Targeting and Processing of Nuclear-encoded Apicoplast Proteins in Plastid Segregation Mutants of Toxoplasma gondii*

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The apicoplast is a distinctive organelle associated with apicomplexan parasites, including Plasmodium sp. (which cause malaria) and Toxoplasma gondii (the causative agent of toxoplasmosis). This unusual structure (acquired by the engulfment of an ancestral alga and retention of the algal plastid) is essential for long-term parasite survival. Similar to other endosymbiotic organelles (mitochondria, chloroplasts), the apicoplast contains proteins that are encoded in the nucleus and post-translationally imported. Translocation across the four membranes surrounding the apicoplast is mediated by an N-terminal bipartite targeting sequence. Previous studies have described a recombinant “poison” that blocks plastid segregation during mitosis, producing parasites that lack an apicoplast and siblings containing a gigantic, nonsegregating plastid. To learn more about this remarkable phenomenon, we examined the localization and processing of the protein produced by this construct. Taking advantage of the ability to isolate apicoplast segregation mutants, we also demonstrated that processing of the transit peptide of nuclear-encoded apicoplast proteins requires plastid-associated activity.

Toxoplasma gondii is an obligate intracellular parasite, a major cause of congenital birth defects in humans and livestock (1, 2), and an important opportunistic infection associated with AIDS (3). This single-cell eukaryotic pathogen contains an unusual organelle that was acquired by horizontal transfer (secondary endosymbiosis) from a eukaryotic alga (4). The apicoplast has been identified in many apicomplexan parasites, including Toxoplasma, Plasmodium, Eimeria, Babesia, Theileria, etc. (5).

Previous studies have shown that the apicoplast is essential for long term parasite viability (6–8). When this organelle is eliminated by either pharmacological or molecular genetic manipulation, parasites are killed with distinctive “delayed death” kinetics. Plastid-deficient parasites are capable of normal growth within and escape from the first host cell, but their replication is inhibited immediately upon invasion into a new host cell. Although the mechanistic basis of the delayed death phenotype remains unexplained, these studies demonstrate that the apicoplast is an essential organelle and therefore a potential target for drug development (9).

The apicoplast possesses a 35-kilobase pair circular organelar genome containing rRNA and tRNA genes and 28 open reading frames (10). All identified genes in the apicoplast genome are predicted to encode housekeeping proteins (RNA polymerase, ribosomal proteins, etc.). However, most apicoplast proteins are thought to be encoded in the nuclear genome, as is well known for other endosymbiotic organelles (mitochondria, chloroplasts, etc.). These nuclear-encoded apicoplast proteins must be imported across the four membranes surrounding this organelle (9, 11). Apicomplexan parasite genome and EST sequence databases (12, 13) provide a useful resource for identifying nuclear-encoded genes destined for the apicoplast. Candidate nuclear-encoded apicoplast proteins include enzymes associated with the biosynthesis of fatty acids (14, 15) and terpenoids (16). Several of these proteins have been localized to the apicoplast by immunostaining and/or fusion with fluorescent protein reporters (14, 16–18). Pharmacological experiments using inhibitors of type II fatty acid biosynthesis (cerulenin, triclosan, and thiolactomycin), 1-deoxy-D-xylulose-5-phosphate reductoisomerase (fructoseomycins), or acetyl-CoA carboxylase (arylxylophenoxypropionates) also support the presence of these enzymes (14–16, 19, 20), although formal proof that these drugs target the apicoplast itself is lacking. To substantiate the association of predicted metabolic pathways with the apicoplast and to further characterize this organelle, we have attempted to purify the apicoplast using various cell fractionation methods.

Nuclear-encoded apicoplast proteins are characterized by a bipartite targeting sequence consisting of a signal peptide at the extreme N terminus followed by a plastid transit domain (11). Mutational studies (21, 22) indicate that this bipartite structure is both necessary and sufficient for targeting proteins into the apicoplast. Targeting appears to proceed via the secretory pathway with sequential removal of the signal peptide and the plastid transit peptide (11). Although removal of the secretory signal sequence (in the endoplasmic reticulum) is difficult to detect by Western blotting, the processing of the plastid-targeting domain is easily observed and presumed to occur within the plastid lumen (22).

We have exploited the ability to target heterologous reporter proteins into the apicoplast to develop fluorescent and enzymatic markers for organelar purification. One recombinant fusion protein was found to disrupt apicoplast segregation (8), yielding plastid-deficient and “super-apicoplast”-containing parasites by fluorescence-activated cell sorting. These mutants allow the examination of apicoplast protein synthesis, targeting, and processing in the presence or absence of the organelle.
Parasites and Host Cells—T. gondii tachyzoites were maintained by serial passage in human foreskin fibroblast cell monolayers cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with heat-inactivated fetal bovine serum at 37°C in a humidified CO2 incubator as described previously (23).

Molecular Methods—Several nuclear-encoded apicoplast proteins have previously been shown to contain a bipartite leader sequence consisting of a secretory signal sequence thought to mediate translocation across one membrane (into the endoplasmic reticulum) followed by a plastid transit domain thought to mediate translocation across the remaining three membranes surrounding the apicoplast (11, 21, 22). (Fig. 1). Two previously validated apicoplast targeting signals from nuclear-encoded apicoplast proteins were employed in this study; these signals were derived from the N-terminal domains of the acyl carrier protein (ACP) (14) or ferrodoxin NADP-reductase (FNR) (24).

Plasmids ACP-GFP, ACP-CAT, and FNR-GFP-mROP1 have been described previously (8, 14). Plasmid FNR-RFP (kindly provided by Dr. Boris Striepen) contains 150 amino acids from the N terminus of ferrodoxin NADP-reductase (FNR) fused to a red fluorescent protein marker (DsRed, CLONTECH). Plasmid ACP-CAT (kindly provided by Dr. Robert G. K. Donald) is a fusion between the ACP leader sequence (ACP) and the chloramphenicol acetyltransferase (CAT). 50 µg of plasmid DNA (Qiagen Maxi-preps) was transfected into 107 freshly lysed-tachyzoites by electroporation as described previously (23). In transient assays, protein expression was typically assayed at 24 h post-infection. Stable transformants were selected in the presence of 20 µM chloramphican.

Microscopy—GFP, RFP, and fluorescein isothiocyanate fluorescence was detected using a Zeiss Axiovert 35 inverted microscope equipped with a 100-W Hg-vapor lamp and fluorescein and rhodamine filter sets. Confocal microscopy was performed on a Zeiss Laser Scanning Microscope (LSM510). For immunofluorescence assays, parasites were fixed with 3.7% paraformaldehyde, permeabilized with 0.25% Triton X-100, and blocked with 1% bovine serum albumin in phosphate-buffered saline (pH 7.4) at room temperature. The expression of CAT was detected using a polyclonal rabbit antisera (1:1000) (5 Prime → 3 Prime, Inc., Boulder, CO) followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:160, Sigma).

Cell Fractionation—Several protocols were evaluated for apicoplast purification. The Stansted cell disrupter (25) was found to be the most effective, as discussed under “Results” (cf. Fig. 2). ACP-CAT parasites were harvested from five heavily infected 175-cm2 tissue culture flasks (~2 × 107 tachyzoites) by filtering through 3-µm pore size polycarbonate filters (Nucleopore) and centrifuged at 1500 × g for 15 min. Parasites were washed twice with homogenization buffer (250 mM sucrose, 5 mM Tris-HCl (pH 7.5), 1 mM EDTA), resuspended in 10 ml of the same buffer supplemented with protease inhibitors (0.1 µg/ml each of aprotinin, pepstatin A, and leupeptin and 0.5 µM phenylmethylsulfonyl fluoride) and DNase I (1 µg/ml), mechanically disrupted in the Stansted biological cell disrupter at ~2000 p.s.i., and centrifuged at 1500 × g at 4°C for 15 min to remove unbroken parasites. The supernatant containing various organelles, vesicles, and other subcellular structures was centrifuged at 30,000 × g at 4°C for 30 min onto a 2.2 M sucrose cushion (buffered with 5 mM triethanolamine-HCl and 1 mM EDTA, pH 7.6). The interface (a crude apicoplast fraction) was collected, mixed with 2.2 M sucrose to 0.6 ml, and loaded at the bottom of a sucrose gradient step gradient (0.3 M each of 1.6, 1.5, 1.4, 1.35, 1.3, 1.25, 1.2, and 1.0 M sucrose buffered with 5 mM triethanolamine-HCl and 1 mM EDTA, pH 7.6). Ultracentrifugation was carried out in a Beckman L8 55 Ultracentrifuge for 1 h at 112,000 × g using an SW55 rotor, and 0.2-ml fractions were collected from the top of the gradient.

Sucrose gradient fractions were assayed as follows. The total amount of protein in each fraction was measured by Bradford assay (Bio-Rad). As high sucrose concentrations may interfere with colorimetry, fractions containing 200,000 × g for 1 h in a Beckman TL-100 tabletop Ultracentrifuge to remove the sucrose before the assay. Protein gel electrophoresis was performed on a 12% SDS-polyacrylamide gel electrophoresis gel. For Western blotting, proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell) using standard protocol (26), and antigens were detected using the ECL system (Amersham Pharmacia Biotech). After exposure to x-ray film (Eastman Kodak Co.), densitometry was performed using the ImageQuant software (Molecular Dynamics). CAT assays were performed using 14C-labeled chloramphenicol and thin layer chromatography on Silica Gel G-coated plates (Fisher) essentially as described previously (23). Quantification was carried out using a Storm PhosphorImager (Molecular Dynamics) equipped with the ImageQuant software. Specific activity in each fraction was calculated as the amount of ACP-CAT, ROP7, or CAT activity (arbitrary units) divided by the total protein in each fraction. Enrichment ratios were defined as the specific activity in each fraction divided by the specific activity of the whole parasite control.

Membrane Extractions—For extraction in sodium carbonate (27), freshly prepared membranes were incubated with 0.2 M Na2CO3 (pH 11.5) on ice for 45 min followed by centrifugation at 100,000 × g at 4°C for 1 h to pellet membranes and insoluble structures. For extraction in Triton X-114 (28, 29), a freshly prepared 10% (v/v) stock solution of Triton X-114 (Sigma) in phosphate-buffered saline was added to membranes to a final concentration of 2%. Extraction was carried out on ice for 16 h following centrifugation at 15,000 g × 4°C for 10 min in a tabletop microcentrifuge or at 100,000 × g × 4°C for 1 h in a Beckman L8 55 Ultracentrifuge to remove Triton-insoluble material. To examine phase separation, the supernatant was incubated for 10 min at 37°C and centrifuged at 15,000 × g × 10 min at room temperature, and the resulting extracts were washed several times by repeating the above procedure. Proteins in the detergent phase were precipitated for 1 h in acetone at ~20°C and solubilized in SDS loading buffer for polyacrylamide gel electrophoresis in parallel with the aqueous phase samples.

Fluorescence-activated Cell Sorting (FACS)—FACS was performed on a Becton Dickinson FACS Vantage SE dual laser flow cytometer equipped with Coherent Innova 305 argon and Coherent Spectrum argon-krypton water-cooled lasers. GFP was excited using the primary argon laser tuned to 488 nm at 200 milliwatts, and GFP fluorescence was detected using a standard 560SP dichroic mirror along with a 530DF30 band-pass filter in FL1. RFP was excited using the secondary argon-krypton laser tuned to 568 nm at 150 milliwatts, and RFP fluorescence was detected using a 710DRLP dichroic mirror along with a 610DF30 band-pass filter in FL5. All filters were purchased from Omega Optical. Light scatter and fluorescence were collected using logarithmic amplification with forward scatter light as the threshold parameter. T. gondii tachyzoites were harvested as above and resuspended in culture medium to 5 × 106 parasites/ml. Extracellular parasites were sorted using standard high speed conditions on the FACS Vantage (70-µm nozzle tip, 45-p.s.i. sheath pressure). This resulted in a drop drive frequency of 71,000 drops/s, a drive level of 6.5 volts, and a drop breakoff of 33.4 drops. At these settings, parasites flow through the cell sorter at a rate of 12,000/s with abort rates typically less than 10%. Pulse Processing Plus was used to generate a forward scatter (pulse width) parameter for aggregate detection. Parasites were sorted into standard 12 × 75-mm polystyrene collection tubes containing 0.5 ml of phosphate-buffered saline (pH 7.4).

RESULTS

Targeting of Heterologous Proteins into the Apicoplast—We have previously shown that N-terminal fusion of the bipartite targeting sequences from various nuclear-encoded apicoplast proteins permits the targeting of GFP into the apicoplast in stable T. gondii transgenics (14) and that this marker can be exploited to follow organellar replication in living parasites (see Ref. 30 and Fig. 1A). To facilitate two-color fluorescence studies, a red fluorescent protein reporter was also targeted into the
Targeting and Processing of Apicoplast Proteins

Fig. 2. Density gradient fractionation of T. gondii organelles. Parasite tachyzoites stably expressing a transgenic ACP-L-CAT reporter were disrupted and separated on a 1.1–1.6 M sucrose density gradient as described under “Experimental Procedures.” Fractions were assayed for ACP-L-CAT (A), rhoptry protein ROP7 (B), and microneme protein MIC3 (not shown) by Western blotting and for enzymatic activity of CAT (C). D, apicoplast and rhoptry enrichment relative to total protein levels (determined by Bradford assay). Two major bands were observed for rhoptry enrichment relative to total protein level (determined by Bradford assay). Two major bands were observed for rhoptry enrichment relative to total protein level.

Apicoplasts, as shown in Fig. 1B. To provide an enzymatic marker for subcellular fractionation, the CAT enzyme was targeted to the apicoplast as well (Fig. 1C). All of these fusion proteins target specifically to the apicoplast, which is visible as a small dot in the apical juxtanuclear region of each tachyzoite (4). The four tachyzoites shown in each panel are the progeny of a single clonal parasite infection; T. gondii tachyzoites replicate synchronously within the intracellular parasitophorous vacuole until host cell lysis (producing 2, 4, 8, 16, etc. daughters/vacuole).

Subcellular Fractionation of the Apicoplast by Density Gradient Ultracentrifugation—Transgenic parasites stably expressing ACP-L-CAT were disrupted by various methods (Dounce homogenizer, French press, Stansted cell disruptor, or homogenization with silicon carbide in a mortar and pestle). Cell fractionation was then carried out through a series of differential and equilibrium density gradient centrifugation steps as described under “Experimental Procedures.” Fractions were assayed for protein concentration, CAT activity, and the abundance of particular proteins by Western blotting with specific antibodies.

The Stansted cell disruptor yielded the cleanest results for several subcellular organelles including the apicoplast. Results from a representative experiment are shown in Fig. 2. Although we consistently observed a high enrichment ratio for rhoptries at ~1.4/1.5 M sucrose (~38-fold in this experiment; see panels B and D) and micronemes (data not shown), the apicoplast was widely distributed across the gradient with no fraction exhibiting >6-fold enrichment (maximum at the 1.25/1.3 M sucrose interface; see panels A and C). Similar distribution of the apicoplast and rhoptries has been observed in many different experiments using sucrose or Percoll gradients. Electron microscopic analysis (not shown) suggests that the distinctive four-membrane architecture that characterizes the apicoplast (4, 9) is very fragile, making the isolation of intact organelles with a uniform density very difficult.

Targeting of a Marker Protein to Apicoplast Membranes—Frustrated by the apparent heterogeneity in apicoplast density, we attempted to develop an alternative strategy based on affinity purification. Such approaches have been successfully applied to chloroplast purification in plant systems (31). As no apicoplast surface antigen has been identified to date (and the outer membrane of the apicoplast may be virtually indistinguishable from the endoplasmic reticulum/Golgi apparatus (11, 32)), we engineered a series of constructs in which proteins containing a bipartite apicoplast-targeting signal were fused to a transmembrane anchor and cytoplasmic epitope tag (33). Unfortunately, however, none of these constructs exhibited efficient targeting to the apicoplast (data not shown).

Interestingly, one construct engineered in the course of these studies (ACP-GFP-mROP1) was found to target specifically to the apicoplast, alter its morphology, and block organelle segregation during parasite mitosis (8). To determine the localization of the ACP-GFP-mROP1 (a fusion of the ACP targeting signal to GFP and a fragment of the rhoptry protein ROP1 (34)), stable transgenic parasites expressing FNRL-RFP within the apicoplast were transiently transfected with ACP-GFP-mROP1, as shown in Fig. 3. In untransfected parasites, each tachyzoite in every parasitophorous vacuole exhibits a red apicoplast attributable to the FNRL-RFP reporter (Fig. 1B). Parasites expressing the ACP-GFP-mROP1 fusion, however, produce only one apicoplast per vacuole (Fig. 3A, compare with Fig. 1). This single apicoplast contains both GFP and RFP, whereas other parasites within the same vacuole exhibit only weak, punctate GFP fluorescence in the apical region and very little RFP fluorescence (despite the continued expression of FNRL-RFP from the stably integrated transgene; see below). Confocal microscopy demonstrates that the ACP-GFP-mROP1 labels only the periphery of the apicoplast, whereas FNRL-RFP labels the organellar lumen (Fig. 3B). RFP and GFP labeling
are mutually exclusive in these parasites, as shown by the quantitative fluorescence profile, suggesting the localization of these markers to different compartments within the same organelle. This observation is consistent with previous immunoelectron microscopy showing ACP-GFP-mROP1 staining near the periphery of the apicoplast (8).

Integral membrane proteins and proteins that are tightly associated with membranes (including glycosylphosphatidylinositol-anchored proteins such as the major T. gondii surface antigen P30) remain associated with membrane fractions after extraction with either carbonate or Triton X-114 (27, 28). To determine whether the ACP-GFP-mROP1 fusion protein is associated with the complex four-membrane structure surrounding the apicoplast, we extracted crude apicoplast fractions with either Na₂CO₃ or Triton X-114. To test the hypothesis that the processing of nuclear-encoded apicoplast proteins occurs within the apicoplast itself (as is the case in chloroplasts (36, 37)), we exploited the apicoplast segregation defect induced by ACP-GFP-mROP1 to isolate plastid-deficient parasites. We developed a two-color strategy to improve on the single-color FACS procedure described previously (8), ensuring high purity FACS sorting.

Transgenic parasites expressing a stable FNR₆-RFP reporter were transiently transfected with the ACP-GFP-mROP1 construct (cf. Fig. 3A). Extracellular parasites were then harvested and analyzed by two-color FACS, as shown in Fig. 5C. Approximately 62% of the parasites in this experiment exhibited RFP fluorescence comparable with the parental FNR₆-RFP line (Fig. 5B) but no GFP fluorescence (upper left quadrant in Fig. 5C). These parasites failed to take up or express the ACP-GFP-mROP1 poison construct. Approximately 4% of the sample exhibited both GFP and RFP fluorescence (upper right). These “superbright” parasites were successfully transfected with the poison construct and retained the apicoplast during parasite division (cf. Fig. 3A), accumulating both ACP-GFP-
mROP1 and FNRL-RFP in the large, nonsegregating organelle (8). Approximately 34% of parasites exhibited RFP expression levels below background levels (lower half of Fig. 5C; compare with wild-type parasites shown in Fig. 5A). These are parasites that failed to retain an apicoplast during mitosis (black parasites in Fig. 3A).

The following peaks were sorted for further analysis. Population R₁ consists of untransfected FNRL-RFP transgenics (red, not green); population R₂ consists of apicoplast segregation mutants containing a super-apicoplast (red and green); and population R₃ consists of apicoplast segregation mutants that lost the apicoplast. Because a significant number of parasites in the stable FNRL-RFP line exhibit weak fluorescence (~18% of the parasites in Fig. 5B fall within the lower left quadrant), apicoplast-deficient parasites were isolated based on both positive staining for GFP and negative staining for RFP (green, not red). The purity of these populations was assessed by inoculation into fresh host cell monolayers, as shown in Table I. Plastid-deficient parasites could be isolated with a purity of >98%; "superplastid"-containing parasites were isolated with a purity of ~77% (although this may underestimate the true value because of the reversion to wild type; see below). Gating conditions used to isolate populations R₂ and R₃ were established to maximize purity without particular concern for yield; each of these subpopulations consisted of ~1% of the total population, permitting the isolation of ~5 × 10⁶ parasite tachyzoites (~50 µg of wet weight) in a 1-h sort on a FACS Vantage instrument.

Processing of Nuclear-encoded Apicoplast Proteins Requires Presence of the Apicoplast Organelle—Proteins isolated from FACS-sorted parasites containing or lacking the apicoplast (Fig. 5 and Table I) were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting to examine the processing pattern of the nuclear-encoded apicoplast markers FNRL-RFP and ACP-GFP-mROP1 (Fig. 6). Two bands of ~42 and ~31 kDa were labeled by anti-RFP antibodies in FNRL-RFP transgenics (lane 2), corresponding to the unprocessed and mature forms of this protein, respectively. (Note that the larger size difference between this doublet and the bands detected in Fig. 4 is attributable to the larger size of the FNR leader domain: ~11 versus 5 kDa for ACP.) As expected, these same two bands were observed in population R₁ (lane 3). The super-apicoplast-containing parasites (R₃) exhibit both the processed and unprocessed forms of ACP-GFP-mROP1 (labeled by anti-GFP antibodies), in addition to both forms of FNRL-RFP (lane 4). As noted above, this is consistent with the microscopical observation of both GFP and RFP in these giant apicoplasts.

### Table I

| Population | Apicoplast type | Purity % |
|------------|----------------|----------|
| R₁         | Wild type      | 0        | 0        | 100      |
| R₂         | Giant          | 34       | 154      | 32       | 77       |
| R₃         | None           | 3        | 197      | 98.5     |

*From Fig. 5.*

(Fig. 3B). In contrast, whereas parasites lacking an apicoplast (R₃ in Fig. 5 and Table I) express near normal levels of the nuclear-encoded apicoplast proteins FNRL-RFP and ACP-GFP-mROP1 (note that four to five times more parasites were loaded in lanes 1–2 than in lanes 3–5), no processing was observed, indicating that the processing of nuclear-encoded apicoplast proteins probably occurs within the apicoplast itself.

### Apicoplast Segregation Resumes As Transient ACP-GFP-mROP1 Expression Declines—The disruption of apicoplast segregation leads to plastid-deficient and super-apicoplast-containing parasites as described above. Although the transient expression of recombinant transgenes is lost over the course of 2–3 days in culture (38), plastid-deficient parasites are unable to recover, presumably because there is no way to regain an apicoplast once this organelle (and its genome) have been lost. Long term culture of super-apicoplast-containing parasites eventually leads to the outgrowth of apparently wild-type parasites, however. Microscopic observation suggests that these represent revertants, although it is also possible that a few individual wild-type parasites were inadvertently isolated along with the mutants. Prolonged cultivation of host cells infected with super-apicoplast-containing parasites revealed vacuoles containing more than one apicoplast (Fig. 7). These vacuoles typically contain one parasite with a single giant apicoplast and one or more parasites harboring a smaller, normal-sized apicoplast. Although it appears to be impossible for a giant, malformed apicoplast to resolve to normal morphol-
The discovery of the apicoplast, a plastid organelle that is essential for apicomplexan parasite viability, offers a potential target for chemotherapy provided that we can identify the biological function(s) of this organelle that make it essential for parasite survival (9). Unfortunately, attempts to purify the apicoplast by density gradient fractionation have failed to yield >6-fold enrichment despite the excellent purification of other subcellular organelles in the same experiments (Fig. 2). The apparent heterogeneity in apicoplast density may be attributable to several factors. Electron microscopic observations indicate that the apicoplast is surrounded by four delimiting membranes (4, 9), supporting the proposed secondary endosymbiotic origin of the organelle (4). It is possible that the procedures required to rupture the complex parasite pellicle (39) disrupt a variable number of plastid membranes, leaving behind free apicoplast remnants of diverse density. The problem of apicoplast fragmentation may be further exacerbated by the complex morphology exhibited by this organelle during its progress through the cell cycle (22, 30). The apicoplast is closely associated with other cell components, particularly the cytoskeletal mitotic apparatus (30), and these associations may inhibit the purification of the apicoplast as a distinct organelle.

Attempts to target a reporter to the apicoplast surface for affinity purification resulted in the development of a fusion construct expressing the bipartite apicoplast targeting domain of ACP, fused to GFP and a fragment of rhoptry protein ROP1. As described previously, the protein product of ACP-GFP-mROP1 targets specifically to the apicoplast, disrupts its morphology, and inhibits apicoplast segregation during parasite division (8). This fusion protein, which lacks a transmembrane domain, has nevertheless been shown to associate with the apicoplast membranes by high-resolution confocal microscopy (Fig. 3), immunoelectron microscopy (8), and carbonate extraction (Fig. 4). Membrane association may be mediated by the mROP1 domain because this protein in its native form associates with the parasitophorous vacuole membrane after secretion from the rhoptries (40). Neither ROP1 nor ACP-GFP-mROP1 contains an obvious transmembrane domain, which is consistent with the insolubility of these proteins in Triton X-114 (Fig. 4). Like ROP1 (41), ACP-GFP-mROP1 exhibits aberrant mobility at ~97 kDa on SDS-polyacrylamide gel electrophoresis gels (Figs. 4 and 6; the predicted molecular size of ACP-GFP-mROP1 is ~68 kDa). The precise mechanism of ACP-GFP-mROP1 association with membranes remains unresolved, as is the case for ROP1 itself (41). ROP1 has been reported to exhibit a low level similarity to rat salivary gland proteins known to form protein complexes, and therefore ACP-GFP-mROP1 may form Triton- and carbonate-insoluble protein aggregates that are trapped within the internal membranes of the apicoplast. Preliminary studies on Triton-X-114-extracted parasites suggest that ACP-GFP-mROP1 forms a high molecular weigh aggregate that pellets rapidly during ultracentrifugation (data not shown).

How the ACP-GFP-mROP1 poison construct mediates the surprising apicoplast segregation defect is likewise unknown. Constructs in which the ROP1 domain of ACP-GFP-mROP1 is replaced with a conventional single-span α-helical transmembrane domain derived from human CD46 (42, 43) or with a glycosylphosphatidylinositol anchor derived from the major parasite surface antigen P30 (44) fail to efficiently target the apicoplast and do not produce the plastid mis-segregation phenotype (data not shown). Regardless of the mechanism responsible for this phenotype, however, the ability to generate plastid-deficient and superplastid-containing parasites (8) and to efficiently isolate these mutants (Fig. 5 and Table I) provides valuable reagents for studies on apicoplast function.

Despite the dramatic changes in apicoplast morphology and inhibition of organelle segregation observed in super-apicoplast-containing parasites, apicoplast DNA replication (8) and protein import/processing (Fig. 6, lane 4) appear to proceed normally. These parasites are also capable of essentially normal replication and cell-cell spread through multiple infectious cycles (8). Presumably, the growth of apicoplasts of ever larger size would ultimately kill even those parasites that retain the organelle, which probably accounts for the inability to isolate even very slow-growing transgenic parasites that stably ex-
press ACP-GFP-mROP1. The prolonged replication of parasites harboring a super-apicoplast ultimately results in the outgrowth of normal parasites, however. The simplest explanation for this observation is that giant, malformed apicoplasts occasionally fragment during cell division (Fig. 7), producing small, effectively wild-type apicoplast organelles containing at least one copy of the apicoplast genome. As the expression of the ACP-GFP-mROP1 poison construct declines, apicoplast fragments are able to segregate normally during cell division. These observations confirm that ACP-GFP-mROP1 expression causes only a transient block in apicoplast segregation without imposing other detrimental effects on the biological functions of this organelle.

Apicoplast-deficient parasites provide the opportunity to explore the targeting of nuclear-encoded plastid proteins in T. gondii. Previous studies have shown that the extreme N terminus of these proteins is necessary for apicoplast targeting and that on its own, this terminus can function as a secretory signal sequence (21, 22). Neither FNR1-RFP nor ACP-GFP-mROP1 was secreted in apicoplast-deficient parasites nor were these reporters retained within the endoplasmic reticulum (45). GFP fluorescence was detected in vesicles in the apical region of parasites lacking an apicoplast, however, suggesting that trafficking to the apicoplast may involve vesicular transport (Fig. 6, lane 5). The inability to observe red fluorescence may be attributable to the pH sensitivity or slow maturation rate of RFP relative to GFP and its derivatives (46).

Apicoplast-deficient parasites also provide the opportunity to explore the processing of nuclear-encoded plastid proteins. Transgenic reporters engineered to contain an N-terminal apicoplast targeting domain are processed as effectively as native proteins, despite nearly normal levels of steady state protein (45). GFP fluorescence was detected in vesicles in the apical region of parasites lacking an apicoplast despite nearly normal levels of steady state protein (Fig. 6, lane 5). The inability to observe red fluorescence may be attributable to the pH sensitivity or slow maturation rate of RFP relative to GFP and its derivatives (46).

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