Most apicomonad parasites harbor a relict chloroplast, the apicoplast, that is critical for their survival. Whereas the apicoplast maintains a small genome, the bulk of its proteins are nuclear encoded and imported into the organelle. Several models have been proposed to explain how proteins might cross the four membranes that surround the apicoplast; however, experimental data discriminating these models are largely missing. Here we present genetic evidence that apicoplast protein import depends on elements derived from the ER-associated protein degradation (ERAD) system of the endosymbiont. We identified two sets of ERAD components in Toxoplasma gondii, one associated with the ER and cytoplasm and one localized to the membranes of the apicoplast. We engineered a conditional null mutant in apicoplast Der1, the putative pore of the apicoplast ERAD complex, and found that loss of Der1Ap results in loss of apicoplast protein import and subsequent death of the parasite.

Apicomplexa are a phylum of obligate parasites that include the causative agents of malaria, toxoplasmosis, and cryptosporidiosis. Recent evidence suggests that apicomplexans evolved from a free-living photosynthetic ancestor (1). This ancestry is reflected in the presence of a chloroplast-like organelle, the apicoplast (2). While no longer engaged in photosynthesis, the apicoplast is essential to parasite survival and home to several critical biosynthetic pathways. Bioinformatic and experimental evidence suggests that the apicoplast is engaged in the synthesis of fatty acids, isoprenoids, and heme (3, 4). Genetic or pharmacological ablation of these pathways blocks parasite growth and the apicoplast, therefore, is currently considered a prime target for antiparasitic drug development (5–7). The on-line version of this article (available at http://www.jbc.org) contains links to supplemental data.

The nucleotide sequences reported in this paper have been submitted to the GenBank™/EBI Data Bank with accession number(s) FJ976521, FJ976522, FJ976518, FJ976516, FJ976520, FJ976519, and FJ976517.

1 Supported by a predoctoral fellowship from the American Heart Association.

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4 The abbreviations used are: Tic, translocon of the inner chloroplast membrane; ERAD, endoplasmic reticulum-associated protein degradation; RACE, rapid amplification of cDNA ends; HA, hemagglutinin; GFP, green fluorescent protein; ACP, acyl-carrier protein; PIPES, 1,4-piperazineethanesulfonic acid; ATP, ATPase; Cdc48, ATP-dependent coiled-coil ATPase of Drosophila melanogaster; Npl4, nuclear protein localization 4; Ufd1, nuclear-encoded polypeptide associated with the ERAD complex; FJ976521, FJ976522, FJ976518, FJ976516, FJ976520, FJ976519, and FJ976517.

5 This work was supported, in whole or in part, by National Institutes of Health Grant AI64671 (to B. S.).

6 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables ST1–ST3, Methods, and Figs. S1–S4.
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MATERIALS AND METHODS

Gene Identification and Gene Tagging—Yeast sequences for Der1, Cdc48, and Ufd1 (GenBank™ IDs P38307, NP_010157, and NP_011562, respectively) were used as query sequences for BLAST searches against the T. gondii genome and GenBank™ databases. Initial RT-PCR experiments indicated that the automated gene predictions for the respective T. gondii homologs did not identify the beginning and end for all genes correctly. We performed 5'- and 3'-RACE using the SMART RACE cDNA amplification kit (BD Biosciences), and the resulting PCR products were cloned and sequenced. The sequences of primers used are listed in supplemental Table ST1. Using this cDNA amplification kit (BD Biosciences), and the resulting PCR products, we experimentally verified the ends of six genes and the 3'-end of Ufd1Ap. The 5'-end of the Ufd1Ap gene was identified by RT-PCR taking advantage of array-based promoter predictions (21). The experimentally validated T. gondii genes described in this study are Der1-1ER (FJ976521), Der1-2ER (FJ976522), Cdc48Cy (FJ976518), Ufd1Cy (FJ976516), Der1Ap (FJ976520), Cdc48Ap (FJ976519), and Ufd1Ap (FJ976517). The full-length coding sequences were amplified from T. gondii cDNA using primers introducing flanking BglII and AvrII restriction sites, subcloned into plasmid pCR2.1 (Invitrogen) and subsequently introduced into the equivalent sites of either plasmid pCATH or pCTM3, placing them under the control of the T. gondii α-tubulin promoter and fusing a 3× HA tag (pCATH) or a 3× c-Myc tag (pCTM3), respectively, to the 3'-end. These constructs were stably introduced into RH strain T. gondii parasites using chloramphenicol selection (22).

To study the localization of Ufd1Ap, we tagged the 3'-end of the gene by targeting the native locus with a cosmid clone of the respective locus (ToxoX83, see ToxoDB and Ref. 23) modified by RT-PCR taking advantage of array-based promoter predictions (21). The experimentally validated T. gondii genes described in this study are Der1-1ER (FJ976521), Der1-2ER (FJ976522), Cdc48Cy (FJ976518), Ufd1Cy (FJ976516), Der1Ap (FJ976520), Cdc48Ap (FJ976519), and Ufd1Ap (FJ976517). The full-length coding sequences were amplified from T. gondii cDNA using primers introducing flanking BglII and AvrII restriction sites, subcloned into plasmid pCR2.1 (Invitrogen) and subsequently introduced into the equivalent sites of either plasmid pCATH or pCTM3, placing them under the control of the T. gondii α-tubulin promoter and fusing a 3× HA tag (pCATH) or a 3× c-Myc tag (pCTM3), respectively, to the 3'-end. These constructs were stably introduced into RH strain T. gondii parasites using chloramphenicol selection (22). For cryo-electron microscopy, infected cells were fixed in 4% paraformaldehyde/0.05% glutaraldehyde (Polysciences Inc.) in 100 mM PIPES buffer. Samples were then embedded in 10% gelatin and infiltrated overnight with 2.3M sucrose/20% polyvinyl pyrrolidone in PIPES at 4 °C. Samples were frozen in liquid nitrogen and sectioned with a cryo-ultramicrotome. Sections were probed with the indicated primary antibodies followed by the appropriate secondary antibody conjugated to 12 or 18 nm colloidal gold, stained with uranyl acetate/methylcellulose, and analyzed by transmission EM as described previously (30).

Pulse-chase Labeling and Immunoprecipitation—Pulse-chase experiments were carried out essentially as described

5 G. G. van Dooren, unpublished data.
6 C. F. Brooks, G. G. van Dooren, and B. Striepen, unpublished data.
previously (12). Briefly, host cell cultures were infected with 2 × 10⁶ parasites and grown in the presence or absence of ATc. 2 days after infection, pulse-chase labeling with 100 μCi/ml [³⁵S]cysteine and [³⁵S]methionine (MP Biomedicals) was conducted as described under “Results.” To test parasites that were exposed to ATc 3 and 4 days, cultures were preincubated with drug in the previous passage for the appropriate time. Proteins of interest were purified by immunoprecipitation, separated by SDS-PAGE, and visualized by autoradiography. Antibodies used were anti-MIC5, a kind gift from Vern Carruthers, University of Michigan (32), anti-lipoic acid (Calbiochem), anti-RFP (Roche Applied Science), and anti-apicoplast-Cpn60 (rabbit serum raised against recombinant protein for this study).

Phylogenetic Analyses—For the phylogenetic analysis of Cdc48, we used sequences from 30 taxa (GenBank™ accession numbers for Cdc48 proteins are provided under supplemental methods) and generated a multiple sequence alignment in ClustalX. 900 unambiguously aligned amino acid positions were used for further analysis (alignments available on request). This data set was subjected to maximum likelihood phylogenetic analysis using RAxML version 7.0.4 (33). A phylogenetic tree was constructed using the GAMMA + P-Invar evolutionary model, and the model parameters were alpha: 0.981286, invar: 0.114318, and Tree-Length: 3.905532. Bootstrap analyses were conducted using 100 replicates (34).

RESULTS

Two Differentially Localized Sets of ERAD Components in T. gondii—Using the ERAD components from yeast and their recently identified cryptophyte homologs (14) as query sequences, we identified the genes for four putative Der1, two Ufd1, and two Cdc48 homologs in the T. gondii genome. Interestingly, the plastid-less apicomplexan Cryptosporidium retains only two Der1 homologs and a single Ufd1 and Cdc48.
The coding sequences of seven of these candidates were amplified by PCR from *T. gondii* cDNA and introduced into parasite expression constructs that resulted in C-terminal fusion with an epitope tag (see "Materials and Methods" and supplemental Table ST1). The resulting constructs were transfected into parasites, and stable transgenic lines were obtained by drug selection. Ectopic expression of proteins from a non-native promoter can potentially impact their localization. To ensure that the observed localization patterns are physiological we also introduced an epitope tag directly into the genomic locus of one gene (Ufd1AP, see "Materials and Methods" for more detail), and we raised antibodies to recombinant protein for a second gene (Cdc48Ap, see supplemental methods).

Using immunofluorescence assays we found that different *T. gondii* ERAD proteins localize to distinct subcellular compartments. Fig. 1, a and b shows the localization of two Der1 homologs to a perinuclear structure that colocalizes with P30-GFP-HDEL, a marker for the *T. gondii* ER (35). One homolog each of Cdc48 and Ufd1 is found in the cytosol. The localization of these four proteins is comparable with the localization of their homologs in yeast and mammalian cells and is consistent with their likely role in the classical ERAD pathway. A second set of ERAD components localized to the apicoplast. Fig. 1 e, f, and g shows localization of Der1Ap-HA, Cdc48Ap-c-Myc, and Ufd1Ap-HA and the apicoplast ACP for comparison. Apicoplast proteins typically contain an N-terminal signal for apicoplast targeting, which is cleaved upon arrival (9, 36).

Sequence analysis predicts the presence of such a signal in the
apicoplast ERAD proteins. Western blot analysis reveals two bands for each protein, suggesting N-terminal processing (Fig. 1, h–j), and the respective molecular masses are consistent with the predicted full-length precursor and the processed smaller mature form of the proteins.

Whereas ERAD proteins localize to the apicoplast, they do not colocalize fully with the luminal marker ACP (see insets in Fig. 1, e–g). In silico modeling of the protein structure of Der1Ap suggests the presence of transmembrane domains, whereas Cdc48Ap and Ufd1Ap are predicted to be soluble. To study their intraorganellar localization, we performed immuno-electron microscopy. As shown in Fig. 2, Der1Ap and Cdc48Ap localize to an organelle that is bound by four membranes and can be labeled for the apicoplast marker ACP. Der1Ap and Cdc48Ap were confined to the periphery of the organelle, where both proteins colocalize, whereas ACP was found mainly in the lumen of the apicoplast. We conclude that a set of ERAD components is likely associated with the apicoplast membranes.

**Der1Ap Is Essential for Parasite Growth**—To genetically dissect the function of apicoplast ERAD components, we engineered a conditional null mutant in Der1Ap, the presumptive membrane pore of the putative translocon. We introduced an epitope-tagged Der1Ap minigene under the control of a tetracycline-regulatable promoter into a parasite strain expressing the tetracycline transactivator protein (37). In this background, we replaced the coding sequence of the native Der1Ap locus with a chloramphenicol acetyl transferase (CAT) selectable marker by double homologous recombination (5, 12). We confirmed disruption of the locus in chloramphenicol-resistant clonal parasites by PCR and Southern blot analysis (Fig. 3). In this mutant line, Der1Ap expression can be suppressed by culture in the presence of ATc. To measure inducible Der1Ap-HA expression, we grew parasites for 0–4 days on ATc, harvested parasites, and detected Der1Ap-HA protein by Western blot. We found that Der1Ap-HA levels were greatly reduced after 1 day on ATc and undetectable after 2 days (Fig. 5a).

We next asked whether Der1Ap was essential for parasite growth. We introduced a red fluorescent protein into both mutant and parental lines and monitored parasite growth by measuring fluorescence intensity. In the absence of ATc, both cell lines grow at the same rate (Fig. 4, a and b). Whereas ATc appears to have no effect on the parental strain, growth of the mutant slows dramatically after 3 days of treatment. Pretreatment for 3 days prior to the assay blocks growth entirely. Growth of the mutant is restored...
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when we reintroduce the Der1Ap coding sequence under a constitutive promoter (Fig. 4c), demonstrating the specificity of the observed phenotype. As a separate measure for parasite growth, we performed plaque assays. In this assay, confluent host cell cultures are infected with a small number of parasites and incubated for 8 days. Repeated rounds of invasion, growth, and egress result in the formation of clearings in the host cell monolayer that can be visualized by crystal violet staining. These experiments demonstrated minimal plaque formation in cultures infected with mutant parasites when these were grown in the presence of ATc, whereas parental and complemented strains showed no attenuation in growth (Fig. 4, d–f). We conclude that Der1Ap function is essential for parasite growth.

Genetic Knockdown of Der1Ap Ablates Apicoplast Protein Import—Whereas recent studies suggest that ERAD-derived proteins are found in several organisms harboring secondary plastids (19, 20, 38), experimental evidence for their function is largely missing. The apicoplast ERAD system could act equivalently to the ER system in quality control-associated protein export from the apicoplast, or alternatively in protein import into the organelle (14). To test the latter possibility, we measured apicoplast protein import in the Der1Ap mutant by assaying the maturation and post-translational modification of cargo proteins (12). We introduced a transgenic marker for the apicoplast lumen, FNR-RFP (29), into the mutant background. This strain was then used to perform pulse-chase labeling experiments. We labeled parasites for 1 h with 35S-labeled amino acids (pulse, P), followed by washout and incubation in nonradioactive medium for 2 h (chase, C). We next measured processing or modification of several apicoplast-targeted and control proteins by immunoprecipitation, gel electrophoresis, and autoradiography. As shown in Fig. 5b, FNR-RFP occurs as a 38-kDa precursor during the pulse and is processed during the chase to yield the mature form of 27 kDa. ATc treatment results in a significant reduction and subsequent loss of FNR-RFP maturation after 1 and 2 days of treatment, respectively (Fig. 5, b and c). Equivalent results were obtained using an antibody against Cpn60, a native luminal apicoplast chaperone, whereas treatment had no apparent effect on the maturation of MIC5, a secretory protein targeted to the micronemes (32) (Fig. 5, b and c). As an independent measure of protein import, we determined the level of lipoylation of the apicoplast pyruvate dehydrogenase E2 subunit (PDH-E2) (5, 12). Protein import is a prerequisite for lipoylation of PDH-E2 as the process requires two apicoplast resident enzymes, and the precursor molecule octanoyl-ACP that is synthesized de novo in the lumen of the organelle by the type II fatty acid synthesis system (5, 39). An antibody was used to immunoprecipitate specifically lipoylated proteins from parasite lysate. After 1 day on ATc, the level of lipoylated PDH-E2 was reduced, falling to undetectable levels after 2 days (Fig. 5, b and c). Several mitochondrial enzymes are similarly lipoylated and modification relies on successful import into the mitochondrion. This process showed no observable defect in the Der1Ap mutant. We conclude that ablation of protein import in the Der1Ap mutant is specific to the apicoplast. To ensure that the observed defects are not an artifact of ATc treatment, we performed control import experiments using the Der1Ap parental strain. In this strain, ATc treatment for 4 days had no apparent effect on the maturation of Cpn60 or the lipoylation of PDH-E2 (see supplemental Fig. S2).

Defects in apicoplast replication have been shown to affect apicoplast protein import (40). To test whether Der1Ap has a role in apicoplast replication, we performed live imaging of Der1Ap mutant parasites expressing FNR-RFP. We observed no defects for the first 2 days, a decrease in plastid numbers after 3 days, and widespread organellar loss after 4 days (Fig. 5d and supplemental Fig. S3). We conclude that Der1Ap does not directly affect apicoplast replication, and we establish the following sequence of consequences of ATc treatment in the Der1Ap mutant: Der1Ap protein levels are severely depleted after 1 day, directly coinciding with a loss of protein import, leading to subsequent defects in organellar biogenesis and parasite growth on days 3 and 4 post-treatment. Our data strongly support a direct role for Der1Ap in protein import into the apicoplast.

The Apicoplast ERAD System Is Derived from the Red Algal Endosymbiont—The apicoplast ERAD system could have evolved by duplication of ERAD genes of the host or alternatively could be derived by horizontal gene transfer from the...
We performed phylogenetic analyses for the *T. gondii* ERAD components. The strong sequence divergence of Der1 proteins precluded the construction of meaningful alignments and trees. However, Cdc48 proteins are highly conserved providing 900 unambiguously aligned residues for robust analysis. We found that the two *T. gondii* proteins were of divergent phylogenetic origin (Fig. 6). The cytoplasmic protein forms a well-supported clade with homologs from chromalveolates (including photosynthetic and plastid-less taxa). This placement is consistent with the current model of vertical evolution of Apicomplexa as established by phylogenetic analyses using numerous protein and ribosomal RNA sequences (41, 42). In contrast, Cdc48AP clusters with proteins from organisms harboring secondary plastids of red algal origin. Whereas analysis of Ufd1 did not provide full resolution of the tree of life because of a lower level of sequence conservation when compared with Cdc48, it fully supported divergent ancestry of the cytoplasmic and apicoplast protein (supplemental Fig. S4). We conclude that the apicoplast ERAD system is likely derived from the red algal endosymbiont, whereas the classical ER resident system was inherited vertically.

**DISCUSSION**

Endosymbiosis is now well established as a mechanism that has played a crucial role in the evolution of eukaryotic cells. One organism, the symbiont, is engulfed by a second organism, the host, and a stable symbiotic relationship ensues in which the endosymbiont loses its independence and gradually evolves into an organelle that is controlled by the host and serves the host metabolic needs. A common hallmark of this process is massive horizontal gene transfer from the endosymbiont to the host (43). This gene transfer affords control to the host but also requires the establishment of mechanisms to reroute proteins that are now encoded and synthesized by the host back into the symbiont. A large body of work on mitochondria and chloroplasts has demonstrated the presence of elaborate protein translocons in the inner and outer membranes of these organelles (44, 45) that specifically recognize targeting information and deliver cargo proteins accordingly to the organelar lumen or various membrane compartments.

The apicoplast is the product of secondary endosymbiosis, the enslavement of a single celled eukaryotic alga. Compared with their primary progenitors, secondary plastids are surrounded by additional membranes that must be traversed by nuclear-encoded proteins (a total of four membranes surround the apicoplast). The evolution of mechanisms to traverse these additional membranes must have occurred early in organelle acquisition, and a simple solution would have been to use existing protein transport complexes. Our previous studies support this model and have shown that transport over the innermost apicoplast membrane is dependent on elements derived from the translocon of the inner chloroplast membrane of the endosymbionts chloroplast (12). The conservation of the Tic complex would make it appear likely that the Toc complex is equally conserved. However, so far genome searches have failed to identify Toc components in Apicomplexa or diatoms (13). The presence of the Toc complex might be masked by a high level of sequence divergence of its components or alternatively indicate that it has been replaced by a different mechanism. How proteins might cross the third or periplastid membrane is of particular interest as this membrane is thought to be a derivative of the endosymbionts plasma membrane. Based on
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the discovery of genes encoding elements of the ERAD system in the nucleomorph in cryptophytes, Sommer et al. (14) proposed that this complex was retooled to function in trafficking of plastid proteins (proteins of ERAD origin have also been speculated to be part of the peroxisomal proteome (46)). Most recently, this model has received support from studies on a variety of organisms bearing secondary plastids including the current study. ERAD-associated proteins are found in the membranous compartment surrounding secondary plastids in the apicomplexans Plasmodium and Toxoplasma ((19, 20) and Figs. 1 and 2 and supplemental Fig. S1), the diatom Phaeodactylum (19), and the cryptophyte Guillardia (14). Light and electron microscopy experiments indicate that these proteins are associated with the outer membranes of the plastid, but they lack resolution to tie the system to a specific membrane. Interestingly, split GFP assays in Phaeodactylum demonstrate the presence of two Der1 homologs in the third membrane. However, it is important to note that these experiments do not exclude the presence of Der1 homologs in other plastid membranes. Additional markers are needed to establish if the ERAD system is limited to the third membrane or might also be found in the second membrane and thus have replaced the Toc complex previously present in this membrane (Fig. 7d).

Whereas the presence of an ERAD translocon in the membranes of plastids was consistent with a role in plastid biology, it was unclear what this role of the ERAD complex might be. Functional data discriminating between a role in protein import versus protein export and quality control have as yet been missing. Whereas symbiont-derived Der1 proteins in Phaeodactylum have been shown to interact at a steady state level with fusion proteins targeted to the periplastid compartment, no interaction was observed with fusion proteins targeted to the plastid lumen (38). In the current study, we have used the ability to construct conditional mutants in T. gondii to devise a rigorous test of the protein import hypothesis. We isolated a T. gondii Der1Ap mutant and demonstrated that this protein is essential for apicoplast protein import and parasite survival. We have previously shown that apicoplast protein import across the innermost membrane is essential for Toxoplasma survival (12). In contrast, genetic disruption of classical ERAD in yeast or mammalian systems does not affect cell viability (18), and mutant cells have to be subjected to stressors resulting in the accumulation of misfolded proteins to produce a viability phenotype. More directly, we have developed and validated assays that track post-translational modifications of reporters that are restricted to the apicoplast lumen and thus require successful import (12). Applying these assays to the current study, we demonstrate a direct correlation between the loss of Der1Ap and a complete loss of apicoplast protein import (Fig. 5). These genetic and biochemical experiments provide the strongest evidence to date that the novel plastid ERAD system has a direct and essential role in plastid protein import.

Our phylogenetic analyses of CDC48 and Ufd1 indicate that the T. gondii ERAD systems are phylogenetically distinct and that the apicoplast system is derived from the red algal endosymbiont (Fig. 6 and supplemental Fig. S4). This is consistent with the presence of homologs in the cryptophyte nucleomorph, a remnant of the algal nucleus (14). It thus appears that the Der1 protein of the red algal symbiont was re-targeted from its original location in the ER to the symbiont to its plasma membrane, where it could now function in importing plastid-targeted proteins from the host secretory pathway (Fig. 7, a–c). This represents a remarkably simple and elegant solution for the complex problem of how to evolve protein exchange between host and endosymbiont at the beginning of their relationship. Once targeted to the endosymbionts cytoplasm proteins could take advantage of established mechanisms, namely the Toc and Tic, to gain access to the chloroplast. We hypothesize that the apicoplast employs a series of specific translocons that reflect the diverse evolutionary origin of the membranes in which they reside (Fig. 7d).

Acknowledgments—We thank Jessica Kissinger for help with phylogenetic analyses, Carrie Brooks for technical assistance, Julie Nelson for help with flow cytometry, and Vern Carruthers, Gary Ward, and Geoff McFadden for antibodies.

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