Dissection of brefeldin A-sensitive and -insensitive steps in apicoplast protein targeting

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Summary

The apicoplast is a relict plastid found in many apicomplexans, including the pathogens *Toxoplasma gondii* and *Plasmodium falciparum*. Nucleus-encoded apicoplast proteins enter the ER, and after cleavage of the signal sequence, are routed to the apicoplast by virtue of a transit peptide, which is subsequently removed. To assess the mechanisms of localization we examined stable transfectants of *T. gondii* for the localization and processing of various GFP fusion proteins. GFP fusions bearing apicoplast targeting sequences targeted efficiently to the plastid, with no retention in the ER, even when an ER retention/retrieval sequence was added. Incubation with brefeldin A, which blocks ER-to-Golgi trafficking by inhibiting a GTP exchange factor required for retrograde trafficking, blocked the processing of the protein. Surprisingly, it did not affect the immunofluorescence pattern. To avoid the potentially misleading presence of pre-existing GFP fusion protein in the apicoplast, we used a ligand-regulated aggregation system to arrest the GFP fusion protein in the ER prior to trafficking. Upon addition of ligand to promote disaggregation, the fusion protein targeted to the plastid, even in the presence of brefeldin A. Ligand release at 15°C, which blocks trafficking of Golgi-routed proteins, also allowed significant localization to the plastid. Our data indicate that apicoplast proteins can localize to the region of the plastid when Golgi trafficking is inhibited, but suggest that some steps in import or maturation of the proteins may require a brefeldin A-sensitive GTP exchange factor.

Key words: Toxoplasma, Apicoplast, Chloroplast, Brefeldin A, Endoplasmic reticulum, Apicomplexa

Introduction

The phylum Apicomplexa contains many pathogens of medical and veterinary importance, including the agents of human malaria (*Plasmodium falciparum* and other species) and toxoplasmosis (*Toxoplasma gondii*). *T. gondii* is a major cause of birth defects and the agent of AIDS-associated *Toxoplasma* encephalitis. These small protozoa have highly developed secretory systems that fulfil many functions in invasion and intracellular parasitism. The cellular architecture of *T. gondii* is highly organized, with many organelles having a defined position in the basal to apical axis of the cell, making it a useful cell biological model for the Apicomplexa (Joiner and Roos, 2002). Among the several destinations of the apicomplexan secretory system are specialized organelles such as rhoptries, micronemes and dense granules, as well as the plasma membrane, the parasitophorous vacuole, and endocytotic organelles. Each parasite also possesses a single non-photosynthetic plastid that is served by the secretory system. The secretory system of *T. gondii* is polarized, with the nuclear envelope serving as an intermediate between the ER and Golgi (Hager et al., 1999). In particular, vesicular traffic from the nuclear envelope is concentrated on the apical side of the nucleus next to the Golgi.

Protein and vesicle trafficking within the *T. gondii* secretory system is homologous to trafficking in other eukaryotes (Ngo et al., 2000). Most proteins leave the ER for the Golgi and from there are sorted to their diverse destinations by virtue of specific targeting sequences (Bonifacino and Traub, 2003; Jurgens and Geldner, 2002; Lippincott-Schwartz et al., 2000). Cargo proteins are packaged within vesicles that bear markers dictating fusion with the destination organelle. Also required are a variety of additional molecules to ensure that vectorial capacity is maintained. Among these molecules are small GTP binding proteins and their corresponding GTP/GDP exchange factors (GEFs). The early steps of ER-to-Golgi trafficking can be disrupted in most organisms by the fungal metabolite brefeldin A (BFA), which inhibits Sec7, a GEF required for ongoing function of the GTP binding protein ARF1 (Donaldson et al., 1992). Although ARF1 functions at multiple sites in the secretory system, most species possess multiple GEFs related to Sec7 that perform specific functions (Geldner et al., 2003; Jackson and Casanova, 2000). Some of these are inhibited by BFA (Donaldson et al., 1992). Treatment of *T. gondii* with BFA causes disruption of the Golgi and distension of the nuclear envelope (Hager et al., 1999).

The apicomplexan plastid, termed the apicoplast, is thought to have arisen from a secondary endosymbiosis with a photosynthetic alga, in part because it is surrounded by four closely apposed membranes (Kohler et al., 1997). The inner two membranes of the apicoplast presumably correspond to the double membranes of the algal chloroplast, the periplastid membrane probably derives from the plasma membrane of the

alga, whereas the outermost membrane corresponds to the ancient vacuole membrane. Chloroplast proteins are directed from the cytosol to the chloroplast by a transit peptide, which is efficiently cleaved by a stromal processing peptidase following import. In contrast, the first step in trafficking of apicoplast proteins is entry into the ER, which is effected by a typical signal sequence (DeRocher et al., 2000; Waller et al., 2000). Cleavage of the signal sequence reveals a transit peptide, which contains all of the information for targeting from the ER to the plastid. Although it is widely assumed that some molecules related to the translocation apparatus of chloroplasts are likely to be involved in the passage through the inner two membranes of the apicoplast, the mechanism by which apicoplast proteins leave the ER and then traverse the outer two membranes is still enigmatic. A stromal processing peptidase homologue, which bears an apicoplast targeting sequence has been identified in *Plasmodium* (van Dooren et al., 2002). Interestingly, immature apicoplast proteins with the transit peptide still attached are easily detected on immunoblots of *T. gondii* cell extracts although the immature chloroplast proteins are virtually never detected in plant extracts.

Here we report studies testing the hypothesis that apicoplast proteins must pass through the Golgi to reach their destination. We have exploited a conditional aggregation domain (CAD) system (Rollins et al., 2000), which was initially used to allow regulated protein secretion in mammalian cells (Rivera et al., 2000). This system uses multiple domains of the FK506 binding protein engineered to aggregate in the absence of a synthetic ligand. Following synthesis in the absence of ligand, secretory proteins aggregate in the ER and are blocked from trafficking. We have successfully transferred this system to *T. gondii* and demonstrate that ligand release allows trafficking of secretory proteins, and that trafficking to the apicoplast is not inhibited by BFA. Similarly, it is not blocked at temperatures that abrogate secretion. However, processing of the apicoplast proteins is inhibited by BFA, suggesting that some functions of apicoplast targeting or import may require a Sec7-like GEF.

Materials and Methods

Cell culture, transfection and BFA treatment

T. gondii were grown in human foreskin fibroblasts according to standard techniques. Among the strains used were RH and its corresponding HXGPRT deletion strain (Donald et al., 1996). Plasmids were introduced by electroporation and stable transfectants selected either with chloramphenicol or mycophenolic acid plus xanthine as required. CAD fusion transfectants were selected in the presence of ligand AP21998 (1 μM) a gift of Ariad Pharmaceuticals (http://www.ariad.com) to prevent aggregation of the fusion proteins.

Brefeldin A (BFA) (Calbiochem, La Jolla CA) was dissolved in methanol to a concentration of 10 mg/ml and stored in single-use aliquots at –20°C. For low-temperature incubations, samples were incubated in a tray cooled by a circulating water bath and monitored to confirm that the proper temperature was maintained.

Constructs

Several GFP fusion constructs based on the signal and transit sequences of apicoplast ribosomal protein S9 and acyl carrier protein (ACP) were used in these studies. GFP fusions with the S9 signal sequence [amino acids 1-42; S9(S)-GFP], the signal plus transit sequence [amino acids 1-159; S9(S+T)-GFP] and the signal plus partial, but functional, transit sequence [1-95; S9(1-95)-GFP] and the ACP targeting sequence [1-103; ACP-GFP] have been previously described (DeRocher et al., 2000). The ACP-GFP and S9 GFP coding sequences were transferred into the Gra1-PCNA1/S65TGFP-DHFR HXGPRT plasmid (gift from Michael White). The *T. gondii* ER retrieval sequence was added to the C-terminus of fusion proteins by site-directed mutagenesis of the above plasmids by oligonucleotide mutagenesis using 5′-GGCATGGATGAGCTATACAAACACGACGAGCTGTGACTGCAGCCCACAGGAGCTG-3′ and 5′-CAGCTTCTGTGGGCTGCAGTCACAGCTCGTCGTGTTTGTATAGTTCATCCATGCC-3′ (HDEL coding sequence underlined). The plasmid pC4S1-F(M)4-FCS-hGH was generously provided by Ariad Pharmaceuticals and used as a source of the conditional aggregation domain (CAD). This plasmid contains four copies of the CAD domain, which were released by cleavage with *Xba*I and *Spe*I and cloned into the *Avr*II site in between the S9 signal sequence (amino acids 1-42) or signal plus transit peptide (amino acids 1-159) and GFP. The constructs are abbreviated here as S9(S)-CAD-GFP and S9(S+T)-CAD-GFP. Constructs were confirmed by DNA sequencing.

Microscopy

For instrinsic fluorescence and immunofluorescence assays (IFAs), parasites were grown overnight within fibroblast monolayers on coverslips. IFAs were performed as described (DeRocher et al., 2000). Following fixation and permeabilization, GFP was visualized using rabbit anti-GFP (Molecular Probes, Eugene OR) plus FITC-goat anti-rabbit IgG (Southern Biotechnology, Birmingham, AL). The ER marker BiP was revealed using a rabbit antiserum (1:200) raised against its trypanosomal homolog, kindly provided by Jay Bangs (Bangs et al., 1993) (no antibody raised against a *T. gondii* ER marker is available). The apicoplast was revealed by DAPI staining or by incubation with quantum red-coupled streptavidin (Sigma, St Louis MO), which detects endogenously biotinylated apicoplast acyl coA carboxylase (Jelenska et al., 2001). Because proteins in the parasitophorous vacuole are not easily fixed for immunostaining, secreted GFP fusion proteins were viewed by intrinsic fluorescence either in live cells or following a 10-minute fixation in 2% paraformaldehyde in PBS. For S9(S+T)-CAD-GFP release studies, cultures were grown for 2-3 days without ligand, and then used to infect monolayers on coverslips.

Samples were examined using a DeltaVision wide-field deconvolution microscopy system (Applied Precision, Issaquah, WA) equipped with an Olympus UplanApo 100× 1.35 NA oil iris objective. Images were deconvolved using SoftWoRX 3.2.3 using the manufacturer's suggested parameters except images of secreted GFP, which were deconvolved for five cycles. A FCS 2 perfusion chamber and objective heater (Bioptechs, Butler, PA) were used to maintain live cells at 37°C and images were acquired on the DeltaVision microscope using an Olympus PlanApo 60× 1.40 NA oil objective.

Pulse-chase analysis and immunoblotting

Approximately 10^7 intracellular parasites were incubated with 100 μCi/ml [^{35}S]trans label (methionine plus cysteine, MP Biomedicals, Irvine, CA, 1175 Ci/mmol) in methionine and cysteine-free medium for the indicated times. The labeling medium was removed and incubation was continued in complete medium as required. The cultures were lysed with 10 μl Laemmli sample buffer, heated in a boiling water bath for 5 minutes to denature the proteins, then diluted with 500 μl SK lysis buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 2 mM EDTA, 1% NP-40, 0.25% deoxycholate, 1.5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 μM pepstatin, 100 μM PMSF). This procedure was found to enhance recovery of the immature forms of the GFP fusion proteins, which were otherwise poorly recovered by immunoprecipitation. Anti-GFP IgG (Molecular Probes, Eugene, OR) or rabbit anti-Mic5 (gift of Vern Carruthers) was added and the

immune complexes were collected with Protein A coupled to magnetic beads (Dynal, Oslo, Norway). Samples were analyzed by SDS-PAGE, transferred to nitrocellulose and subjected to phosphorimaging. Blots containing immunoprecipitated samples or total cell lysates (4×10^6 cells) were probed with rabbit antibodies to GFP (1:10,000) (gift from Jim Cregg), Mic5, or nucleoside triphosphate hydrolase (NTPase) (1:2000, gift of Timothy Stedman and Keith Joiner) (Bermudes et al., 1994). Bound antibodies were detected with protein A conjugated to horseradish peroxidase, using the ECL Plus system (Amersham, Piscataway, NJ). Signals were quantified by phosphorimaging to assess steady state levels of the corresponding proteins.

Results

Addition of an ER retrieval sequence does not abrogate apicoplast targeting.

Proteins with an ER retrieval sequence at the C-terminus are retrieved from the pre-Golgi or *cis*-Golgi compartment and recycled back to the ER. If apicoplast proteins traverse the Golgi on the way to the apicoplast, one would expect a C-terminal ER retrieval/retention sequence to block their transport to the apicoplast. The *T. gondii* ER retrieval sequence HDEL (Hager et al., 1999) was added to the C-terminus of known apicoplast-targeted fusion proteins ACP-GFP, S9(S+T)-GFP (where S stands for 'signal' and T for 'transit'), and S9(1-95)-GFP, as well as a secreted protein S9(S)-GFP. The slightly larger size of the protein on immunoblots (ACP-GFP-HDEL, see Fig. 1B) or a PCR amplification product from genomic DNA (other constructs, data not shown) confirmed that the fusion constructs retained the added sequence in the stable transfectants. Transfectants were stained with anti-GFP or anti-BiP (an ER marker), quantum red streptavidin and DAPI. The apicoplast genome can often be seen as a small dot on the apical side of the nucleus, which colocalizes with quantum red streptavidin. Streptavidin binds acyl coA carboxylase in the apicoplast (Jelenska et al., 2001), and also weakly detects biotinylated proteins in the host cell. As in previous studies (Hager et al., 1999), when only a signal sequence was present (no transit peptide), addition of HDEL to the C-terminus of the protein caused retention in the ER (Fig. 1A, S9(S)-GFP-HDEL). The GFP did not colocalize with the apicoplast in the *x*, *y* or *z* dimensions and its staining pattern was similar to the anti-BiP staining pattern (Fig. 1A). In contrast, ACP-GFP-HDEL (Fig. 1A), S9(1-95)-GFP-HDEL and S9(S+T)-GFP-HDEL (not shown) were detected only in the apicoplast, as determined by colocalization with the apicoplast genome and quantum red streptavidin (Fig. 1A). To confirm that HDEL-tagged proteins were in the apicoplast and not an adjacent compartment, serial sections of the plastid were examined. GFP was found to colocalize with the streptavidin marker in *x*, *y* and *z* dimensions (Fig. 1A, merge and *Y-Z*).

Immunoblot analysis was used to determine whether the transit peptide was proteolytically cleaved in the ACP-GFP-HDEL transfectants. The transit peptide of ACP is predicted to be approximately 72 amino acids in length. As shown in Fig. 1B, the mature form at 30 kDa is present in both ACP-GFP and ACP-GFP-HDEL transfectants. The precursor form is also abundant in both lines (~36 kDa). Over multiple experiments, somewhat less processing was seen in the ACP-GFP-HDEL parasites than in ACP-GFP parasites, with an average of 30% of the immune detected ACP-GFP-HDEL in the mature form, compared to 50% of the ACP-GFP. It seems unlikely that this effect is due to a difference in the efficiency of the stromal

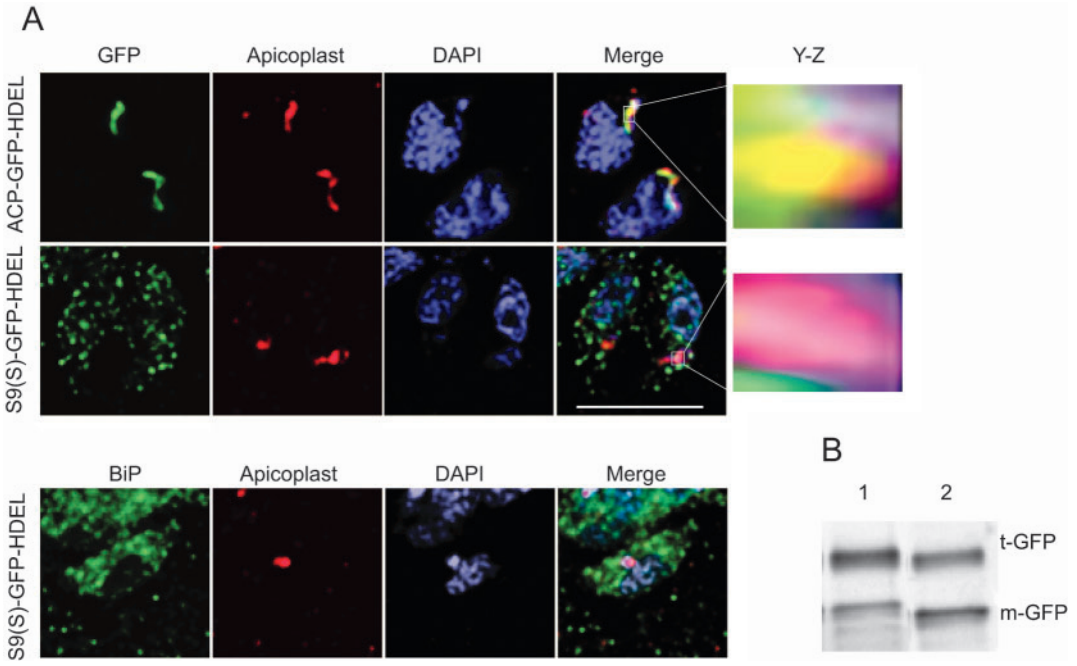

Fig. 1. An ER retrieval signal does not block targeting to the apicoplast. (A) *T. gondii* expressing ACP-GFP-HDEL and S9(S)-GFP-HDEL were analyzed by IFA and visualized with anti-GFP, and anti-BiP [S9(S)-GFP-HDEL only], the apicoplast marker quantum red streptavidin and DAPI. A merge of anti GFP or anti BiP, quantum red streptavidin and DAPI is also shown. A merged projection of the marked apicoplasts in the *y*-*z* dimension is shown. (B) Anti-GFP immunoblot analysis of protein from ACP-GFP-HDEL (lane 1) and ACP-GFP (lane 2) parasites. t-GFP indicates the species containing the transit peptide and m-GFP the mature form lacking the transit peptide. Bar, 5 μm.

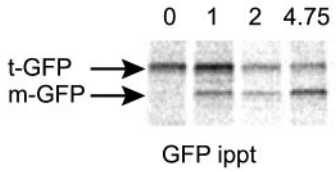

Fig. 2. Kinetics of cleavage of the apicoplast transit peptide. Intracellular *T. gondii* expressing ACP-GFP were labeled with ^{35}S amino acids for 1 hour, and chased for 0, 1, 2 and 4.75 hours in control medium. GFP was immunoprecipitated, proteins resolved by SDS-PAGE, and visualized with a phosphorimager. Transit (t-GFP) and mature (m-GFP) forms of ACP-GFP were detected.

peptidase action on the target proteins as they are identical except for the C-terminal four amino acids. It possibly reflects a delay in the import pathway.

BFA affects processing but not steady state localization of apicoplast-targeted GFP

As a prelude to testing the effects of BFA on apicoplast protein processing, we examined the kinetics of transit peptide cleavage. ACP-GFP expressing parasites were pulse-labeled for one hour with [^{35}S]methionine + cysteine and chased for the indicated time (Fig. 2). The abundance of the immature, transit peptide-GFP form declined over several hours with a corresponding increase in the mature form lacking the transit peptide. The average half-life of the transit peptide form was three hours ± 45 minutes, based on three independent experiments. This half-life is somewhat longer than that seen for *P. falciparum* ACP-GFP in trophozoites and schizonts (van Dooren et al., 2002).

BFA is effective at blocking transport in mammalian cells at concentrations of 50-100 ng/ml, and has generally been used at concentrations of 5-10 μg/ml for studies in *T. gondii* (Brydges et al., 2000; Soldati et al., 1998; Stedman et al., 2003), although 1 μg/ml has been shown to prevent secretion of GRA1 (Coppens et al., 1999). Preliminary studies showed that *T. gondii* were able to proliferate following treatment with 1 μg/ml BFA for 4 hours, but not if the concentration was raised to 10 μg/ml (not shown). Radiolabeled methionine and cysteine were still incorporated into protein between 3 and 4 hours of BFA treatment at the lower concentration (not shown), indicating that the *T. gondii* were still metabolically active. This concentration of BFA also inhibited secretion of P30-GFP into the vacuole when added 5 minutes after host cell invasion (not shown) and blocked the processing of pulse-labeled microneme protein Mic5 from the intermediate form (lacking the signal sequence) to the mature form (Fig. 3), which occurs in a post-Golgi compartment (Brydges et al., 2000).

BFA completely blocked the appearance of the mature form of ACP-GFP. Following pulse-labeling for 30 minutes in the absence of BFA, we chased for 4 hours in the presence of BFA at various concentrations (Fig. 3). Samples chased in 10 ng/ml BFA showed similar amount of processing to the no BFA control, whereas samples chased with 100 ng/ml and 1 μg/ml showed very little processing. Processing of Mic5 showed a similar sensitivity to BFA. Interestingly, concentrations of BFA that blocked cleavage of the transit peptide also stabilized the

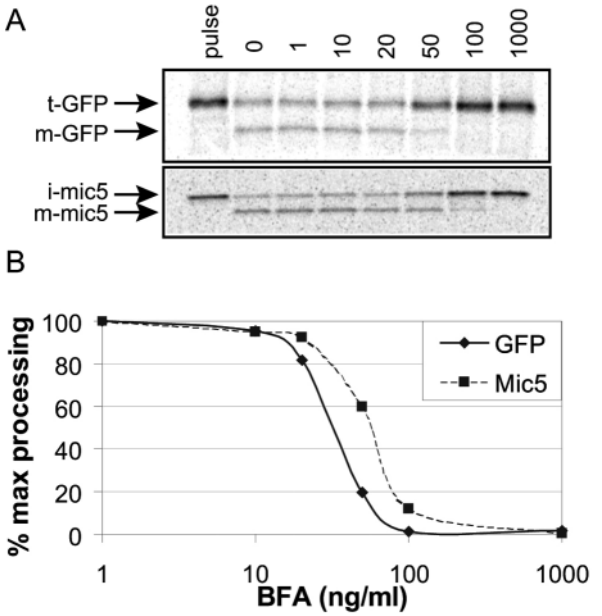

Fig. 3. BFA blocks transit peptide cleavage. (A) Intracellular *T. gondii* expressing ACP-GFP were labeled with ^{35}S amino acids for 30 minutes (pulse) then chased for 4 hours in medium containing the indicated concentrations (ng/ml) of BFA. GFP and Mic5 were immunoprecipitated, resolved by SDS-PAGE and detected by phosphorimaging. Transit peptide (t-GFP) and mature forms (m-GFP) of GFP as well as intermediate (i-mic5) and mature forms (m-mic5) of Mic5 are indicated. (B) Quantification of GFP (diamonds) and Mic5 (squares) processing calculated based on the maximum processing measured in the experiment shown in A.

unprocessed forms of both ACP-GFP and Mic5 (Fig. 3, compare pulse label, 1 ng/ml BFA and 1000 ng/ml BFA).

We investigated the timing of the BFA-sensitive step in apicoplast targeting relative to the cleavage event (Fig. 4). ACP-GFP parasites were pulse-labeled for 1 hour in the presence of BFA (1 μg/ml) to prevent processing during the labeling period. The cells were then rinsed and chased in BFA-free medium for various times prior to harvest (left series). In some samples, BFA was added at these times, and the chase continued for a total time of 4 hours. ACP-GFP was immunoprecipitated and the relative proportion of radiolabeled precursor and mature protein was determined. When BFA was present during both the labeling and chase, no transit peptide processing was observed. Following a 1 hour chase in the absence of BFA, only 15% of the protein was processed. If BFA was added at 1 hour and the incubation continued for another 3 hours, 62% of the protein was processed. These data suggest that there is a BFA-sensitive step prior to processing but that BFA does not directly inhibit processing of apicoplast proteins. Similar results were obtained in four replicate experiments. Once the chase without BFA started, the time required for the transit peptide to be cleaved was compressed relative to Fig. 2 in which no BFA was used. This might reflect ACP-GFP intermediates accumulating just prior to the BFA-sensitive step during pulse-labeling, and then passing through the pathway en masse once the BFA block was released.

The ability of BFA to inhibit apicoplast protein maturation indirectly could be due to a requirement for apicoplast proteins

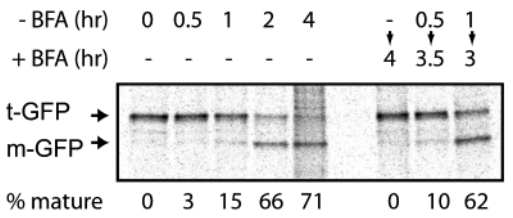

Fig. 4. The BFA-sensitive step in apicoplast protein import occurs before transit peptide cleavage. Intracellular *T. gondii* expressing ACP-GFP were labeled with ^{35}S amino acids in the presence of 1 μg/ml BFA for 1 hour (pulse) then chased in control medium for various times (–BFA, hours). Alternatively (three right lanes), the samples were chased without BFA for the indicated times (–BFA, hours) and then BFA was added to 1 μg/ml and the incubation continued for an additional period (+BFA, hours). In these BFA add-back experiments, the total incubation time was 4 hours. GFP was immunoprecipitated and resolved by SDS-PAGE; t-GFP marks the species containing the transit peptide, m-GFP marks the mature form in which lacks the transit peptide. The amount of precursor and mature protein was quantified by phosphorimaging and the percentage of protein in the mature form is indicated below each lane.

to traverse the Golgi en route to the apicoplast. If so, then BFA should block transport to the plastid and apicoplast protein precursors should accumulate in the ER. ACP-GFP and ACP-GFP-HDEL transfected cells were treated with BFA for 4 hours and processed for immunofluorescence analysis (Fig. 5). Surprisingly, no accumulation of GFP outside the apicoplast was observed in either cell line. Similar results were reported as unpublished data in a review (Joiner and Roos, 2002).

Conditional aggregation can modulate protein trafficking to the apicoplast

Interpreting the lack of effect of BFA on the steady state localization of proteins to the apicoplast was complicated by the abundance of pre-existing GFP in the organelle. As robust regulation of gene expression is not available in *T. gondii*, we used the conditional aggregation domain (CAD) system developed by Rivera (Rivera et al., 2000) to circumvent this problem. These workers showed that proteins bearing tandem CAD repeats are soluble in the presence of synthetic ligand AP21998, but when ligand is withdrawn, the CAD domain proteins form large aggregates that cannot exit the ER. These aggregates dissociate to monomers when ligand is added and resume trafficking.

Sequences encoding regions of leader sequence (signal alone or signal plus transit) of apicoplast ribosomal protein S9 and CAD-GFP fused and constructs were transfected into *T. gondii.* Clonal lines expressing the GFP fusions were established in the presence of ligand. Although GFP fluorescence could be easily seen in the S9(S)-CAD-GFP line grown either with or without ligand, it could only be seen in the S9(S+T)-CAD-GFP cells grown with ligand. However, in all cases the CAD-GFP fusions were easily detected by anti-GFP. Perhaps the combination of CAD-induced aggregation and the long transit peptide prevented proper folding of GFP.

We examined the ability of CAD to modulate protein targeting in *T. gondii* using multiple transfectants expressing S9(S+T)-CAD-GFP (Fig. 6A) and S9(S)-CAD-GFP (Fig. 6B).

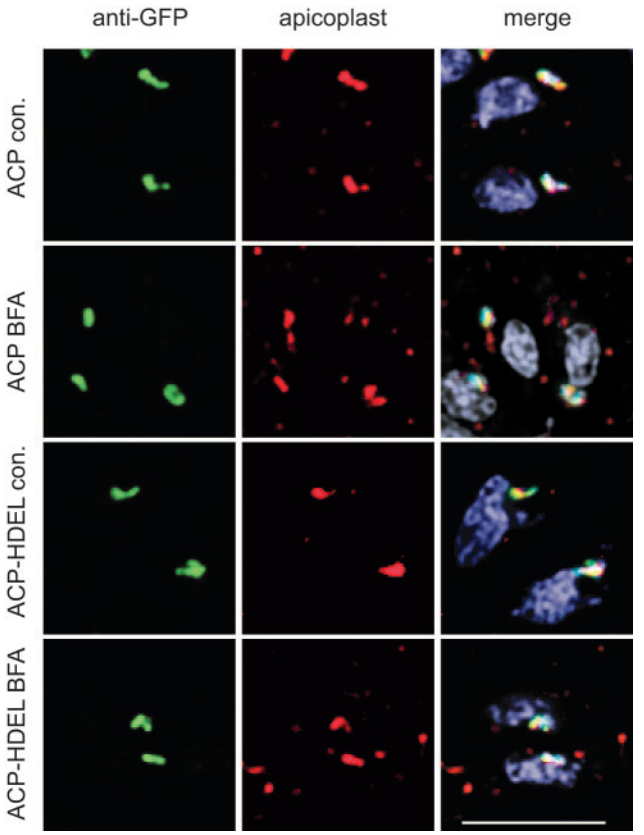

Fig. 5. Extended treatment with BFA does not impede GFP localization to the apicoplast. *T. gondii* expressing ACP-GFP, or ACP-GFP-HDEL were grown in control medium (con.) with or without 1 μg/ml BFA for 4 hours. Shown are IFAs probed with anti-GFP and the apicoplast marker quantum red streptavidin. A merge of anti-GFP, quantum red streptavidin and DAPI is shown. Bar, 5 μm.

When ligand was present, all cell lines showed trafficking similar to lines expressing the same construct but without the aggregation domains. When ligand was withdrawn, GFP was distributed in small clumps throughout the parasite, in a pattern like that seen for the ER marker BiP (Fig. 1) except that it is more punctate in appearance, consistent with CAD-mediated aggregation of GFP.

We identified a cell line in which little expression of the CAD fusion protein was observed in the apicoplast after growth for 3 days in the absence of ligand (Fig. 6A, *t*=0). Sometimes a spot would colocalize with apicoplast markers, but such spots were generally no brighter than other foci of GFP within the cell. Within 5 minutes after addition of ligand, there was a noticeable increase in the amount of GFP that colocalized with the apicoplast and the amount of GFP that colocalized with the apicoplast continued to increase over the course of an hour (Fig. 6A). We saw no evidence that the plastid-targeted proteins passed through the vacuole, as proposed for *P. falciparum* (Cheresh et al., 2002).

One trivial possibility was that adding ligand allowed the cell to degrade the aggregated protein and the decline in GFP in the ER was due to degradation instead of targeting. To test this possibility, we used immunoblot analysis to compare the amount of GFP in S9(S+T)-CAD-GFP cells grown without ligand versus after 40 minutes with ligand (Fig. 6C). Protein

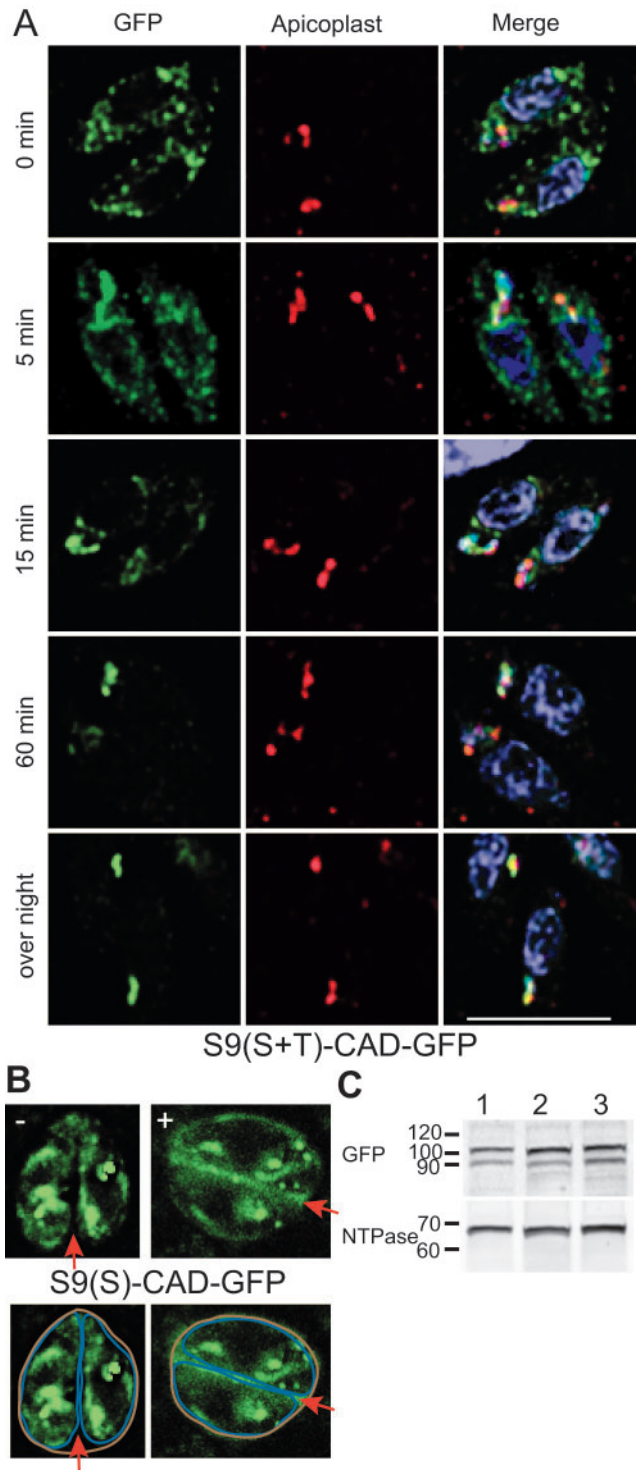

Fig. 6. The conditional aggregation domain system can modulate protein trafficking from the ER. (A) *T. gondii* stably transfected with S9(S+T)-CAD-GFP were grown overnight in the absence of ligand, and then ligand was added for the indicated times before fixation. Shown are IFAs probed with anti-GFP and quantum red streptavidin. A merge of anti-GFP, quantum red streptavidin and DAPI is shown. (B) GFP fluorescence of S9(S)-CAD-GFP transfected *T. gondii* grown without ligand (–) and overnight with ligand (+). Arrow indicates parasitophorous vacuole. Lines were drawn on the same images to assist in identification of the parasites and the parasitophorous vacuole membrane (lower panels). (C) Protein was extracted from S9(1-159)-CAD-GFP parasites that had been treated with ligand for 40 minutes (lane 1), grown overnight without ligand (lane 2), and grown overnight in ligand (lane 3). Samples were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-GFP and anti-NTPase antibodies. Bar, 5 μm.

amounts were normalized to NTPase (Bermudes et al., 1994). There was a modest, 20% decline in the amount of GFP after ligand was added, not enough to account for the dramatic decrease in ER staining that we observed. Interestingly, two immunoreactive species were observed, which migrated identically on SDS-PAGE whether or not ligand was present (see Fig. 6C, similar results were obtained on a 7.5% gel). The smaller of the two was modestly increased in abundance when ligand was present overnight. However, its presence in the absence of ligand suggests that the transit peptide of this fusion protein is susceptible to non-specific proteolysis in the ER. The appearance of the lower band in pulse-labeled cells was unaffected by treatment with the cell-permeable cysteine protease inhibitor E64d (not shown). The S9 transit sequence contains redundant information that allows partial transit peptides to function in sorting, but is inefficiently cleaved when fused directly to GFP (DeRocher et al., 2000; Yung et al., 2003).

BFA does not block CAD-released protein trafficking to the apicoplast

We used the CAD system to examine whether BFA blocks a step in apicoplast targeting between the ER and the apicoplast or a step more closely localized to the apicoplast. S9(S+T)-CAD-GFP cells were grown without ligand (Fig. 7A). To one set of samples, BFA was added but no ligand; to a second set BFA was added and then ligand added 5 minutes later; to a third set only ligand was added. Samples with BFA alone were incubated for 1 hour, samples with ligand were incubated 20 minutes or 4 hours before fixation. In the absence of ligand, BFA did not visibly alter GFP distribution (compare top panels of Fig. 7A with top panels of Fig. 6A). After 20 minutes in ligand, samples with or without BFA both showed noticeable accumulation of GFP in the apicoplast. After incubating with ligand for 4 hours, samples with or without BFA showed very strong accumulation of GFP in the apicoplast, with excellent colocalization in most cells (in *x*, *y* and *z* dimensions). Indeed, blinded analysis of microscopic images comparing the GFP localization with that of the apicoplast stromal marker was unable to discern which samples were exposed to BFA and which were not. Cells with elongated plastids often showed regions with red or green predominating. However, this staining pattern did not correlate with the presence or absence of the inhibitor, and was also observed in some cells expressing ACP-GFP or ACP-GFP HDEL (compare Fig. 1A top panel with Fig. 6A, 60 minute panel). The precise colocalization of GFP and the apicoplast in S9(S+T)-CAD-GFP expressing cells in the presence of BFA supports the contention that the BFA-sensitive step in apicoplast targeting occurs after proteins reach the apicoplast, as defined within the limits of deconvolution microscopy.

In contrast to the apicoplast-targeted GFP, secretion of S9(S)-CAD-GFP was sensitive to BFA (Fig. 7B). Without

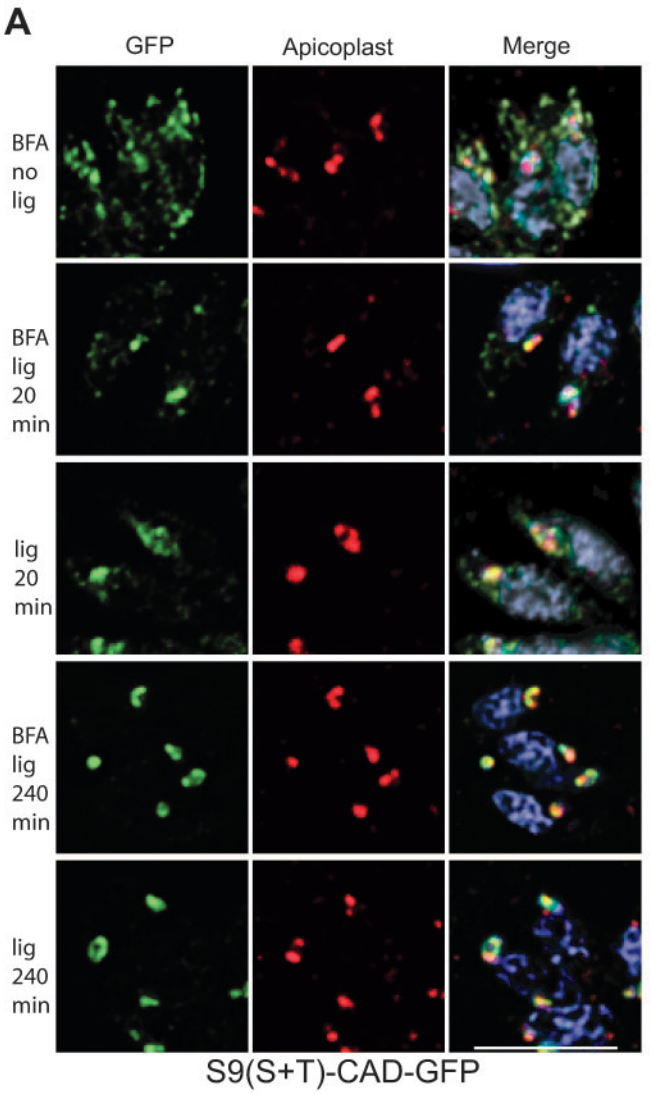

Fig. 7. BFA does not impede ligand-mediated localization of S9(S+T)-CAD-GFP to the apicoplast. (A) Effect of BFA on apicoplast targeting. *T. gondii* stably transfected with S9(S+T)-CAD-GFP were grown for 3 days in the absence of ligand. Samples were incubated in one of several conditions prior to fixation as indicated: BFA, no ligand for 1 hour; BFA for 5 minutes followed by ligand for 20 minutes or 240 minutes; ligand alone for 20 minutes or 240 minutes. Samples were prepared for IFA, anti-GFP and quantum red streptavidin are shown, and a merge of anti-GFP, quantum red streptavidin and DAPI. (B) Effect of BFA on secretion. *T. gondii* stably transfected with S9(S)-CAD-GFP were grown overnight in the absence of ligand. GFP fluorescence in live cells was viewed at time 0 and then in the same cells 30 minutes after the addition of ligand or ligand plus BFA. As with apicoplast proteins, BFA was added 5 minutes before ligand. Arrow indicates parasitophorous vacuole. Lines were drawn on the same images to facilitate the localization of parasites and the parasitophorous vacuole membrane (lower panels). Bar, 5 µm.

ligand, this protein also aggregated in a reticular pattern, and when ligand was added a substantial fraction of the protein was secreted into the parasitophorous vacuole within 30 minutes. However, in the presence of BFA, the ligand-released protein was retained within the parasite, accumulating in a diffuse compartment apical to the nucleus. Karsten and colleagues (Karsten et al., 1998) stated that 50 µg/ml BFA was necessary to cause complete dispersal of the *T. gondii* Golgi. Therefore, we interpret this observation as GFP accumulating in a transport incompetent Golgi or an intermediate compartment. Thus, BFA exerted a block on secreted proteins but did not affect localization to the region of the apicoplast. Taken together, these results suggest BFA blocks a step after the protein arrives at the apicoplast but before transit peptide cleavage.

Low temperature does not block CAD-released protein trafficking to the apicoplast

Incubating mammalian cells at 15°C causes proteins to accumulate in a compartment between the ER and the Golgi (Kuismanen and Saraste, 1989) and has been shown to block trafficking of rhoptry proteins in *T. gondii* and *P. falciparum* (Howard and Schmidt, 1995; Soldati et al., 1998). If protein trafficking from the ER to the apicoplast proceeds through the Golgi, it should be blocked at 15°C. *T. gondii* transfectants expressing CAD fusions in the absence of ligand were pre-chilled, ligand was added, and the incubation continued at 15°C. As expected, no GFP was detected in the parasitophorous vacuole in cells expressing S9(S)-CAD-GFP after 3.5 hours in the presence of ligand at the lower temperature (Fig. 8B). In contrast, after 1 hour at 15°C, S9(S+T)-CAD-GFP was easily detected in the apicoplast (Fig. 8A). Even more GFP localized to the apicoplast after 4 hours. Thus, neither of two conditions that block secretion abrogate protein trafficking to the region of the apicoplast.

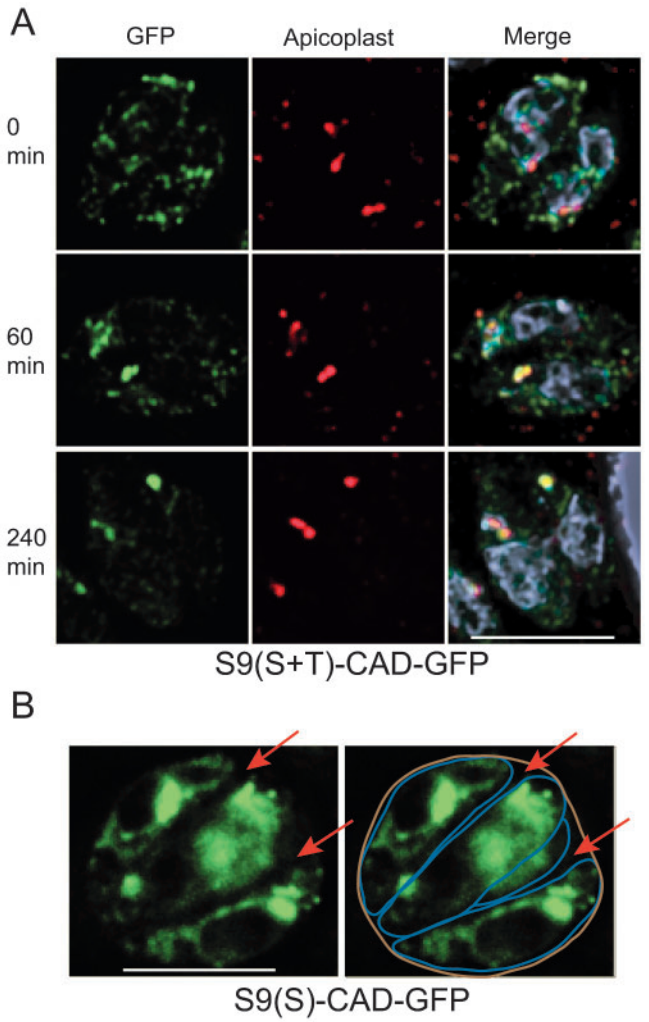

Fig. 8. Ligand-mediated relocalization of S9(S+T)-CAD-GFP to the apicoplast occurs at low temperature. (A) Effect of low temperature on apicoplast targeting. *T. gondii* stably transfected with S9(S+T)-CAD-GFP were grown for 3 days without ligand, and transferred to a water bath equilibrated at 15°C. After 15 minutes, pre-chilled medium containing ligand was added and the cells incubated for the indicated times. Samples were prepared for IFA and probed with anti-GFP and quantum red streptavidin. A merge of GFP, quantum red streptavidin and DAPI is shown. (B) Effect of low temperature on secretion. *T. gondii* stably transfected with S9(S)-CAD-GFP were grown overnight in the absence of ligand and transferred to a water bath equilibrated at 15°C. After 15 minutes, ligand was added as above and the cultures were incubated for 3.5 hours before mild fixation. Cells were viewed for GFP fluorescence and transmitted light. Arrows indicate parasitophorous vacuole. Cartoons are overlaid on the images to facilitate identification of the parasites and the parasitophorous vacuolar membrane. Bar, 5 μm.

Discussion

Several groups of organisms possess secondary plastids that originated from either a red or green alga (Funes et al., 2002). Proteins destined for all known secondary plastids commence their journey in the ER, but mechanisms for targeting from the ER to secondary plastids differ. In Euglenoids (green lineage), proteins are targeted to the chloroplast via the Golgi and contain a stop-transfer sequence after the transit peptide (Sulli et al., 1999; Sulli and Schwartzbach, 1995). In the case of enslaved chloroplasts derived from a red alga, Cavalier-Smith has suggested that proteins traffic directly from the ER (Cavalier-Smith, 2003). Heterokonts (red lineage) show membrane connections between the ER and the outer membrane of chloroplasts (Gibbs, 1979). The preponderance of sequence comparison agrees with a red algal origin for the apicoplast (Fast et al., 2001; Waller et al., 2003), although a few have studies suggested a green algal origin (Kohler et al., 1997). The ambiguity concerning the origin of the apicoplast allows multiple hypotheses for the trafficking mechanism to be entertained. Among possibilities proposed for apicoplast targeting are: (1) passage into the Golgi and subsequent sorting (the route for most secretory proteins and plastids of green algal origin); (2) vesicular traffic directly from the ER to plastids (Foth et al., 2003); and (3) direct import of soluble proteins owing to contiguity between the ER and outer apicoplast membrane (Joiner and Roos, 2002).

We have presented three different types of experiment that argue against the first possibility. First, we have shown that the addition of an ER retrieval sequence does not block targeting to the plastid as cited elsewhere as unpublished data (Joiner and Roos, 2002). Second, we have shown that BFA does not block the localization of proteins to the apicoplast, although it does block removal of the transit peptide (presumed to occur in the plastid lumen). Third, we have shown that low temperature, which blocks protein secretion, does not block targeting to the apicoplast. These results, as well as others in the literature, highlight several key features in apicoplast targeting.

Trafficking from the ER to the plastid appears to be rapid because under steady state conditions apicoplast proteins cannot be detected in the ER and proteins released by adding ligand to the CAD system are seen in the apicoplast within 5 minutes. We found that adding an ER retrieval sequence to apicoplast-targeted GFP fusions did not alter steady state localization of the protein. The majority of the ER retrieval receptor ERD2 is localized to the pre-Golgi in crown group eukaryotes and *P. falciparum* (Elmendorf and Haldar, 1993). Hence, these data argue against trafficking through the Golgi. However, some interactions with Erd2 may occur. The decreased processing of ACP-GFP-HDEL as compared to ACP-GFP suggests a delay in reaching the apicoplast lumen, possibly resulting from interactions with Erd2. More compellingly, a mutant of mitochondrial *T. gondii* SODB2 mistargets to the apicoplast, yet can be retained in the ER by an ER retention sequence (Brydges and Carruthers, 2003). Finally, in the diatom *Phaeodactylum tricornutum* (red lineage) GFP fusions to a truncated, but functional, plastid targeting sequence can be retained in the ER by an ER retrieval sequence, whereas fusions with longer targeting sequences cannot (Apt et al., 2002). These data suggest that the transit peptide receptor and Erd2 compete for the same substrates, which becomes more apparent when the plastid targeting sequence is compromised. Whereas native apicoplast targeting sequences interact strongly with their receptor, overcoming any HDEL interaction with Erd2, the weak interaction of mistargeted or mutant proteins with the transit peptide receptor would allow retrieval back to the ER. We propose that this competition occurs in a novel pre-plastid compartment, and

represents a logical extension of the function of Erd2 in retrieving mistargeted proteins.

We used a regulated secretion system (Rivera et al., 2000) to dissect protein trafficking to the apicoplast. This system uses a synthetic ligand to regulate aggregation of proteins bearing multiple repeats of an aggregation domain. We adapted this system to *T. gondii* and showed trafficking of both secreted and apicoplast proteins is strongly reduced in the absence of ligand. In this regulated system, the ligand-released protein trafficked to the apicoplast even when BFA was present. Localization of the apicoplast-targeted protein was less sensitive to reduced temperature than a secreted protein, suggesting that fewer vesicular fusion events are required to reach the apicoplast. Both of these findings also support the hypothesis that proteins do not need to traverse the Golgi to reach the apicoplast. In studies of *P. falciparum*, immunoelectron microscopy detected NSF1, a component of complexes that catalyze vesicular fusion, associated with membranes of the apicoplast (Hayashi et al., 2001). This observation is consistent with vesicular fusion and hence a GEF being required for ongoing protein import into the organelle. Our work indicates that if vesicles transport proteins from the ER to apicoplast, this process uses a BFA-resistant GEF.

BFA does however, inhibit the appearance of processed apicoplast proteins. Experiments similar to those shown in Fig. 4, but in which no BFA was used during pulse labeling, indicate that ACP-GFP traverses this novel BFA-sensitive step within 30-60 minutes of synthesis (our unpublished results). Our data indicate that the ability of BFA to block transit peptide cleavage is neither due to a Golgi-mediated reduction in targeting (shown by immunofluorescence) nor to a direct inhibition of the processing enzyme (shown by pulse-chase). The inhibition may be an indirect effect of Golgi disruption or due to an off-target action. However, a more intriguing possibility is that BFA inhibits a Sec7-related protein required for an intermediate step in apicoplast targeting. Once a protein reaches the outer membrane of the apicoplast, inhibition of passage through the various membranes would not be easily detected upon microscopic analysis (the apicoplast has a diameter of approximately 250 μM). Although vesicles have been observed in the periplastid space of several heterokonts (Gibbs, 1979), there is no evidence that they contain proteins en route to the chloroplast stroma (Apt et al., 2002) nor that they require a GEF for trafficking. Another possibility is that a GEF may have been recruited to a different function in the apicoplast. At the chloroplast translocon, the target protein associates with the receptors Toc34 and Toc159 in their GTP-bound state. GTP hydrolysis promotes translocation (Becker et al., 2004). Resetting the translocon requires the GDP to be replaced by GTP (Schleiff et al., 2003), but to date there are no reports of GEFs that catalyze this step. In scans of the *T. gondii* genome, we have identified multiple proteins that contain Sec7 domains. Although none of these proteins have canonical apicoplast targeting domains as defined by current paradigms, their targeting to an intermediate compartment may require a unique signal.

The final step in apicoplast targeting is cleavage of the transit peptide domain. The abundance of plastid precursor proteins containing transit peptides in apicomplexan protein extracts, as compared to their scarcity in plant protein extracts, suggests a bottleneck either in the cleavage itself or in final import into the lumen of the plastid. In either case it is clear that an hour or more can elapse from when apicoplast protein passes the BFA-sensitive point in the pathway to when the transit peptide is cleaved. Reconciling these benchmarks in apicoplast import with the molecular mechanisms that affect them provides an exciting challenge in understanding the biology of apicomplexan parasites.

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