Toxoplasma gondii Tic20 is essential for apicoplast protein import

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Apicomplexan parasites harbor a secondary plastid that has lost the ability to photosynthesize yet is essential for the parasite to multiply and cause disease. Bioinformatic analyses predict that 5–10% of all proteins encoded in the parasite genome function within this organelle. However, the mechanisms and molecules that mediate import of such large numbers of cargo proteins across the four membranes surrounding the plastid remain elusive. In this work, we identify a highly diverged member of the Tic20 protein family in Apicomplexa. We demonstrate that Tic20 of *Toxoplasma gondii* is an integral protein of the innermost plastid membrane. We engineer a conditional null-mutant and show that *Tg*Tic20 is essential for parasite growth. To characterize this mutant functionally, we develop several independent biochemical import assays to reveal that loss of *Tg*Tic20 leads to severe impairment of apicoplast protein import followed by organelle loss and parasite death. *Tg*Tic20 is the first experimentally validated protein import factor identified in apicoplasts. Our studies provide experimental evidence for a common evolutionary origin of import mechanisms across the innermost membranes of primary and secondary plastids.

Apicomplexa | plastid | chloroplast

Organelle acquisitions through endosymbiotic events have been major drivers of eukaryotic evolution. The incorporation of a cyanobacterium into a heterotrophic eukaryote led to the formation of plastids (e.g., chloroplasts), enabling eukaryotes to become autotrophic. It is thought that a single, so-called "primary," endosymbiotic event led to the acquisition of chloroplasts in a lineage that later evolved into eukaryotic phyla such as red algae, green algae, and plants (1). An alternative means by which eukaryotes have obtained plastids is through a process of "secondary" endosymbiosis. Here, a eukaryote containing a primary plastid is incorporated into a heterotrophic eukaryote. Secondarily derived plastids are found in numerous lineages of ecologically, economically, and medically important organisms, including diatoms, dinoflagellates, and Apicomplexa. Apicomplexa are a phylum of obligate intracellular parasites that include *Plasmodium* species, the causative agents of malaria, and *Toxoplasma gondii*, which causes severe encephalitis upon congenital infection and in immunocompromised patients. The plastids of apicomplexans are known as apicoplasts and are thought to function in several essential metabolic pathways such as fatty acid, heme, and isoprenoid biosynthesis (2, 3). Because of the phylogenetic and biochemical divergence of these pathways from their host counterparts, they are heavily pursued as potential targets for antiparasitic drugs.

A key step in the conversion of an endosymbiont into a full-fledged plastid is the transfer of endosymbiont genes to the nucleus of the host. This action affords the host cell control over its endosymbiont but requires the evolution of molecular machinery to enable the import of proteins encoded in the nuclear genome back into the organelle to carry out their role. This targeting process has been well studied in plants, where multisubunit protein complexes mediate protein translocation across both membranes that bound the organelle (4). However, protein translocation into secondary plastids is a fundamentally different process. Secondary plastids are surrounded by three or four membranes and reside within the endomembrane system, with plastid-targeted proteins requiring an N-terminal hydrophobic signal peptide to mediate the first step of protein import (5). Although the protein motifs required to direct proteins into secondary plastids are reasonably well characterized (6), little is known about the molecular mechanisms that mediate import. A fascinating evolutionary question is whether protein import into secondary plastids required the evolution of novel machinery or whether organisms were able to retool existing mechanisms to accommodate novel means of protein targeting. Four membranes surround the apicoplast of *Toxoplasma gondii*, and in this work we demonstrate that protein import across the innermost membrane of this secondary plastid requires machinery derived from primary plastids.

Results and Discussion

Tic20 Homolog in Apicomplexa. We performed iterative BLAST searches to identify homologs of the plant inner chloroplast membrane translocase component Tic20 in apicomplexan parasites. We identified Tic20 homologs from all of the available genomes of apicomplexans, with the noted exception of the plastidless genus *Cryptosporidium*. Alignments of apicomplexan Tic20 homologs with plant, red algal, diatom, and cyanobacterial counterparts [supporting information (SI) Fig. S1] reveal the presence of an N-terminal extension with characteristics of a bipartite apicoplast targeting sequence. Similarity to plant and algal Tic20 homologs resides in the C-terminal portion of the protein, although very few residues are conserved between all homologs depicted (Fig. S1).

***Tg*Tic20 Is an Integral Protein of the Inner Apicoplast Membrane.** Cloning of the full ORF of *Tg*Tic20 revealed the presence of three introns and a predicted protein size of 43.4 kDa. We generated a transgenic parasite line expressing *Tg*Tic20 fused to a C-terminal HA tag and monitored its localization by immunofluorescence assay (Fig. 1*A*). *Tg*Tic20-HA (green) localized to a small, apical organelle that overlapped with acyl carrier protein (ACP; red), a marker for the apicoplast stroma. A Western blot of cells expressing the *Tg*Tic20-HA transgene revealed a major protein species of ≈23 kDa and a less abundant species of ≈40 kDa (Fig. 1*B*). This finding suggests that the N-terminal portion of the protein is cleaved to yield the mature protein of ≈20 kDa, consistent with the N terminus of the protein functioning as an apicoplast-targeting domain that is processed upon import into the apicoplast (7, 8). *In silico* modeling of the protein structure of *Tg*Tic20 suggests the presence of four transmembrane domains in *Tg*Tic20, found in close

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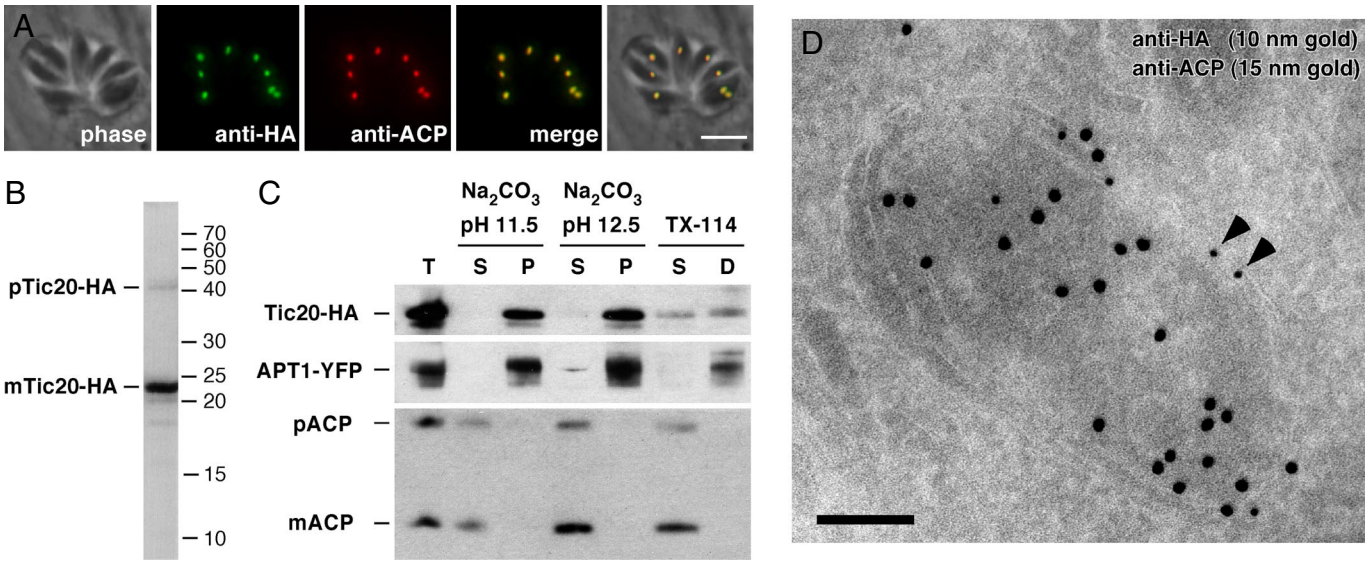

Fig. 1. *Tg*Tic20 is an apicoplast integral membrane protein. (*A*) Immuofluorescence assay depicting an eight-cell *T. gondii* vacuole. *Tg*Tic20-HA (green) colocalizes with the apicoplast marker ACP (red). (Scale bar: 5 μm.) (*B*) Western blot of protein extracts from the *Tg*Tic20-HA line with anti-HA antibodies. A mature *Tg*Tic20 protein species is labeled at ≈23 kDa, whereas a weaker precursor band is labeled at ≈40 kDa. (*C*) Proteins were extracted from the *Tg*Tic20-HA/APT1-YFP line and fractionated into soluble (S) and membrane pellet (P) fractions by sodium carbonate treatment, or into soluble (S) and detergent (D) phases by Triton X-114 phase partitioning. Total protein extracts (T) are shown in the first lane. (*D*) Transmission electron micrograph of the *Tg*Tic20-HA cell line, where *Tg*Tic20-HA is labeled with 10-nm gold beads and ACP with 15-nm gold beads. Arrowheads show *Tg*Tic20-HA labeling at the membranes of the organelle. (Scale bar: 100 nm.)

succession at the C terminus of the protein (Fig. S1). To determine whether *Tg*Tic20 is an integral membrane protein, we performed sodium carbonate extractions and Triton X-114 phase partitioning. Sodium carbonate extractions resulted in *Tg*Tic20 localizing to the membrane fraction, much like the characterized apicoplast phosphate transporter [APT1 (9)] and unlike the soluble ACP (Fig. 1*C*). Much, but not all, *Tg*Tic20 localized to the detergent (i.e., membrane) phase during Triton X-114 phase partitioning, again consistent with a membrane localization for *Tg*Tic20.

To characterize further the subcellular localization of *Tg*Tic20, we performed transmission immunoelectron microscopy on parasites expressing *Tg*Tic20-HA, labeling with anti-HA and anti-ACP antibodies. This action revealed the localization of *Tg*Tic20-HA to membrane-bound organelles that also contained ACP (Fig. 1*D*), consistent with the apicoplast localization of this protein. Localization of *Tg*Tic20-HA within the apicoplast was generally confined to the membranes of the organelle, consistent with the membrane localization of *Tg*Tic20, whereas ACP was distributed throughout the entire organelle.

Four membranes surround the apicoplast, and until now it has been difficult to determine to which membrane a given apicoplast membrane protein localizes. To determine the membrane to which *Tg*Tic20 localizes, we made use of an established self-assembling split pea green fluorescent protein (GFP). In this system, the C-terminal β-strand of GFP (GFP-11) was removed from the remaining 10 β-strands (GFP 1–10) of the molecule. This GFP-11 was engineered with the ability to self-assemble with GFP 1–10 if both molecules localize to the same compartment (10). As a proof of principle, we first targeted GFP 1–10 to the apicoplast stroma by adding the N-terminal apicoplast-targeting domain of ferredoxin-$NADP^+$ reductase (FNR). By itself, this protein was unable to fluoresce (data not shown). We next fused GFP-11 to the C terminus of ACP and transfected this into the FNR-GFP 1–10 line. The resultant parasites revealed GFP fluorescence in the apicoplast (Fig. 2*A*), which indicated that when both components of the split GFP are targeted to the apicoplast stroma, we observe apicoplast fluorescence. It is possible that the two GFP domains interact in the secretory pathway before entry into the apicoplast, thus limiting the predictive value of the assay. To test this, we generated constructs where we fused both GFP 1–10 and GFP-11 to the C terminus of P30, a established secretory marker protein for *T. gondii* (11). Expressed together, secreted GFP 1–10 and GFP-11 result in fluorescence in the parasitophorous vacuole that surrounds *T. gondii* parasites (Fig. 2*C*), indicating that the split GFP domains are capable of interacting when targeted to the same compartment in the secretory system. However, when we express the P30-GFP-11 construct in the FNR-GFP 1–10 line, we see no fluorescence (data not shown), suggesting that any interaction of the split GFP components in the secretory pathway does not result in mistargeting of the component proteins.

Having established the split GFP assay for determining whether proteins are localized to the apicoplast stroma, we next fused GFP-11 to the C terminus of *Tg*Tic20 and transfected this into the FNR-GFP 1–10 cell line. The resultant line revealed fluorescence that colocalized with an apicoplast red fluorescent protein (RFP) marker (Fig. 2*B*), consistent with the C terminus of *Tg*Tic20 residing in the apicoplast stroma. Flow cytometric analyses of the various cell lines described above supported the results obtained by microscopic analysis (Fig. S2*A*). One concern was that the observed apicoplast fluorescence might result from retention of FNR-GFP 1–10 in an outer membrane. To control for this possibility, we monitored cleavage of the apicoplast-targeting leader of FNR-GFP 1–10, a measure for whether proteins are accessible to the stromal processing peptidase enzyme that likely resides in the apicoplast stroma (8). We found no difference in processing of FNR-GFP 1–10 whether expressed by itself or with interacting components (Fig. S2*B*), indicating that most FNR-GFP 1–10 protein resides in the stroma and consequently that *Tg*Tic20-GFP-11 does not prevent targeting of FNR-GFP 1–10 to the stroma.

We conclude that *Tg*Tic20 is an integral protein of the inner apicoplast membrane, with its C terminus residing in the apicoplast stroma. Assuming that the predictions of four transmembrane domains are correct, the N terminus would also be in the stroma, resulting in the model for *Tg*Tic20 topology presented in Fig. 2*D*. Recently, candidate proteins that likely localize to outer membranes of the apicoplast have been identified (9, 12), and the split GFP assay may help to pinpoint the residence of these and other proteins to a specific membrane or apicoplast compartment.

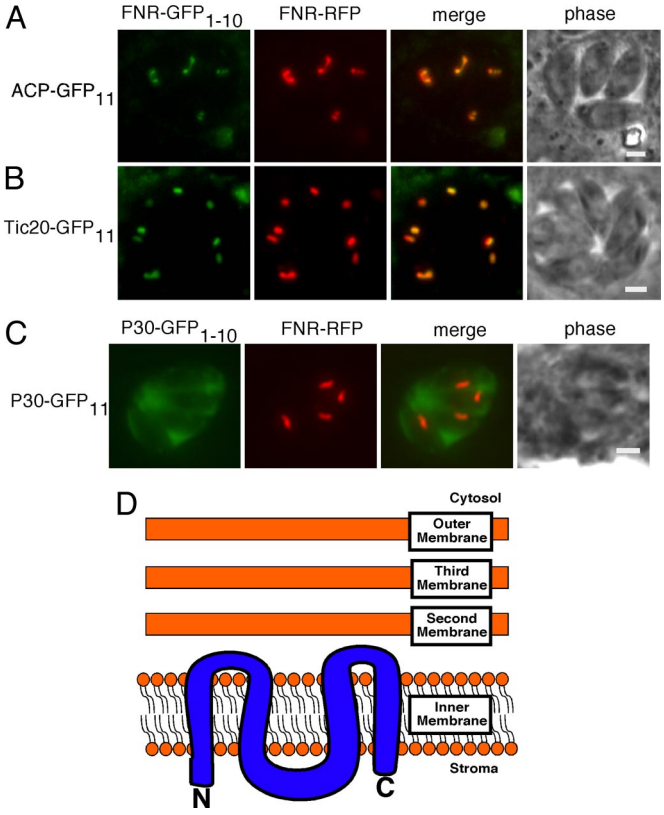

Fig. 2. *Tg*Tic20 localizes to the inner apicoplast membrane. (*A* and *B*) Live images of *T. gondii* cells expressing FNR leader fused to GFP 1–10, where GFP-11 was fused to the C terminus of either ACP (*A*) or *Tg*Tic20 (*B*). In both cell lines, green fluorescence colocalizes with the apicoplast stromal marker FNR-RFP. (Scale bar: 2 μm.) (*C*) Live image of *T. gondii* parasites expressing the secretory marker protein P30 fused to both GFP 1–10 and GFP-11. Green fluorescence localizes to the parasitophorous vacuole. (*D*) Model for localization of *Tg*Tic20. We predict that *Tg*Tic20 localizes to the inner membrane of the apicoplast, with the C terminus in the stroma. *In silico* predictions suggest the presence of four transmembrane domains (Fig. S1).

***Tg*Tic20 Is Essential for Parasite Viability.** To characterize the function of *Tg*Tic20, we generated a conditional *Tg*Tic20 mutant parasite cell line by using a recently described tetracycline-based system (2, 13). We generated a parental cell line (iTic20/eTic20) that contains both endogenous (eTic20) and inducible (iTic20) copies of the *Tg*Tic20 gene (Fig. S3*A*), where transcription of inducible genes can be down-regulated by the addition of the tetracycline analog anhydrotetracycline (ATc) to the growth medium. We generated a conditional *Tg*Tic20 mutant cell line (iTic20/ΔTic20) by disrupting the endogenous gene through homologous replacement of native *Tg*Tic20 with a selectable marker, verifying successful disruption of the native locus through PCR-based screening and Southern blotting (Fig. S3 *B* and C).

To establish whether *Tg*Tic20 is essential for parasite growth and viability, we measured parasite growth in the parental and knockout cell lines by using a real-time fluorescence assay (14). We introduced tandem tomato red fluorescent protein constructs into parental and knockout cell lines. Parasites were added to a 96-well plate, and wells were monitored daily for overall fluorescence intensity, a correlate of parasite growth. Both strains initially grew at similar levels in both the absence and presence of ATc (Fig. 3 *A* and *B*; green diamonds and blue squares). However, preincubation of parasites in ATc for 3 days before the growth assay revealed reduced parasite growth in the knockout cell line but not in the parental cell lines (Fig. 3 *A* and *B*, red triangles). After 6 or 7 days of incubation in ATc, the conditional mutant ceases to grow. To

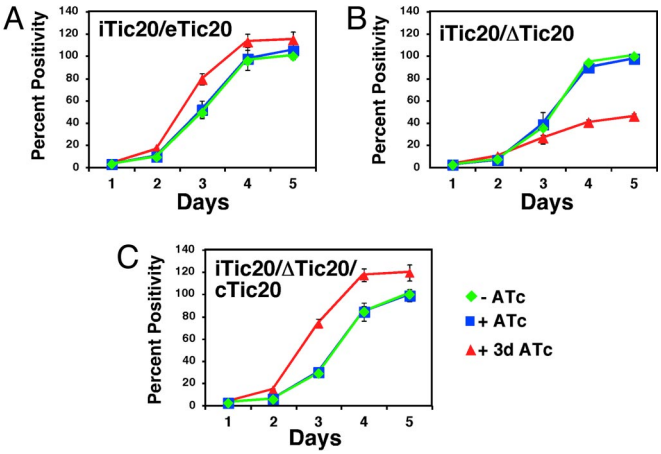

Fig. 3. *Tg*Tic20 is essential for growth of *T. gondii* parasites. (*A–C*) We monitored growth of parental (*A*), knockout (*B*), and complemented (*C*) parasite lines expressing tandem tomato RFP by a fluorescence growth assay. Cells were grown in the absence (green diamonds) or presence (blue squares) of ATc or incubated for 3 days in ATc before beginning the growth assay in the presence of ATc (red diamonds). Values are presented as percentage positivity, and error bars reflect 1 SD from the mean.

confirm that this defect was specifically caused by disruption of *Tg*Tic20, we complemented the conditional mutant by ectopically expressing *Tg*Tic20 from a constitutive promoter (iTic20/ΔTic20/cTic20). Doing so restored parasite growth in the presence of ATc (Fig. 3*C*). We also measured growth of the parental, knockout, and complemented cell lines by plaque assay (Fig. S3 *D–F*). These data supported the conclusions of the fluorescence assays that *Tg*Tic20 is essential for parasite growth.

***Tg*Tic20 Is Essential for Apicoplast Protein Import.** Having established that *Tg*Tic20 is an essential protein of the inner apicoplast membrane, we sought to determine its function. First, we measured the time frame for down-regulating *Tg*Tic20 expression in the mutant cell line. We harvested parasites after growing them for 0–4 days in ATc and monitored protein levels by Western blotting. Growth on ATc resulted in swift down-regulation of expression of the inducible *Tg*Tic20 protein (Fig. 4*A*, *Top*). As a more sensitive measure for *Tg*Tic20 abundance, we immunoprecipitated *Tg*Tic20 protein from $\approx 10^7$ parasites. We measured immunopurified protein levels by Western blotting and found that after 2 days growth on ATc, we could no longer detect *Tg*Tic20 protein in the mutant cell line (Fig. 4*A Bottom*).

We hypothesized that *Tg*Tic20 may function in protein import into the apicoplast. To test this possibility, we established several assays for successful protein import into apicoplasts (Fig. 4*B*). First, we examined processing of the N-terminal targeting domain of apicoplast proteins, a process that likely occurs subsequent to import into the organelle stroma. Apicoplast-targeted proteins typically reveal two differently sized molecular species: a slow migrating band corresponding to the precursor protein and a faster migrating mature protein where the N-terminal targeting leader has been cleaved (7, 8). We asked whether leader processing was affected in the conditional *Tg*Tic20 mutant. To facilitate these studies, we generated a cell line in the conditional *Tg*Tic20 mutant background that expressed a "synthetic" apicoplast-targeted protein, consisting of the apicoplast-targeting leader of *Tg*FNR fused to mouse dihydrofolate reductase [DFHR; a reporter protein typically used for organellar import assays in other systems (15)] and a C-terminal HA tag for detection. To gain a dynamic measure for the timing of defects on protein import in the *Tg*Tic20 mutant, we conducted pulse–chase labeling experiments. We incubated mutant parasites growing in host cells for 0, 2, 3, 4, and 5 days on ATc

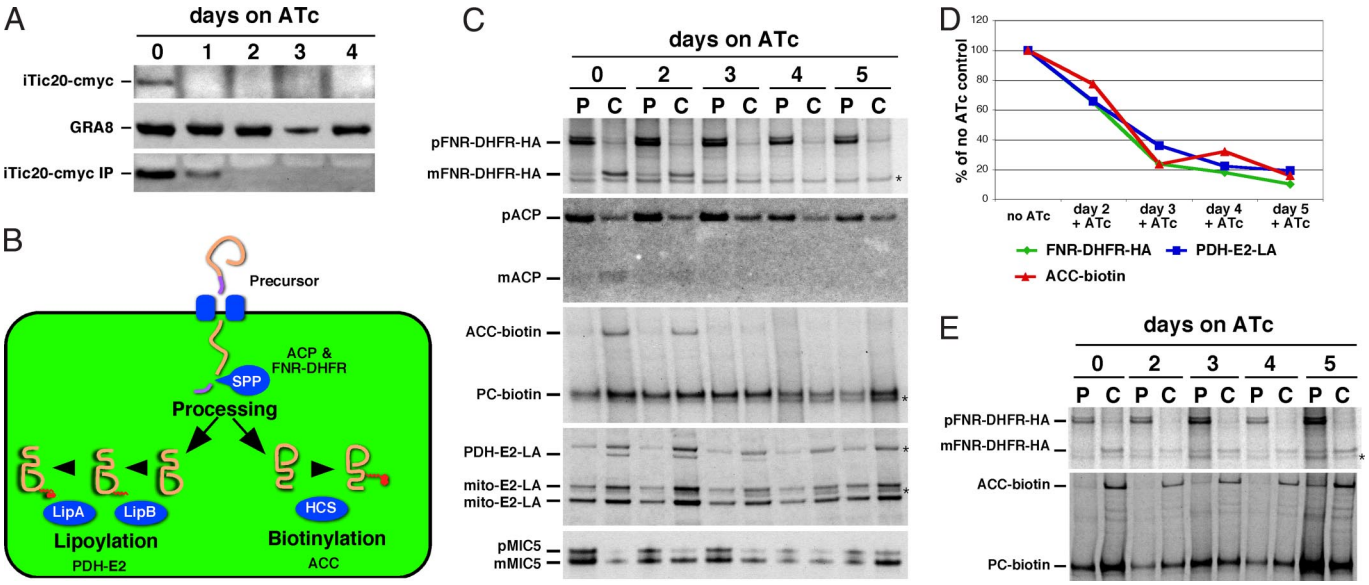

Fig. 4. *Tg*Tic20 is essential for apicoplast protein import. (*A*) Regulation of the inducible *Tg*Tic20-c-myc protein. iTic20/ΔTic20 parasites were grown for 0–4 days on ATc. Proteins were extracted and subjected to Western blotting with either anti-c-myc or anti-GRA8 antibodies (as a loading control) or subjected to immunoprecipitation of the inducible *Tg*Tic20-c-myc protein followed by Western blotting with anti-c-myc antibodies (*Bottom*). (*B*) Schematic depiction of the three protein import assays used in this work. We measured cleavage of preprotein leader sequences by the stromal processing peptidase (SPP), biotinylation of ACC by a holocarboxylase synthetase (HCS), and lipoylation of PDH-E2 by LipB and LipA. All three processes are thought to occur in the apicoplast stroma. (*C*) Pulse–chase analysis of proteins from the *Tg*Tic20 knockout line grown for 0, 2, 3, 4, or 5 days on ATc. Infected host cells were incubated in medium containing ^{35}S-amino acids for 1 h and either harvested (P) or further incubated in nonradioactive medium for 2 h (C). After detergent solubilization, proteins were purified by immunoprecipitation or affinity purification and separated by SDS/PAGE before detection by autoradiography. Protein bands marked by an asterisk in lanes containing biotinylated and lipoylated proteins represent contaminating host cell proteins. The band marked by an asterisk in HA pulldown lanes likely results from the use of an alternative internal start codon representing a shorter cytosolic version of FNR-DHFR-HA. (*D*) Quantification of bands in *C*. FNR-DHFR-HA values (green diamonds) were quantified as the percentage of mature protein in the chase compared with the precursor protein after the pulse. ACC-biotin values (red triangles) were quantified as a percentage of the intensity of PC in the same lane. PDH-E2-LA values (blue squares) were quantified as a percentage of the intensity of the lowermost mito-E2 band in the same lane. Values for each day are expressed as a percentage of the no-ATc value. (*E*) Pulse–chase analysis of proteins from the *Tg*Tic20 parental (iTic20/eTic20) line, performed in an identical manner to *C*.

and radiolabeled proteins with ^{35}S-amino acids for 1 h (pulse). We then washed out the radiolabel and incubated in medium containing an excess of unlabeled amino acids for an additional 2 h (chase). We purified proteins of interest by immuno- or affinity purification, separated them by SDS/PAGE, and detected them by autoradiography. After 5 days growth on ATc, precursor FNR-DHFR-HA protein was made at levels similar to that formed in cells grown in the absence of ATc, indicating that knockdown of *Tg*Tic20 does not affect synthesis of apicoplast-targeted proteins (Fig. 4*C*). However, after 2 days growth in ATc there is a 35% reduction in formation of mature, processed FNR-DHFR-HA, decreasing to undetectable levels at day 4 (Fig. 4*C*; Fig. 4*D*, green diamonds).

We also monitored processing of the native apicoplast protein ACP in the *Tg*Tic20 mutant. Mature ACP contains only one sulfur-containing amino acid, making detection difficult. The experiment shown in Fig. 4*C* suggests that ACP is processed at day 2 on ATc and not beyond, but detection levels are too low to draw a definitive conclusion. As a control, we monitored processing of microneme protein MIC5, which occurs in a post-Golgi compartment of the secretory pathway (16). Even after 5 days of incubation on ATc, MIC5 is processed (Fig. 4*C*), suggesting that *Tg*Tic20 knockdown does not affect other parts of the secretory pathway.

Although we suspect that precursor protein cleavage is a solid marker for whether proteins are able to traffic into the apicoplast stroma, it has not been formally shown that the processing event occurs here. Therefore, we sought to establish independent measures for successful protein targeting to the apicoplast. Several apicoplast enzymes are modified posttranslationally by cofactors after import into the stroma. One such modification is the biotinylation of acetyl-CoA carboxylase [ACC (17) Fig. 4*B*], a protein involved in biosynthesis of fatty acids. We purified biotinylated proteins by using an immobilized streptavidin column. Radiolabeled biotinylated ACC is not yet detectable after the 1-h pulse. In the absence of ATc, we observe robust biotinylation of ACC during the 2-h chase (Fig. 4*C*). Biotinylated ACC is reduced after 2 days of incubation in ATc and severely reduced after 3 days (Fig. 4*C*; Fig. 4*D*, red triangles), consistent with the results of the leader-processing assay. *T. gondii* contains a second major biotinylated protein, the mitochondrial pyruvate carboxylase (PC) enzyme (17). Levels of biotinylated PC remain unchanged after incubation in ATc.

A second postimport modification is lipoylation of the E2 subunit of pyruvate dehydrogenase (PDH-E2). Lipoylation of PDH-E2 is solely mediated by apicoplast-targeted LipA and LipB and requires a substrate synthesized *de novo* within the apicoplast stroma [octanoyl-ACP (2, 18) Fig. 4*B*]. In addition to apicoplast PDH-E2, *T. gondii* contains several lipoylated E2 subunit proteins in the mitochondrion [mito-E2 (2, 18)]. The mitochondrion contains a specific protein (LplA) that functions in the addition of the lipoyl moiety to the E2 enzymes (18), suggesting that, much like the apicoplast, lipoylation can only occur after successful import into the organelle. We purified lipoylated proteins by using an antibody against lipoic acid. After the 1-h pulse, mito-E2 enzymes are labeled, consistent with rapid import into mitochondria (Fig. 4*C*). As with biotinylated ACC, lipoylated PDH-E2 is not observed until the 2-h chase. Lipoylation of the apicoplast PDH-E2 is reduced after 2 days of growth on ATc and severely reduced after 3 days, whereas modification of mitochondrial enzymes was not affected, even after 5 days of incubation on ATc (Fig. 4*C*; Fig. 4*D*, blue squares).

Together, these data indicate that knockdown of *Tg*Tic20 impairs import of apicoplast-targeted proteins into the stroma of the organelle, but does not impair targeting of proteins to other destinations of the secretory pathway or to the mitochondrion. To rule out the possibility that defects in apicoplast protein import

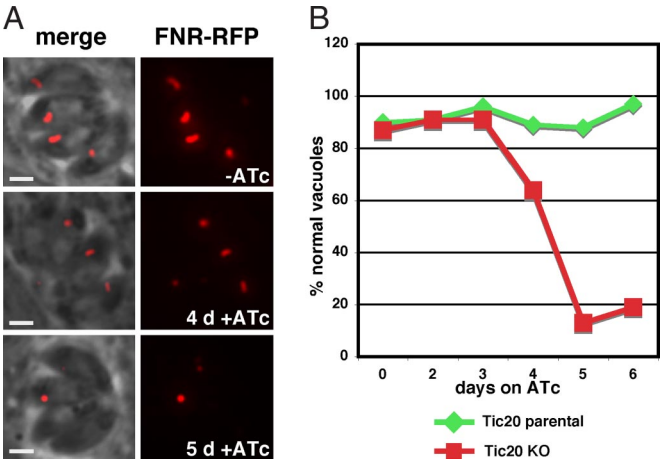

Fig. 5. *Tg*Tic20 is required for apicoplast biogenesis. (*A*) *Tg*Tic20-knockout parasites containing apicoplast-targeted RFP (FNR-RFP) were grown for 0, 4, or 5 days on ATc and subjected to live-cell imaging. (Scale bars: 2 μm.) (*B*) *Tg*Tic20 parental (green diamonds) or knockout (red squares) parasites containing apicoplast-targeted RFP were grown for 0–6 days on ATc. One hundred four-cell vacuoles were imaged at each time point. We graphed the percentage of vacuoles at each time point where every parasite in that vacuole contained an apicoplast.

resulted from nonspecific effects of ATc, we performed pulse–chase analysis on the parental strain grown on ATc for up to 5 days. These results indicate that neither processing of the FNR-DHFR-HA leader nor biotinylation of ACC is affected by ATc alone (Fig. 4*E*).

Preliminary experiments revealed that *Tg*Tic20 knockdown results in defects in apicoplast segregation or biogenesis. This raised the possibility that the inhibition of apicoplast protein import may be a consequence of the loss of apicoplasts from significant numbers of parasites rather than a specific effect on apicoplast import. Alternatively, lack of protein import could lead to biogenesis defects. To visualize the apicoplast and allow us to establish the sequence of events, we targeted RFP to the apicoplast of *Tg*Tic20 mutant parasites. After prolonged incubation in ATc, we observed vacuoles where not all plastids contained visible apicoplasts (Fig. 5*A*). Quantification revealed that major defects in apicoplast biogenesis occurred after 5 days of incubation in ATc, subsequent to the observed defects in apicoplast protein targeting (Fig. 5*B*).

We were able to identify a sequential series of phenotypes in the *Tg*Tic20 mutant. Two days after initiation of the mutant phenotype, *Tg*Tic20 protein is no longer detectable. Coincident with *Tg*Tic20 knockdown is a reduced efficiency of apicoplast protein import, which increases to essentially complete inhibition 4 days after the addition of ATc. Five days after initiation, we observe major defects in apicoplast biogenesis, with arrest of parasite growth ≈6 or 7 days after the addition of ATc. It is likely that death of the parasites results from the loss of apicoplasts and impairment of apicoplast functions. Apicoplasts are thought to perform several essential functions, such as the biosynthesis of fatty acids and isoprenoids (2, 3). It is likely that the failure to target biosynthetic proteins correctly (such as ACP, ACC, and PDH-E2; Fig. 4*C*) leads to ablation of these pathways, resulting in inhibition of parasite growth. Indeed, the apparent universal defects seen in apicoplast protein import in the *Tg*Tic20 mutant suggest that we are simultaneously impairing all apicoplast proteins, making this mutant an attractive candidate to identify apicoplast functions (e.g., through proteomic and metabolomic approaches).

Our results indicate that *Tg*Tic20 is required for apicoplast protein import. What, then, is the function of *Tg*Tic20 in this process? The precise role of Tic20 in chloroplast import in plants has been elusive. Knockdown of plant Tic20 by antisense RNA results in a reduced efficiency of chloroplast protein import (19). Based on its integral membrane localization, it has been postulated that plant Tic20 forms part of the protein import channel of the inner chloroplast membrane (20), although no direct experimental evidence supports this. Our results suggest that knockdown of *Tg*Tic20 protein expression does not immediately ablate import. Two days after the addition of ATc, the amount of *Tg*Tic20 is below our limits of detection. At this time point, apicoplast protein import is clearly affected (as measured by three independent assays), yet still occurs at between 65 and 77% the level of wild-type cells (Fig. 4*D*). This argues against *Tg*Tic20 functioning directly in an inner membrane import channel because the lack of an import channel would likely result in immediate ablation of import into the apicoplast. We considered the possibility that *T. gondii* harbors proteins that can partly complement the function of *Tg*Tic20. Plants contain multiple Tic20 paralogs and may also have additional nonrelated proteins with functions similar to Tic20 (21, 22). However, we did not identify *Tg*Tic20 paralogs in the *T. gondii* genome. Another possibility is that *Tg*Tic20 is an accessory or regulatory component of a putative import complex in the inner membrane. In such a scenario, *Tg*Tic20 may influence the efficiency of protein import through this complex, assembly of the complex, or be involved in a separate process that is essential for functioning of the inner membrane import complex. Identifying and characterizing additional inner membrane import components should allow us to address these questions.

Concluding Remarks. During their intracellular development, apicomplexan parasites such as *T. gondii* must target large numbers of proteins to their apicoplast. Protein targeting occurs via the secretory pathway and requires proteins to cross four membranes before reaching the organelle stroma (5). There has been considerable speculation about how protein targeting across these four membranes is mediated (e.g., 5, 23), but there has been a distinct lack of functional evidence for the various models.

Emerging evidence suggests that *T. gondii* and other Apicomplexa belong to a eukaryotic "supergroup" known as the Chromalveolata (24, 25). Chromalveolates include other major eukaryotic groups such as dinoflagellates and heterokonts (including diatoms and brown algae). A distinguishing feature of chromalveolates is the presence of a plastid that was derived by secondary endosymbiosis from a red alga. Chromalveolate plastids, then, represent a cellular *ménage a trois* of three "founder" organisms: a cyanobacterium, a red alga, and a heterotrophic eukaryote. An early requirement in the acquisition of plastids is the evolution of protein import machinery. An intriguing evolutionary question is which of these founders "donated" the import machinery and whether the origin of individual translocons is tied to the origin of the membrane they cross. Three types of translocons of have been speculated to potentially act in apicoplast protein import: primary plastid-derived Tic and Toc complexes and, more recently, Der1-containing complexes retooled from their original role in protein retrotranslocation across the ER membrane (12). In this work, we show that the innermost apicoplast membrane is crossed using machinery derived (at least in part) from the inner membrane Tic translocation complex of the red algal chloroplast, and we note that Tic homologs are present in other chromalveolates such as diatoms [Fig. S1 (23)]. Rather than evolving a fundamentally different means of protein import into secondary plastids, Apicomplexa and their chromalveolate cousins made use of the machinery already available from their primary plastid progenitors. It remains to be determined whether components of the Toc and Der1 complexes mediate import across other apicoplast membranes. The approaches for characterizing and localizing candidate apicoplast import proteins that we describe here provide an experimental framework to test these hypotheses conclusively.

Materials and Methods

Parasite Culture and Manipulation. Parasites were passaged in human foreskin fibroblasts and genetically manipulated as described in ref. 26. GenBank accession number for *Tg*Tic20 is EU427503. Plasmid construction and flow cytometry techniques are described in detail in *SI Materials and Methods*. All parasite strains described in this work were cloned by either limiting dilution or flow cytometry. Where indicated, parasites were grown in anhydrotetracycline (IBA) at a final concentration of 0.5 μg/ml.

Immunoprecipitation, SDS/PAGE, and Immunoblotting. For pulse–chase analyses, infected host cells were radiolabeled with 100 μCi/ml [^{35}S]methionine/cysteine (GE Healthcare) for 1 h. Cells were either harvested (pulse) or washed twice and incubated in parasite growth medium lacking radioactive amino acids for 2 h (chase) before harvesting. Proteins of interest were purified by immunoprecipitation or affinity purification and separated by SDS/PAGE using standard procedures (8) and detected by autoradiography or PhosphorImaging (GE Healthcare). Immunoblotting, sodium carbonate extractions, and Triton X-114 phase partitioning were performed by standard procedures. Detailed protocols are described in *SI Materials and Methods*.

Microscopy. Immunofluorescence assays and light microscope imaging were performed essentially as described in ref. 26. Detailed procedures for electron microscopy are included in *SI Materials and Methods*.

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