum* genome database (Gardner, 1999), and have not been able to amplify any FtsZ-related sequences from either *T. gondii* or *P. falciparum* DNA using degenerate PCR primers based on conserved FtsZ domains. Furthermore, targeting of the *A. thaliana* plastid-targeted FtsZ1-1 to the *T. gondii* apicoplast (as a GFP fusion) produced no distinctive suborganellar localization and failed to affect apicoplast division (M.J. Crawford and D.S. Roos, unpublished observations). As electron microscopic studies show no evidence for a fission ring in apicomplexan parasites, it is possible that these parasites are able to divide the apicoplast through a combination of force generated by the mitotic spindle and growth of the daughter pellicle.

In higher plants, plastid genome copy number may reach >100 per organelle, and this DNA is concentrated in multiple nucleoids (Kuroiwa et al., 1998). Current models favor partitioning via attachment to the plastid envelope, although the recently discovered complexity of bacterial nucleoid division suggests that this might be an oversimplified view (Kuroiwa et al., 1998; Jensen and Shapiro, 1999). We have shown that the apicoplast genome is organized in a single nucleoid body, and divides early during organellar replication (Figs. 3 and 9), via association with the ends of the organelle. It is not clear whether the nucleoid (located within the four membranes of the apicoplast) and the centrosome (outside this organelle) identify the ends of the

apicoplast independently, or if they are physically linked. Whether DNA segregation to the ends of the apicoplast is directly dependent on centrosome association is currently under investigation, as such an association would provide a simple explanation for the problem of precise DNA segregation, particularly in the large reticulate plastid of schizonts. Association of organellar DNA with the cellular division machinery is not without precedent: segregation of the mitochondrial genome in *Trypansoma brucei* has been shown to be associated with basal body movements (Robinson and Gull, 1991).

How has this mechanism of plastid division, very different from that found in plants, evolved? One attractive model speculates that it may be a consequence of the endosymbiotic origins of the apicoplast. One presumes that the proto-apicoplast was an alga that took up residence within the parasite's endosomal compartment. Because sorting endosomes are tightly associated with centrioles (Ren et al., 1998), the machinery that couples the sorting endosome to the centrosome could have been harnessed for apicoplast replication. Functional compartmentalization of the apicoplast within the endomembrane system is consistent with observations that proteins target to the apicoplast via the secretory pathway (Roos et al., 1999b; DeRocher et al., 2000; Waller et al., 2000).

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