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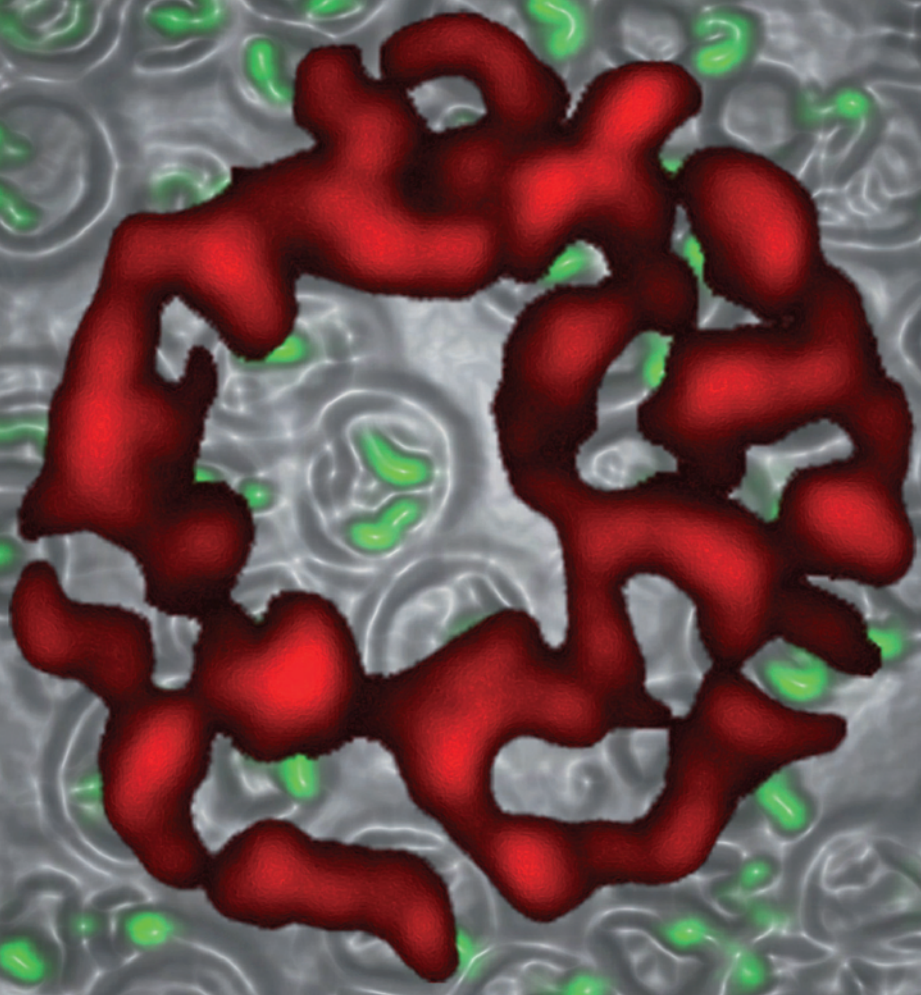
On the cover: Malarial plastid assembly



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Evidence for Golgi-independent transport from the early secretory pathway to the plastid in malaria parasites

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Summary

The malaria parasite *Plasmodium falciparum* harbours a relict plastid (termed the apicoplast) that has evolved by secondary endosymbiosis. The apicoplast is surrounded by four membranes, the outermost of which is believed to be part of the endomembrane system. Nuclear-encoded apicoplast proteins have a two-part N-terminal extension that is necessary and sufficient for translocation across these four membranes. The first domain of this N-terminal extension resembles a classical signal peptide and mediates translocation into the secretory pathway, whereas the second domain is homologous to plant chloroplast transit peptides and is required for the remaining steps of apicoplast targeting. We explored the initial, secretory pathway component of this targeting process using green fluorescent reporter protein constructs with modified leaders. We exchanged the apicoplast signal peptide with signal peptides from other secretory proteins and observed correct targeting, demonstrating that apicoplast targeting is initiated at the general secretory pathway of *P. falciparum*. Furthermore, we demonstrate by immunofluorescent labelling that the apicoplast resides on a small extension of the endoplasmic reticulum (ER) that is separate from the *cis*-Golgi. To define the position of the apicoplast in the endomembrane pathway in relation to the Golgi we tracked apicoplast protein targeting in the presence of the secretory inhibitor Brefeldin A (BFA), which blocks traffic between the ER and Golgi. We observe apicoplast targeting in the presence of BFA despite clear perturbation of ER to Golgi traffic by the inhibitor, which suggests that the apicoplast resides upstream of the *cis*-Golgi in the parasite's endomembrane system. The addition of an ER retrieval signal (SDEL) – a sequence recognized by the *cis*-Golgi protein ERD2 – to the C-terminus of an apicoplast-targeted protein did not markedly affect apicoplast targeting, further demonstrating that the apicoplast is upstream of the Golgi. Apicoplast transit peptides are thus dominant over an ER retention signal. However, when the transit peptide is rendered non-functional (by two point mutations or by complete deletion) SDEL-specific ER retrieval takes over, and the fusion protein is localized to the ER. We speculate either that the apicoplast in *P. falciparum* resides within the ER directly in the path of the general secretory pathway, or that vesicular trafficking to the apicoplast directly exits the ER.

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Introduction

Malaria is one of the world's most devastating diseases, infecting well over 300 million people annually and killing between 2 and 3 million (Snow *et al*., 2005). *Plasmodium falciparum* is the most deadly malaria-causing parasite, and increasing parasite resistance to many existing drugs is exacerbating disease (Gelb and Hol, 2002). A decade ago it was discovered that *Plasmodium* parasites harbour a relict, non-photosynthetic plastid (McFadden *et al*., 1996; Wilson *et al*., 1996; Köhler *et al*., 1997). Research into the malaria plastid – commonly known as the apicoplast – has revealed a set of novel biochemical pathways, which could be exploited to develop new antimalarial compounds directed specifically against this deadly human pathogen (Ralph *et al*., 2004; Surolia *et al*., 2004; Wilson, 2005).

The apicoplast has four surrounding membranes and is believed to have arisen by a process known as secondary endosymbiosis (van Dooren *et al*., 2001). A consequence of secondary endosymbiosis is the creation of multiple membranes (either three or four) around the secondary plastid (van Dooren *et al*., 2001). In secondary endosymbiosis the endosymbiont apparently ends up within the lumen of the host cell's endomembrane system (McFad-

den, 2001; Keeling, 2004), either the rough endoplasmic reticulum (ER) in the case of heterokont algae or an undefined endomembrane compartment in several other organisms including the apicoplast of malaria (and other apicomplexan) parasites.

Most proteins that function in secondary plastids are encoded in the nucleus and must make their way back through the multiple membranes (Deane *et al*., 2000; McFadden and van Dooren, 2004). The first step of this complex routing of proteins into any secondary endosymbiont involves the secretory pathway, and all secondary plastid-targeted proteins identified thus far possess a signal peptide on their N-terminus (Grossman *et al*., 1990; Bhaya and Grossman, 1991; Sulli and Schwartzbach, 1996; Lang *et al*., 1998; Waller *et al*., 1998; Wastl and Maier, 2000; Nassoury *et al*., 2003; Bachvaroff *et al*., 2004; Hackett *et al*., 2004). In *Plasmodium* this signal peptide is believed to mediate translocation across the rough ER membrane via the Sec apparatus depositing the protein into the lumen of the ER wherein the signal peptide is removed by signal peptidase (van Dooren *et al*., 2000a; 2002). At this stage the second component of the bipartite leader, the so-called transit peptide, takes over and is responsible for transport into the endosymbiont across the remaining bounding membranes (Waller *et al*., 2000). This two-step process of protein translocation into secondary plastids has been further demonstrated in the related parasite *Toxoplasma gondii* (DeRocher *et al*., 2000; 2005; Yung *et al*., 2001; Harb *et al*., 2004), and also holds true for other organisms with multimembrane plastids (Sulli *et al*., 1999; Wastl and Maier, 2000; Apt *et al*., 2002; Nassoury *et al*., 2003; Patron *et al*., 2005). Such molecular dissection of this two-part leader of organisms with secondary plastids indicates that targeting to all secondary plastids proceeds via the secretory pathway (van Dooren *et al*., 2000b).

Several key questions about the first steps of apicoplast targeting in malaria parasites remain unresolved. First, it is not clear whether apicoplast-destined proteins bear canonical signal peptides or signal peptides with specific apicoplast-targeting information. It has been suggested that secondary plastid proteins perhaps bear special signal peptides that work by diverting the plastid proteins into a distinct domain of the endomembrane system (Schwartzbach *et al*., 1998). Second, it is not understood whereabouts within the endomembrane system the apicoplast lies and hence which parts of the endomembrane system proteins traverse *en route* to the plastid. Electron microscopy localized the plastid within the ER in heterokont algae, haptophytes and cryptomonads, clearly showing that the outer plastid membrane is continuous with the nuclear envelope and studded with ribosomes. This means that the endosymbiont resides within the lumen of the rough ER in these algae (Gibbs, 1979). The location of the malaria parasite apicoplast is less obvious because the outer membrane is smooth and no permanent connection with other components of the endomembrane system has yet been visualized (Hopkins *et al*., 1999; Kohler, 2005). Thus, the apicoplast could be pre or post-Golgi and could lie along the default secretory pathway to the parasitophorous vacuole (PV) or be one of numerous terminal destinations in the parasite's secretory pathways (van Dooren *et al*., 2000a). In the related parasite *T. gondii* the apicoplast sits just anterior to the nucleus adjacent to the ER and Golgi (McFadden and Waller, 1997; McFadden and Roos, 1999).

In this paper we investigate the route of *P. falciparum* apicoplast-targeted proteins. We show the efficacy of non-apicoplast signal peptides in mediating apicoplast targeting *in vivo* by exchanging them into a bipartite apicoplast leader in conjunction with a reporter protein (GFP). We also explore the site of the apicoplast within the endomembrane system and demonstrate that the apicoplast shares a close association with the ER. We provide evidence that apicoplast proteins do not pass through the Golgi by showing that targeting and ER/apicoplast association persists in the presence of the ER to Golgi transport inhibitor Brefeldin A (BFA). Finally, we set up an *in vivo* competition assay between an apicoplast transit peptide and the C-terminal ER retention motif (SDEL), which is recognized in the *cis*-Golgi. We show that a wild-type transit peptide is dominant over the ER retention motif whereas a dysfunctional (or absent) transit peptide allows the ER-retention motif to dominate thereby suggesting that the apicoplast is pre-Golgi.

Results

Apicoplast proteins traffic via the general secretory pathway

To investigate whether apicoplast signal peptides contain specific targeting information we fused *P. falciparum* signal peptides not normally involved in apicoplast targeting with an apicoplast transit peptide in the context of a GFP fusion protein (Fig. 1A). First, we fused the signal peptide from EBA175 (Erythrocyte Binding Antigen 175 kDa) to ACP transit peptide [ACP(t)] to create the chimeric apicoplast leader EBA175(s)-ACP(t)-GFP (Fig. 1Aii). In the second construct the KAHRP (Knob Associated Histidine Rich Protein) signal peptide was fused to ACP(t) to create KAHRP(s)-ACP(t)-GFP (Fig. 1Aiii). KAHRP has an unusual, so-called 'recessed' signal peptide (Triglia *et al*., 1987) that directs the protein into the parasite's secretory pathway (Wickham *et al*., 2001). An additional pentameric motif located downstream of the KAHRP signal peptide cleavage site then directs the protein out of the parasite and into the cyto-

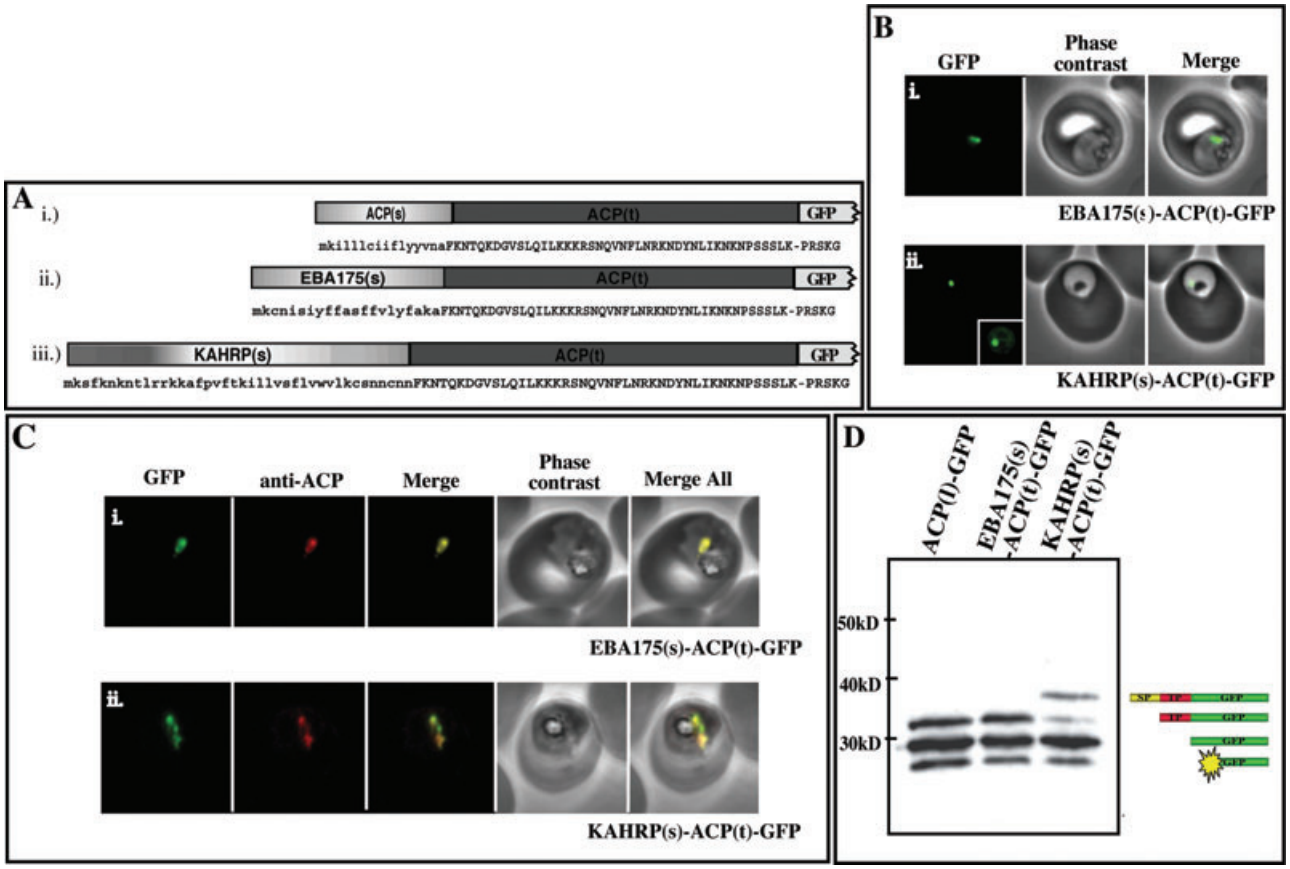


Fig. 1. Signal peptides from apicoplast and non-apicoplast proteins are interchangeable.
A. Schematic diagram and sequence of ACP(l) (i) and signal peptide chimeric constructs EBA175(s)-ACP(t) (ii) and KAHRP(s)-ACP(t) (iii). Signal peptides are outlined in light grey and their corresponding sequence in lowercase letters. ACP transit peptide [ACP(t)] is in dark grey and its sequence is in uppercase.
B. Live images of transgenic parasites expressing EBA175(s)-ACP(t)-GFP (i) and KAHRP(s)-ACP(t)-GFP (ii), which display a typical fluorescence pattern of an apicoplast localization (Waller *et al*., 2000). Overexposed images of KAHRP(s)-ACP(t)-GFP expressing parasites (ii – inset) show extra-apicoplast staining, which is the ER (not shown).
C. IFA of parasites expressing chimeric apicoplast leaders. EBA175(s)-ACP(t)-GFP (i) and KAHRP(s)-ACP(t)-GFP (ii) expressing parasites labelled with anti-ACP antibody. In both cases GFP colocalizes with ACP further outlining that chimeric apicoplast leaders correctly target to the apicoplast lumen.
D. Western blot of ACP(l)-GFP, EBA175(s)-ACP(t)-GFP and KAHRP(s)-ACP(t)-GFP expressing parasites. Schematic diagram to the right shows each bands predicted identity. All lanes show bands corresponding to GFP with transit peptide still attached, GFP with transit peptide processed and a GFP degradation product (Waller *et al*., 2000; Wickham *et al*., 2001). Cleavage of the transit peptide most likely occurs in the apicoplast (Sato *et al*., 2004; van Dooren *et al*., 2005) and therefore transit peptide processed GFP is a sign of apicoplast targeting. KAHRP(s)-ACP(t)-GFP expressing parasites also display a higher molecular mass species that is the right size for the signal peptide to be still attached (see text).

plasm of the host blood cell (Marti *et al*., 2004). The pentameric export motif was not included in our KAHRP(s)-ACP(t)-GFP construct (Fig. 1Aiii).

Analysis of resulting EBA175(s)-ACP(t)-GFP live transgenic parasites shows a typical apicoplast localization (Fig. 1Bi). KAHRP(s)-ACP(t)-GFP expressing parasites also show much the same pattern of GFP fluorescence (Fig. 1Bii), but in addition show faint fluorescence in a structure reminiscent of the ER (Fig. 1Bii, inset). Indirect immunofluorescence assay (IFA) colocalization of the reporter constructs with the native apicoplast protein ACP demonstrates trafficking of EBA175(s)-ACP(t)-GFP and KAHRP(s)-ACP(t)-GFP to the apicoplast (Fig. 1C, i and ii). The additional fluorescence observed in early-stage KAHRP(s)-ACP(t)-GFP expressing cells colocalizes with the ER marker BiP but not the *cis*-Golgi marker ERD2 (not shown).

Trafficking of apicoplast proteins involves a proteolytic cleavage event that removes the transit peptide inside the apicoplast (van Dooren *et al*., 2002) and therefore this is another indication of apicoplast targeting. We monitored proteolytic cleavage of EBA175(s)-ACP(t)-GFP and KAHRP(s)-ACP(t)-GFP on a western blot and compared this with the proteolytic profile of parasites expressing

ACP(l)-GFP (Fig. 1D) (van Dooren *et al*., 2005). This western blot shows that both chimeric apicoplast leaders are faithfully processed from a higher molecular weight to a mature form (Fig. 1D) and we also see a protein species previously identified as a GFP degradation product (Fig. 1D) (Waller *et al*., 2000; Wickham *et al*., 2001). Additionally, KAHRP(s)-ACP(t)-GFP expressing parasites show a low-abundance, higher-molecular-weight precursor at a size that is consistent with the KAHRP signal peptide still being attached (Fig. 1D). We are not usually able to detect signal peptide-bearing apicoplast-destined proteins, probably because signal peptidase removes the signal peptide during translation/translocation into the ER (von Heijne, 1999). Exactly why a KAHRP signal peptide-bearing precursor in KAHRP(s)-ACP(t)-GFP expressing parasites exists is unknown. We postulate that this chimeric apicoplast leader is cleaved kinetically slower, which results in the small amount of GFP we observe in the ER in this parasite line (Fig. 1Bii, inset); however, we cannot discount any other possibility.

The apicoplast has a close association with the ER

We wished to investigate the relationship between the apicoplast and the parasite's secretory apparatus. Unfortunately, current fixing and preparative techniques for electron microscopy yield very poor membrane preservation in *P. falciparum*, and the identity of the outermost apicoplast membrane has remained elusive (Hopkins *et al*., 1999; Kohler, 2005). To address the relationship between the apicoplast and the parasite's secretory system we performed simultaneous immunofluorescent localization of the apicoplast and the ER, in late ring and early trophozoite stage parasites, in which these structures are non-branched and in their simplest forms. Parasites expressing ACP(l)-GFP [in which GFP exclusively localizes to the apicoplast (Waller *et al*., 2000)] were labelled with an antibody directed towards the ER marker BiP (Elmendorf and Haldar, 1993) and extensive confocal microscopy was performed on a large number of cells (representative samples in Fig. 2 and Fig. S1). In early erythrocytic stages the ER in *P. falciparum* shows a simple perinuclear structure with two small distensions at one end of the cell (commonly towards the growing food vacuole; van Dooren *et al*., 2005). In all cells examined the apicoplast was in close association with one of these two distensions of the ER (Fig. 2A and B and Fig. S1). Figure 2A shows a parasite in which apicoplast appears to be 'cupped' by the ER, as viewed by stacked optical sections (Fig. 2Ai). When focusing on the apicoplast in a single optical section, the apicoplast and the ER are very close (Fig. 2Aii). Figure 2B shows an infected red blood cell that contains three parasites (although one contains no ER and therefore is probably dead). Viewed by stacked optical sections strands of ER meet up with each of the small apicoplasts (Fig. 2Bi). By focusing separately on each apicoplast in single optical sections a strand of ER is observed to be very close to each of the apicoplasts (Fig. 2Bii). Reconstruction of serial optical sections rotated around a 360° axis of both specimens also shows the close position of the ER and apicoplast (*Supplementary material*, Movies S1 and S2). More images of single optical sections showing the location of the ER and its intimate relationship to the apicoplast can be viewed in the Fig. S1.

Brefeldin A does not inhibit apicoplast targeting in P. falciparum

To investigate further the relationship between the secretory pathway and the apicoplast we treated cells with BFA – an inhibitor of ER to Golgi transport – and observed the effect on apicoplast targeting. Based on our morphological observations outlined above, we formed the hypothesis that the apicoplast – like some other secondary plastids – is located slightly distal to the ER but proximal to the Golgi. Accordingly we predicted that BFA would not block the transport of proteins to the apicoplast.

We tested the effect of BFA on targeting to the apicoplast by using three transgenic cell lines: two apicoplast leader mutants ACP(s)-GFP (Waller *et al*., 2000) and ACP(l)K18E_K22E-GFP (Foth *et al*., 2003) that secrete GFP via the Golgi out to the PV surrounding the parasite in a red blood cell; and ACP(l)-GFP (Waller *et al*., 2000), which faithfully targets GFP to the apicoplast. ACP(s)-GFP and ACP(l)K18E_K22E-GFP cell lines were tightly synchronized and then treated with BFA immediately after invasion. At this stage, these cells are devoid of fluorescence because GFP in the PV is lost at the previous schizont egress, thus avoiding the problem of GFP protein accumulated in the compartment of interest prior to application of the ER to Golgi transport inhibitor (BFA). After BFA treatment, the fluorescence pattern of all parasite lines was observed (Fig. 3A). BFA-treated ACP(s)-GFP and ACP(l)K18E_K22E-GFP expressing parasites no longer showed fluorescence around the periphery of the parasite (Fig. 3Ai and iii respectively) but instead showed an internal GFP localization (Fig. 3Aii and iv respectively) consistent with the structure of the ER in early-stage parasites after treatment with BFA (Wickham *et al*., 2001).

Visualising the fluorescence pattern of ACP(l)-GFP cells after BFA treatment showed no difference in the localization pattern of GFP (Fig. 3A, v and vi). To address whether the lack of change in fluorescence pattern reflected an insensitivity of apicoplast targeting to BFA or simply a pool of stable GFP targeted to the

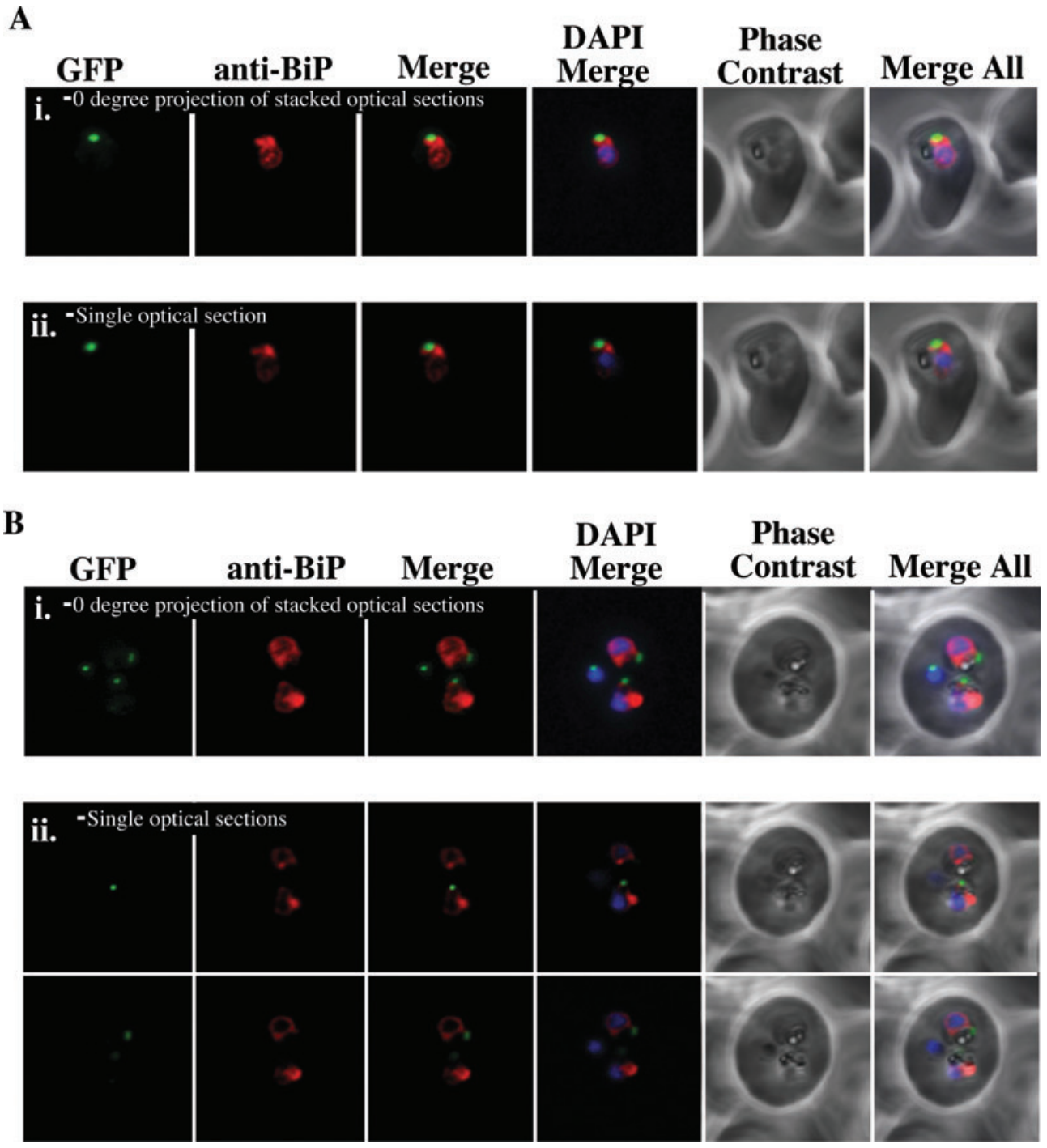


Fig. 2. The apicoplast has a close association with the ER. ACP(I)-GFP expressing parasites (GFP in the apicoplast) were analysed by IFA with anti-BiP antibody labelling to assess the relationship between the ER and the apicoplast.
A. Anti-BiP IFA of a ring-stage parasite expressing ACP(I)-GFP. (i) A 0° projection of stacked optical sections demonstrating that the apicoplast is 'cupped' by a strand of ER. (ii) A single optical section demonstrating that the apicoplast and ER are closely juxtaposed. A 360° rotation of this sample can be viewed in *Supplementary material* (Movie S1).
B. Anti-BiP IFA of multiple ACP(I)-GFP expressing parasites within the same erythrocyte. (i) A 0° projection of stacked optical sections showing that each apicoplast is closely associated with a strand of ER. (ii) Two single optical sections focus on each parasites apicoplast and show that the apicoplast and the ER are closely apposed. The third parasite in the erythrocyte is devoid of ER and is probably dead. A 360° rotation of this sample can be viewed in *Supplementary material* (Movie S2).

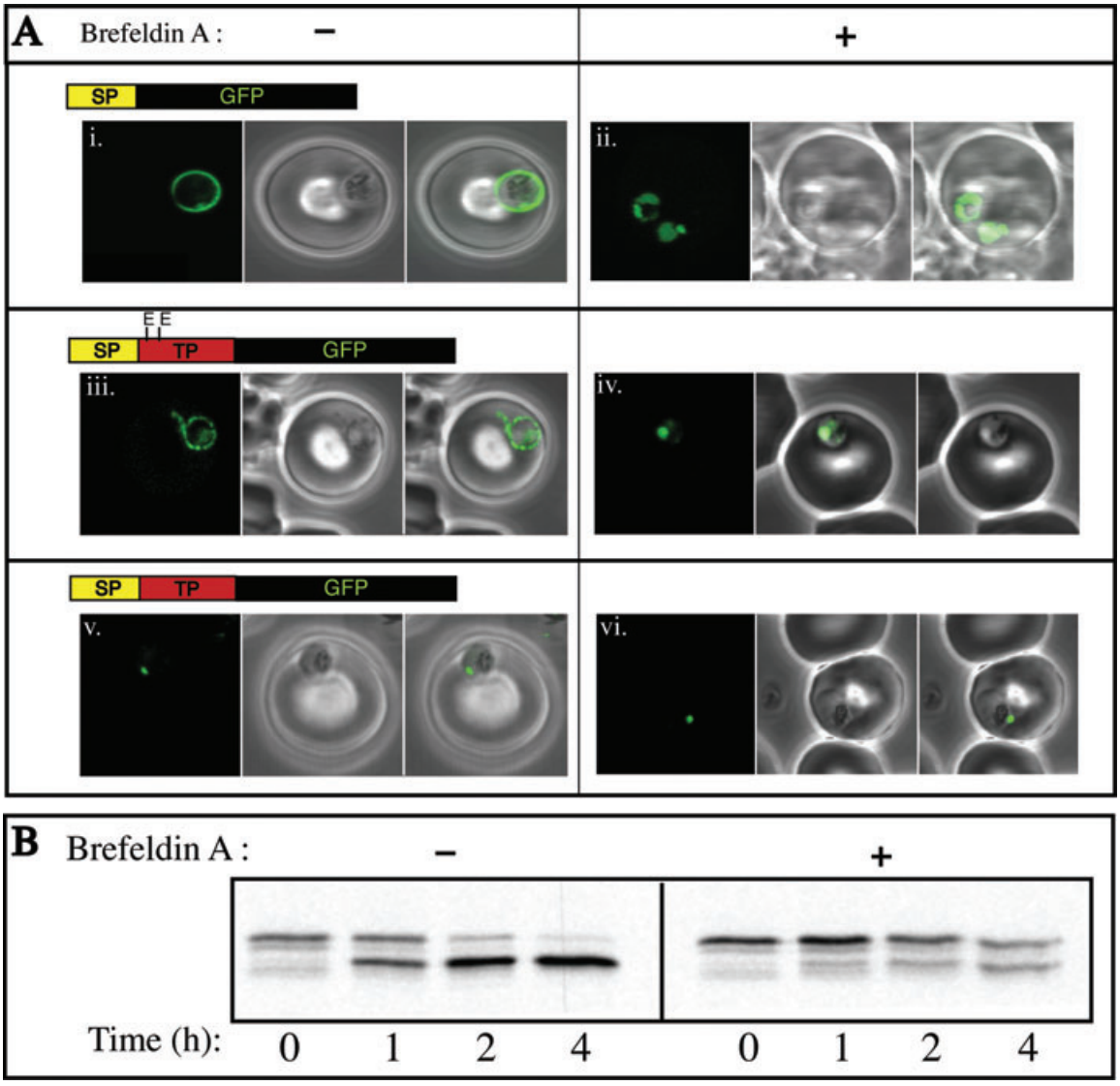


Fig. 3. The effect of BFA on apicoplast targeting.
A. The localization of GFP in the cell lines ACP(s)-GFP, ACP(l)K18E_K22E-GFP and ACP(l)-GFP (Waller *et al*., 2000) in the presence (+) and absence (–) of 5 μg ml^{-1} of BFA was examined. ACP(s)-GFP and ACP(l)K18E_K22E-GFP parasites were forced to re-synthesise GFP at the start of a new cycle in the presence of BFA. (i) An ACP(s)-GFP expressing parasite – untreated BFA control (–). GFP is localized to the PV. (ii) Two ACP(s)-GFP parasites in a single red blood cell with BFA (+). In the presence of BFA GFP fluorescence is retained within the parasite, demonstrating that targeting of protein to the PV is sensitive to the effects of BFA. (iii) An ACP(l)K18E_K22E-GFP expressing parasite – untreated BFA control (–). GFP is localized to the PV and an extension of the PV known as the tubovesicular network. (iv) An ACP(l)K18E_K22E-GFP expressing parasite treated with BFA (+). In the presence of BFA GFP fluorescence is retained within the parasite, also demonstrating that targeting of protein to the PV is sensitive to the effects of BFA. (v) An ACP(l)-GFP expressing parasite – untreated BFA control (–). GFP is localized to the apicoplast. (vi) An ACP(l)-GFP expressing parasite treated with BFA (+). In the presence of BFA GFP localization does not change in comparison with the control.
B. Pulse-chase and anti-GFP immunoprecipitation of ACP(l)-GFP expressing parasites. Cells were treated in the presence (+) or absence (–) of 5 μg ml^{-1} of BFA and then pulsed with [^{35}S] methionine (45 min) and chased over a 3 h time period. Apicoplast protein targeting was then assayed by observing the proteolytic cleavage of the transit peptide under such conditions. Using this sensitive method it can be seen that BFA has only a partial effect on apicoplast targeting, compared with untreated (–). Although transit peptide processing slows down over a period of 4 h, it does not stop.

apicoplast that had persisted before drug treatment, we also analysed apicoplast protein targeting using a pulse-chase protocol (Fig. 3B). Previously, it has been shown that apicoplast targeting involves the removal of the transit peptide, and that this processing event can be monitored using a [^{35}S]-methionine pulse-chase approach coupled with immunoprecipitation (van Dooren *et al*., 2002). Using such an approach in the presence of BFA (added 3 h earlier) we monitored the effect of BFA by analysing the protein processing profile. Although

BFA slowed processing, processing clearly occurs over the 4 h chase (Fig. 3B). Partial inhibition of processing by BFA could be due to general retardation of secretory pathway flow or even parasite growth during the chase period because the secretory system is largely disabled in this experiment (Elmendorf and Haldar, 1993). Alternatively, a certain portion of the target molecules for BFA may only be partially inhibited by this fungal metabolite. Nevertheless, we conclude that BFA treatment, using concentrations that inhibit secretory traffic to the PV, permits substantial trafficking to the apicoplast. Failure of BFA to abrogate apicoplast targeting is consistent with our morphological findings that the apicoplast is proximal to the Golgi in the secretory pathway. This conclusion is based upon two assumptions, the first is that native protein trafficking occurs in the ACP(l)-GFP transgenic cell line, and second that removal of the transit peptide occurs post import into the apicoplast, which seems reasonable because the stromal processing peptidase (which removes the transit peptide) localizes to the apicoplast stroma (van Dooren *et al*., 2002; Sato *et al*., 2004).

Treatment with BFA does not disrupt the apicoplast-ER association

Given that BFA is ineffective in stopping apicoplast protein targeting, and that the apicoplast and the ER share a close association, we were interested to observe the morphology of these two compartments in the presence of BFA (Fig. 4). ACP(l)-GFP transgenic parasites were treated with BFA and then fixed, permeabilized and the ER observed using anti-BiP antibodies (Fig. 4). Cell images were viewed as both 0° projection of stacked optical sections (Fig. 4Ai and Bi), single optical sections (Fig. 4Aii and Bii), or reconstructed into a 3D image rotating through 360° (*Supplementary material*; Movies S3 and S4).

In the presence of BFA the ER changes morphology and relocalizes to the periphery of the parasite (Fig. 4). Nevertheless, the apicoplast and the ER remain closely associated in the presence of BFA as judged from stacked optical sections (Fig. 4Ai and Bi) and single optical sections (Fig. 4Aii and Bii) and by viewing and 3D reconstructions (*Supplementary material*; Movies S3 and S4). This observation is consistent with apicoplast targeting continuing in the presence of BFA (as demonstrated above).

Apicoplast transit peptide function is dominant over an ER retention signal

On the basis of the preceding data we suggest that apicoplast proteins do not pass through the Golgi but rather exit straight from the ER. To further test this hypothesis we fused the C-terminal *P. falciparum* ER retention signal – SDEL – that is recognized by the *cis*-Golgi protein ERD2, to ACP(l)-GFP to yield ACP(l)-GFP-SDEL (Fig. 5Ai). We also fused SDEL to two apicoplast leader mutants ACP(s)-GFP and ACP(l)K18E_K22E-GFP to yield ACP(s)-GFP-SDEL and ACP(l)K18E_K22E-GFP-SDEL respectively (Fig. 5Bi and Ci). We then assayed apicoplast targeting by microscopy and western blot analysis (Fig. 5).

Transgenic parasites expressing ACP(l)-GFP-SDEL were analysed by microscopy (Fig. 5A). Live parasites showed GFP in a single distinct spherical organelle (Fig. 5Aii) in early-stage parasites that then developed in later stage parasites into a large reticulated structure (Fig. 5Aiv) characteristic of the apicoplast (Waller *et al*., 2000). Additionally in late rings and early trophozoites GFP extends into a structure resembling the ER (Fig. 5Aiii). Immunofluorescence analysis with anti-ACP antibodies shows that ACP(l)-GFP-SDEL localizes to the apicoplast (Fig. 5Av). The additional fluorescence in early-stage parasites colocalizes with the ER marker BiP (Fig. 5Avi), which may signify a slowing down in apicoplast targeting due to transitory interactions with ER-located ERD2 (see below). Intriguingly, the fluorescence pattern of ACP(l)-GFP-SDEL in early stages appears to be continuous between the ER and the apicoplast (Fig. 5Aiii, v and viii), also suggesting a possible connection between these two organelles. It was therefore also important to look at the localization of ACP-GFP-SDEL in relation to ERD2.

ERD2 is the receptor for the ER retention motif SDEL, and ERD2 functions by recognizing XDEL (X being a species-specific amino acid which happens to be either S or I in the case of *P. falciparum*) containing proteins in the *cis*-Golgi and moving them back via retrograde transport to the ER. Therefore, the greatest concentration of ERD2 is in the *cis*-Golgi, but it is also found in the ER (Struck *et al*., 2005). We performed careful analysis of ACP-GFP-SDEL expressing parasites labelled with anti-ERD2 and found that although GFP colocalizes with a portion of ERD2, which resembles the ER (Struck *et al*., 2005), the most intense spot of ERD2 staining is separate from all GFP including that of the apicoplast (Fig. 5Avii). This is most easily seen looking at a single optical section focusing on the apicoplast (Fig. 5Aviii). In such an optical section the intense spot of the ERD2 staining is not present (compare Fig. 5Avii and viii) and looking at a 360° rotation it is clear that the *cis*-Golgi sits adjacent to the apicoplast (*Supplementary material*, Movie S5). Using our analysis we cannot, however, discount that a small amount of GFP is in the *cis*-Golgi. Western blot analysis of ACP(l)-GFP-SDEL (Fig. 5D) shows a molecular weight species consistent with a processing event similar to that observed with other apicoplast-targeted proteins (Fig. 1 and Waller *et al*., 2000). It should be noted, however, that

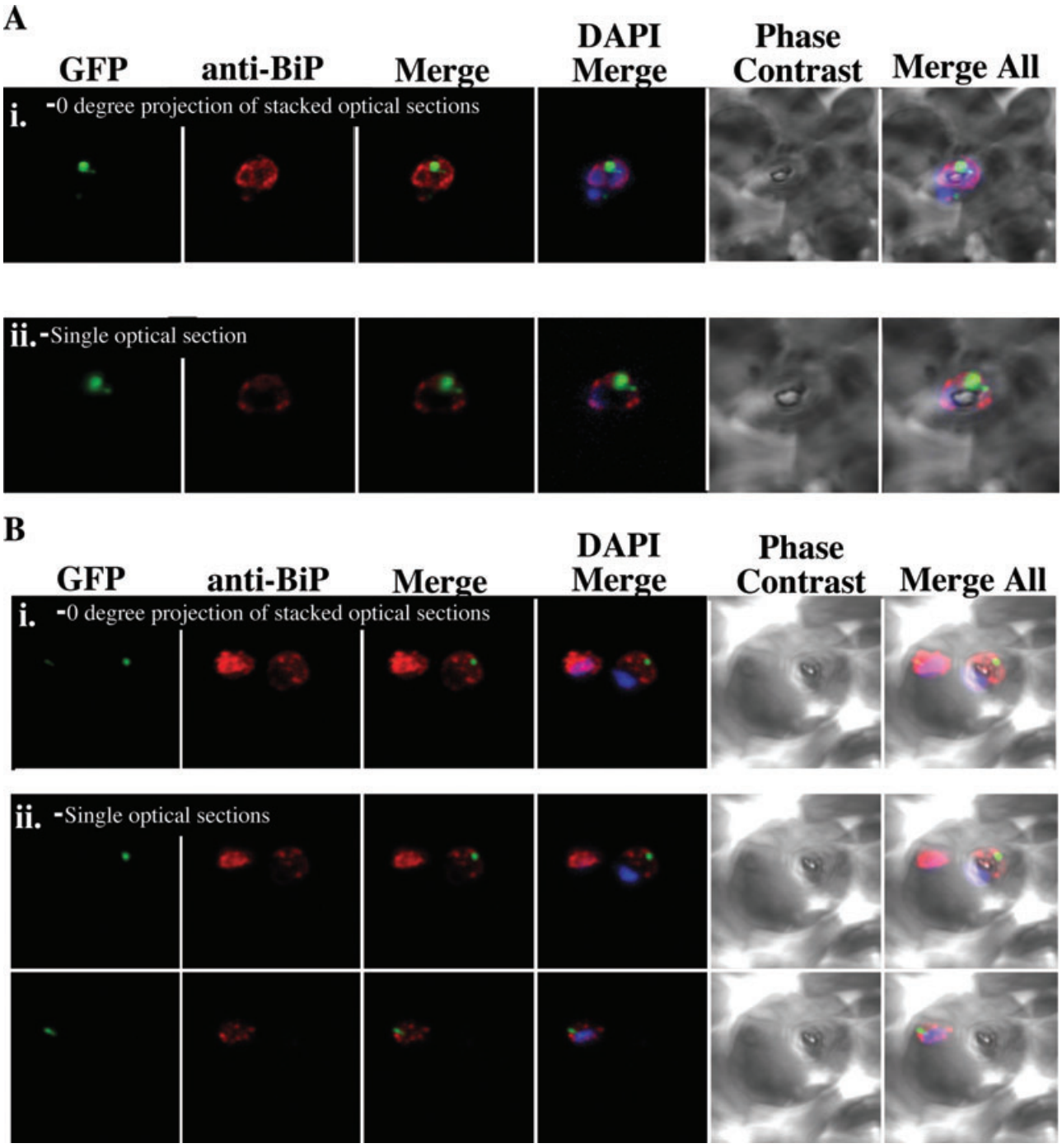


Fig. 4. BFA treatment does not abolish the apicoplast–ER association. ACP(I)-GFP expressing parasites were treated with 5 μg ml^{-1} of BFA for 3 h and then analysed by IFA with anti-BiP antibody labelling to assess the relationship that the ER and apicoplast in the presence of BFA.
A. Anti-BiP IFA of a BFA-treated ACP(I)-GFP expressing parasite. (i) 0° projection of stacked optical sections demonstrating that the ER has changed shape and now shows a more peripheral localization. However, the apicoplast and ER still share common space. (ii) A single optical section demonstrating that the even in the presence of BFA the apicoplast and ER are still closely juxtaposed. A 360° rotation of this sample can be viewed in *Supplementary material* (Movie S3).
B. Anti-BiP IFA of two ACP(I)-GFP expressing parasites treated with BFA. (i) A 0° projection of stacked optical sections demonstrating that each apicoplast is closely associated with a strand of ER. (ii) Two single optical sections demonstrate that both parasites apicoplasts are closely opposed with the ER. A 360° rotation of this sample can be viewed in *Supplementary material* (Movie S4).

there is a higher proportion of unprocessed (transit peptide containing) product than normally seen by western blot (Fig. 1D), this is also seen in *T. gondii* (DeRocher *et al*., 2005) and again this may be due to a slowing down of targeting due to transitory interactions with ERD2 in the ER (also see below) and therefore more transit peptide-containing product.

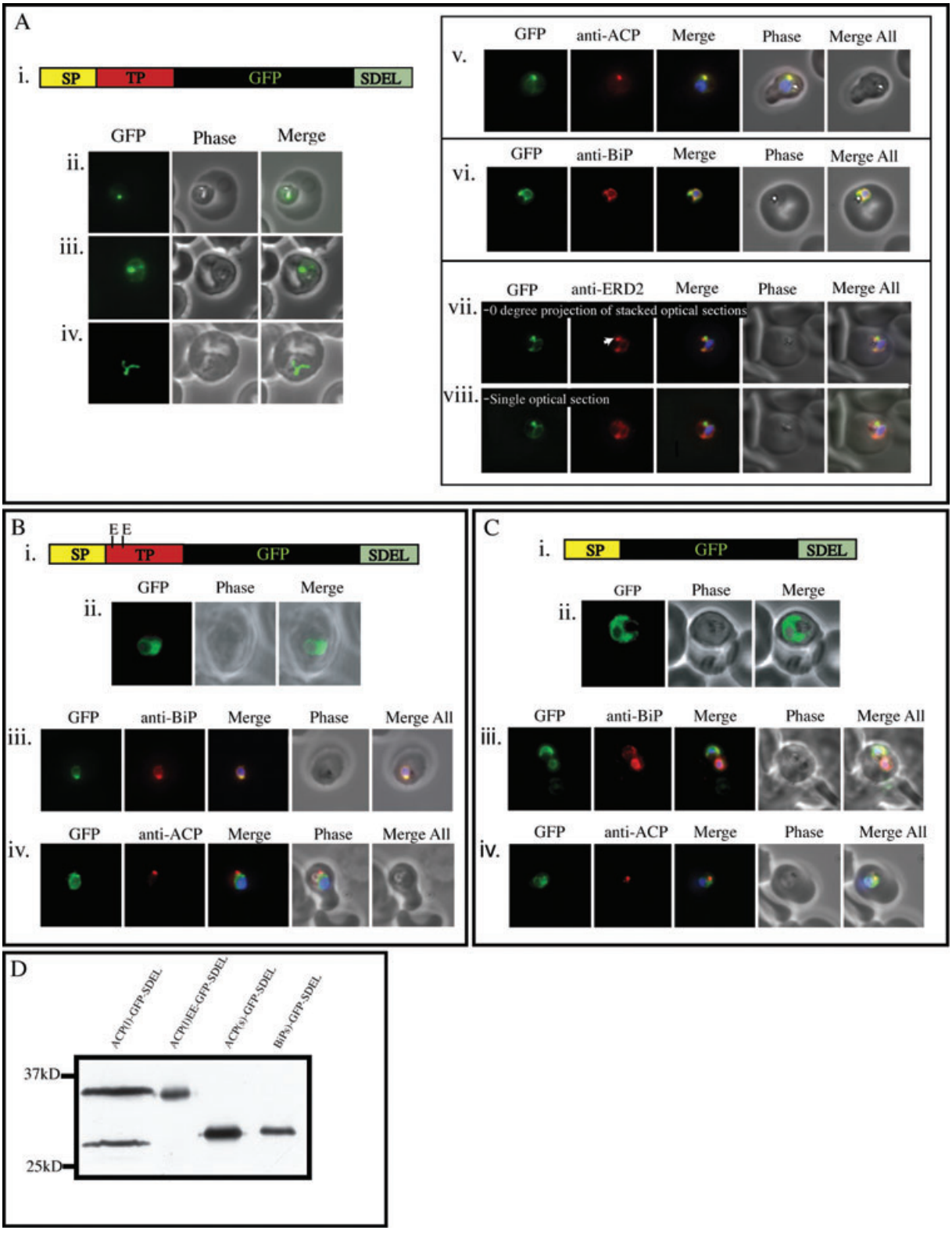
We combined two dysfunctional transit peptides with the ER retention signal to confirm that the SDEL motif would resume dominance in the absence of apicoplast targeting. When the ACP transit peptide function is ablated by mutating the first two positively charged lysines to negatively charged glutamic acids, or by removing the transit peptide all together (Fig. 5B and C respectively), the SDEL retention motif takes over and the GFP fusion protein becomes perinuclear with two 'horn-like' structures (Fig. 5Bii and Cii respectively) typical of an ER localization (van Dooren *et al*., 2005; Struck *et al*., 2005). Indeed, both ACP(l)K18E_K22E-GFP-SDEL and ACP(s)-GFP-SDEL fluorescence colocalizes with the ER marker BiP (Fig. 5Biii and Ciii) and is close, but does not overlap with anti-ACP (which corroborates our previous observations regarding the apicoplast – ER close association) (Fig. 5Biv and Civ). Furthermore, western blot analysis in both fusion proteins shows an absence of a processed (transit peptide removed) form. ACP(l)K18E_K22E-GFP-SDEL is the same size as pre-processed GFP of ACP(l)-GFP-SDEL (Fig. 5D) whereas ACP(s)-GFP-SDEL is the same size as BiP(s)-GFP-SDEL (Fig. 5D), another GFP fusion protein that we have previously shown to localize to the ER (van Dooren *et al*., 2005). Both the IFA and western blot results are consistent with ACP(l)EE-GFP-SDEL and ACP(s)-GFP-SDEL remaining in the ER and not making it into the apicoplast lumen. Overall, these reporter constructs demonstrate that the transit peptide (and recognition by its cognate receptor) is dominant over the recognition of the SDEL ER retention motif by ERD2. This strongly suggests that the apicoplast is situated upstream of the *cis*-Golgi in the secretory pathway, before ERD2 affects anterograde secretory traffic.

Discussion

At least 12 subcellular and extracellular destinations have been identified for secretory pathway proteins in *Plasmodium* parasites (van Dooren *et al*., 2000b). Targeting motifs for some of these destinations have been revealed (Waller *et al*., 2000; Foth *et al*., 2003; Gilberger *et al*., 2003 #101; Marti *et al*., 2004; van Dooren *et al*., 2005) while others still remain elusive. It is possible that the signal peptide itself encodes some targeting information. Some parasite proteins targeted out to the red blood cell are unusual in that the hydrophobic domain of their signal peptide is indented from the most N-terminus, but the function, if any, for this feature is unknown. We have tested whether any information contributing to apicoplast targeting may be held within the apicoplast protein's signal peptide. We fused two different signal peptides to the ACP transit peptide [ACP(t)] to create two different chimeric apicoplast leaders Fig. 1. In the first case the signal peptide from EBA175 was fused to ACP transit peptide. This so-called 'classical' EBA175 signal peptide normally plays a role in transport of its appended protein to specialized invasive organelles called the micronemes where EBA175 is then subsequently involved in merozoite invasion into the host erythrocyte (Reed *et al*., 2000; Gilberger *et al*., 2003). The second signal peptide was taken from KAHRP and fused to ACP(t). The KAHRP signal peptide has its hydrophobic domain somewhat indented from the N-terminus and is involved in trafficking of the nascent protein into the red blood cell (Wickham *et al*., 2001), where it plays a critical role in cytoadherence of the infected erythrocyte (Crabb *et al*., 1997a).

Analysis of the resulting transgenic parasites harbouring either EBA175(s)-ACP(t)-GFP or KAHRP(s)-ACP(t)-GFP shows GFP fluorescence within the apicoplast and a similar proteolytic cleavage pattern on western blot (Fig. 1). This demonstrates that heterologous signal peptides are interchangeable with a given apicoplast signal peptide and can mediate apicoplast targeting just as efficaciously. Thus, no specific targeting information is located within the apicoplast signal peptide. Furthermore, the interchangeable nature of EBA175(s), KAHRP(s) and ACP(s) suggests that erythrocyte, micronemal and apicoplast-targeted proteins commence their journey at the same place in the secretory pathway. Indeed, given our results it is likely that all proteins bearing a signal peptide begin anterograde transport in a similar manner.

Plasmodium falciparum seems to contain a somewhat 'stripped down' version of the 'classical' eukaryotic secretory apparatus. The parasite's ER has a simple perinuclear structure (van Dooren *et al*., 2005), and the Golgi has been shown to be in an unstacked conformation (Van Wye *et al*., 1996; Struck *et al*., 2005). Furthermore, no classical endosomal trafficking pathway has yet been characterized, although haemoglobin is delivered to the food vacuole by phagocytosis (Klemba *et al*., 2004). *P. falciparum* also seems to lack conventional peroxisomes (McIntosh *et al*., 2005). We were interested to locate the position of the apicoplast within the parasite's rudimentary secretory system. We employed immunofluorescence coupled with confocal microscopy to analyse the relationship between the ER and apicoplast (Fig. 2). Our extensive microscopy clearly positions the apicoplast at the tip of one of the previously described, 'horn-like' extensions of the ER that project from the nuclear envelop in early-stage parasites (van Dooren *et al*., 2005). The



close apposition of the ER and apicoplast has also been seen by electron microscopy in both *P. falciparum* and the closely related parasite *T. gondii* (Hopkins *et al*., 1999; McFadden and Roos, 1999). Other four-membrane plastids also share a close association with the ER. Indeed, the outer plastid membrane in heterokont, haptophyte and

Fig. 5. The apicoplast transit peptide is dominant over an SDEL ER retention motif. An SDEL ER retention motif was appended to the end of three apicoplast leader – GFP fusions. A wild-type ACP(I) transit peptide, ACP(I)EE, which contains a mutant transit peptide and ACP(s), which completely lacks a transit peptide. These constructs were designed to test whether the transit peptide or SDEL is recognised first. This competitive experiment allows inference of the position of the apicoplast relative to the cis-Golgi, which houses the SDEL receptor ERD2. Apicoplast targeting was monitored by western blot and fluorescence pattern of GFP.
A. (i) Schematic representation of ACP(I)-GFP-SDEL and (ii–viii) localisation of GFP of parasites harbouring this construct. GFP fluorescence shows a typical apicoplast localisation (ii–iv) but additionally in late ring and early trophozoite stage parasites shows fluorescence in a structure typical of the ER (iii). (v–viii) Anti-ACP, anti-BiP and anti-ERD2 IFA's on ACP(I)-GFP-SDEL expressing parasites assess the location of GFP. A large amount of GFP co-localises with ACP (v) demonstrating apicoplast targeting, whereas the rest of GFP fluorescence co-localises with the ER marker BiP (vi). GFP however, largely is absent from the cis-Golgi (vii – arrowhead) as ascertained by ERD2 staining (vii–viii). ERD2 localises to the cis-Golgi and also to the ER as it retrieves XDEL containing proteins from the cis-Golgi. Fluorescence from ACP(I)-GFP-SDEL expressing parasites does not overlap with the most intense spot of ERD2 staining (as ascertained by single optical section – viii) suggesting that the apicoplast is not in the cis-Golgi.
B. Transgenic parasites were created harbouring ACP(I)EE-GFP-SDEL (i). The pattern of GFP in live parasites was reminiscent of an ER localisation (ii) (van Dooren *et al*., 2005). All GFP co-localises with anti-BiP (iii) and not with the apicoplast marker ACP (iv).
C. Transgenic parasites expressing ACP(s)-GFP-SDEL (i). ACP(s)-GFP-SDEL has a typical ER localization in live parasites (ii), which also colocalizes with anti-BiP (iii) and not with anti-ACP (iv).
D. A western blot of parasites expressing ACP(I)-GFP-SDEL, ACP(I)EE-GFP-SDEL, ACP(s)-GFP-SDEL and BiP(s)-GFP-SDEL (van Dooren *et al*., 2005). ACP(I)-GFP-SDEL is processed and infers apicoplast targeting whereas ACP(I)EE-GFP-SDEL and ACP(s)-GFP remain unprocessed which confirms the microscopy observations that GFP does not enter the apicoplast when the transit peptide is ineffectual or absent.

cryptomonad algae is studded with ribosomes and is continuous with the nuclear envelope/rough ER (Gibbs, 1979; 1981). In these organisms the endosymbiont is literally situated within the lumen of the rough ER. No ribosomes are observed on the outermost apicoplast membrane, so it is not rough ER. Nevertheless the close association between apicoplast and ER suggests an intimate relationship that probably involves transient continuity – either by vesicle traffic or ephemeral connections – that mediates traffic of presumably soluble, transit peptide-bearing proteins to the apicoplast.

Brefeldin A is a fungal metabolite used to study secretory traffic in eukaryotes. BFA binds the sec7 domain of some ADP-Ribosylation Factor – Guanine nucleotide Exchange Factors (ARF-GEFs) and disrupts interaction with their ARF partners (Mossessova *et al*., 2003). ARFs and ARF-GEFs are essential for vesicle formation at the ER–Golgi interface and therefore their inhibition stops anterograde transport from the ER to Golgi; retrograde transport then redistributes the Golgi back onto the ER. We assayed the effect of BFA on apicoplast targeting to understand the relationship that the apicoplast shares with the secretory pathway. We treated cells expressing ACP(I)-GFP (GFP targeted to the apicoplast) with 5 $\mu g\ ml^{-1}$ of BFA and monitored apicoplast targeting by the transit peptide proteolytic processing event. We showed that apicoplast targeting can still take place in the presence of BFA, albeit at a somewhat reduced rate. Significantly, the localization of two apicoplast leader mutants, ACP(s)-GFP and ACP(I)K18E_K22E-GFP, which normally target GFP out into the PV, is drastically affected and shows a redistribution of GFP to the parasite interior, which resembles the collapsed ER/Golgi complex (Wickham *et al*., 2001). Moreover, we also showed that the relationship between the apicoplast and the ER is maintained in the presence of BFA.

Recently, DeRocher *et al*. (2005) investigated the pathway of apicoplast proteins in the related parasite *T. gondii*. Similar to us, DeRocher *et al*. as well as a study by Brydges and Carruthers (2003), showed that the function of the transit peptide acts before the function of an ER retention motif. DeRocher *et al*. also demonstrate that apicoplast protein trafficking is not affected by treatment with BFA in *T. gondii*. However, in contrast to our findings they observed that BFA does inhibit apicoplast protein proteolytic processing. Indeed, they demonstrate that apicoplast proteolytic processing is inhibited at 1 $\mu g\ ml^{-1}$, which is one-fifth of the concentration (5 $\mu g\ ml^{-1}$) that we used in *P. falciparum*. DeRocher *et al*. suggest that one possibility from these findings is that sensitivity of apicoplast processing – but not apicoplast targeting – to BFA in *T. gondii* could occur because of several differentially BFA-sensitive molecules such as the ARF-GEFs that are involved in the apicoplast trafficking process (DeRocher *et al*., 2005). DeRocher *et al*. suggest a vesicle-mediated step from ER to the apicoplast that is performed by a BFA-resistant ARF-GEF but that a second vesicle-mediated step – suggested to be intra-apicoplast transport (between the apicoplast membranes) – acts through a BFA-sensitive ARF-GEF (DeRocher *et al*., 2005). This partial targeting would not deliver the precursor protein into the stroma of the apicoplast where the stromal processing peptidase, which removes the transit peptide, resides (van Dooren *et al*., 2002; Sato *et al*., 2004) and therefore that is why transit peptide processing is not observed. It would be interesting to see whether apicoplast-targeted proteins in BFA-treated *T. gondii* accumulate around the periphery of the apicoplast, which has been shown previously with other apicoplast-targeting mutants (He *et al*., 2001; Harb *et al*., 2004). Indeed, more work is needed to understand whether BFA in *T. gondii,* and *P. falciparum,* acts only on ARF-GEF

proteins or has other targets and how these relate to the apicoplast and apicoplast targeting.

Recently, Kilian and Kroth (2005). investigated the effect of BFA on protein targeting in another plastid with four membranes, namely the heterokont diatom *Phaeodactylum tricornutum*. Kilian and Kroth (2005) showed that BFA treatment resulted in plastid-targeted protein accumulating in a 'blob'-like structure, which sits between the two lobes of the plastid. This compartment was suggested to lie between the inner and outer plastid membranes and to be the result of blockage of an intraplastid trafficking process, which could involve a vesicle-mediated step reliant on a BFA-sensitive ARF-GEF (Kilian and Kroth, 2005). However, unlike our studies Kilian and Kroth assayed plastid targeting by GFP microscopy rather than observing the transit peptide cleavage event. It will be interesting to learn if the partially trafficked GFP in diatom plastids retains the transit peptide as it does in *T. gondii* (DeRocher *et al*., 2005).

Why is apicoplast transport largely insensitive to BFA in *P. falciparum* but sensitive in *T. gondii* and *P. tricornutum*? Differing sensitivities of apicoplast/secondary plastid targeting to BFA may be due to different sensitivities of this drug to certain ARF-GEFs involved in the targeting process. This serves to remind us that while the molecular mechanism of BFA is well characterized on human cells and yeast, the effect on *P. falciparum*, and indeed most other eukaryotes, is not known in any detail (however, see Baumgartner *et al*., 2001; Wiek *et al*., 2004).

The trafficking to most cellular destinations requires specific targeting or retention signals. Eukaryotes use a C-terminal penultimate XDEL motif (in which the X amino acid is species specific) to retain proteins in the ER (Nilsson and Warren, 1994). The XDEL motif is recognized by the membrane-bound *cis*-Golgi protein ERD2 (Semenza *et al*., 1990), which then transports the attached protein back to the ER via retrograde transport (Nilsson and Warren, 1994). *P. falciparum* ER-located proteins have either an SDEL or IDEL C-terminal motif (Elmendorf and Haldar, 1993; La Greca *et al*., 1997; Gardner *et al*., 2002). We wished to investigate whether the *P. falciparum* SDEL ER retention signal was recognized before or after an apicoplast transit peptide to further understand the position of the apicoplast within the secretory pathway. To do this we created ACP(l)-GFP-SDEL – a fusion protein with both an apicoplast leader sequence and an SDEL *cis*-Golgi retrieval motif (Fig. 5). If resulting parasites showed fluorescence in the ER, then SDEL would be recognized by ERD2 before the transit peptide, and this would infer that the apicoplast is a post *cis*-Golgi compartment. However, as expected, this fusion protein is located in the apicoplast and transit peptide processing occurs (Fig. 5), suggesting that the reverse is true – that the transit peptide is recognized first and that the apicoplast is pre-*cis*-Golgi. We also show that upon perturbation of the transit peptide – either by changing the first two positive charges to negative charges, or by complete transit peptide ablation – the SDEL retrieval motif is recognized, and GFP is retained in the ER. These fusion proteins do not make it to the apicoplast nor are they proteolytically cleaved to remove the transit peptide (Fig. 5). This demonstrates that even in the context of an apicoplast leader, albeit a non-functional one, the SDEL retrieval sequence can still be recognized. In addition, an ER localization for ACP(l)K18E_K22E-GFP strongly suggests that the apicoplast must recognize the N-terminal positive charge component of the transit peptide before ERD2 encounters the SDEL signal.

It is interesting to note that in early-stage parasites expressing ACP(l)-GFP-SDEL [like in KAHRP(s)-ACP(t)-GFP expressing cells; discussed earlier] some GFP remains in the ER and that this fluorescence looks continuous with the apicoplast. Furthermore, this fluorescence does not overlay with the *cis*-Golgi (Fig. 5). Normally GFP in transit through the secretory pathway cannot be detected and only becomes visible once it accumulates at the target destination. This anomaly was also seen in *T. gondii* and DeRocher *et al*. suggest that it could be due to the slowing of trafficking due to transient associations with small amounts of ERD2 in the ER (DeRocher *et al*., 2005) – we also think this is true for ACP(l)-GFP-SDEL in *P. falciparum*. It is tempting to suggest that the observed continuous pattern of GFP fluorescence between the apicoplast and the ER in ACP(l)-GFP-SDEL expressing cells reflects an association/continuity between these two organelles. However, this conclusion is tempered by the fact that the connection is only observed in cells expressing artificial reporter gene constructs.

In the diatom *P. tricornutum* Apt *et al*. (2002) also investigated the response to appending an ER retention signal to the end of a plastid-targeted GFP-targeted protein. They show that in the context of a full plastid leader fused to GFP there is efficient targeting to the plastid (Apt *et al*., 2002). However, one severely truncated transit peptide that competently targets GFP to the plastid fails to do so when fused to an ER retention signal, with the reporter protein accumulating in the ER (Apt *et al*., 2002). This may suggest that there is some kind of competition between the plastid transit peptide and an ER retrieval sequence in diatoms. Competition studies between mutant apicoplast leaders and the SDEL ER retrieval, and indeed, other organellar targeting signals in *P. falciparum* will help resolve this conundrum and may shed additional light on the position of the apicoplast and other organelles along the secretory pathway.

In this study we show that the apicoplast shares an intimate relationship with the ER and provide evidence

that proteins destined for the apicoplast do not pass through the Golgi. In a recent study by Cheresh *et al*. (2002) it was argued that apicoplast proteins traffic via the PV and are BFA sensitive. Using a transgenic system that expresses apicoplast leader–GFP fusion very early in the erythrocytic cycle they argued that GFP was not imported directly into the apicoplast, but rather was first secreted outside the parasite into the parasitophorous vacuolar space (Cheresh *et al*., 2002). As the parasites matured, this pool of unprocessed (transit peptide attached) GFP disappeared from the PV and processed protein began accumulating in the apicoplast (Cheresh *et al*., 2002). Cheresh *et al*. (2002) interpreted this to mean that the secreted protein was re-routed back inside the parasite to the apicoplast and duly processed. Using this same transgenic line they also argued that apicoplast targeting is sensitive to the effects of BFA. In this study we also show that GFP fusion proteins destined for the PV but not the apicoplast are sensitive to BFA. However, in direct contrast to their results we show that in the presence of a functional transit peptide, apicoplast targeting is largely BFA insensitive. Cheresh *et al*. (2002) only assayed the effects of BFA on apicoplast protein trafficking by microscopy and without an endogenous apicoplast marker. We believe the pulse-chase method to be more robust in detecting the apicoplast's insensitivity to BFA. It would be interesting to test apicoplast targeting using our methods on the Cheresh apicoplast GFP cell line.

In our study we have used a range of techniques to demonstrate that the apicoplast has a close association with the ER, is located proximal to the Golgi, and is in the default secretory pathway. Given the now widely accessible techniques such as transgenic parasites and ever improving cell biology in malaria parasites it will be very interesting to investigate the finer points of apicoplast targeting, such as how proteins transverse the four surrounding membranes and the identification of components involved in such processes. Considering the array of secretory destinations in *Plasmodium* parasites, it will also be very interesting to extend our study and begin to dissect protein targeting pathways to generate a 'road map' of intracellular and extracellular protein traffic.

Experimental procedures

Construction of GFP chimeras

EBA175(s)-ACP(t)-GFP and KAHRP(s)-ACP(t)-GFP were made using the *P. falciparum* Gateway™ plasmids described in Tonkin *et al*. (2004), in which the fusion proteins are driven by the Hsp86 promoter. Hsp86 is expressed throughout the life cycle, but peaks in late trophozoites (Bozdech *et al*., 2003). Points of fusion between the ACP transit peptide and the two heterologous signal peptides were based on their predicted signal peptide cleavage sites of SignalP (http://www.cbs.dtu.dk/services/SignalP). The chimeric leaders were then checked again for *in silico* cleavage by SignalP.

Both chimeric apicoplast leaders were made by two successive PCR amplification steps. EBA175(s)-ACP(t) was amplified using primers 1 and 5 and then with primers 2 and 5, while KAHRP(s)-ACP(t) was amplified using primers 3 and 5 followed by primers 4 and 5 (Table 1). In both cases ACP(l)-pHC1 (Waller *et al*., 2000) was used as a template. Products were cut using AvrII and BglII and ligated into the equivalent sites of pHGB (Tonkin *et al*., 2004). Resulting plasmids were cultivated and mixed with pCHD-1/2 in a Gateway™ LR clonase reaction (Invitrogen). Recombination reaction produced transfection plasmids with the GFP expression cassette and the hDHFR expression cassette in a head-to-tail arrangement. Large quantities of plasmid were isolated using Qiagen Maxi columns.

Leader–GFP-SDEL constructs were assembled using vectors for *P. falciparum* based around Invitrogen's multisite gateway system (van Dooren *et al*., 2005). GFP was fused to the SDEL *P. falciparum* ER retention signal by adding the SDEL DNA sequence to the 3′ primer(s), amplifying by PCR and cloning into the 3′ entry clone – pENTR2/3 (van Dooren

Table 1. List of primers used in this study.

Number	Primer (5′ to 3′) (features underlined)	Features
1	TATATATTTTTTTGCTTCCTTCTTTGTGTTATATTTTGCAAAAGCTTTTAAAAATACACAAAAAGATGG	NA
2	CACCAGATCTTGTGCATACAATGAAATGTAATATTAGTATATATTTTTTTGCTTCC	BglI site
3	TTTCCCTGTTTTTACTAAAATTCTTTTAGTCTCTTTTTTAGTATGGGTTTTGAAGTGCTTTAAAAATACACAAAA AGATGGAGTGTCC	NA
4	AGTCAGATCTATGAAAAGTTTTAAGAACAAAAATACTTTGAGGAGAAAGAAGGCTTTCCCTGTTTTTACTAA AATTC	BglI site
5	TGGACCTAGGTTTTAAAGAGCTAGATGGG	AvrI site
6	GGGGACAGCTTTCTTGTACAAAGTGGCACCTAGGAGTAAAGGAGAAGAACTTTTCACTGGAG	attB2 site
7	TTATAATTCGTCACTATCTACAAGCTTTTTGTATAGTTCATCCATGCCATGTGTAATCCC	NA
8	GGGGACAACTTTGTATAATAAAGTTGCGGTACCTTATAATTCGTCACTATCTACAAGCTTTTTG	attB3 site
9	CACCATTCAAAAATGAAACAAATTAGG	NA
10	GTTTGAGTCAACGGCACTTATAAA	NA
11	CACCTTATTAGAATGAAGATCTTATTACTTT	NA
12	TGTATTTTTAAAAGCGTTAACATAATATAG	NA
13	TTTTAAAGAGCTAGATGGG	NA

et al., 2005). Amplification took place using primers 6 and 7 in conjunction with primer 8 in a two-step amplification using pHGB as a template (Tonkin *et al*., 2004) (Table 1). The resulting PCR product was purified and recombined with pDONRP2R-P3 using BP clonase mix (Invitrogen) to yield GFP-SDEL-pENTR2/3.

Leaders to fuse to GFP-SDEL were amplified using the following primers and then recombined into pENTR-D-TOPO. The ACP leader was amplified using primers 11 and 13 using ACP(l)-pHC1 (Waller *et al*., 2000) as a template, while ACP(l)K18E_K22E was amplified using primers 11 and 13 with ACP(l)K18E_K22E-pHH2 (Foth *et al*., 2003) as a template (Table 1). ACP signal peptide [ACP(s)] was amplified using primer 11 and 12 with ACP(l)-pHC1 as a template. PCR products were ligated in pENTR-D (Invitrogen) using Topoisomerase-based ligation. Resulting entry clones were mixed with GFP-SDEL-pENTR2/3 and an entry clone consisting of the HSP86 5′ region; HSP86 5′ –pENTR4/1 (van Dooren *et al*., 2005). To each combination of three entry clones the destination clone pCHDR-3/4 was added (van Dooren *et al*., 2005). pCHDR-3/4 contains an expression cassette for hDHFR that confers resistance to WR99210. Briefly, equimolar concentrations of super-coiled plasmid were mixed in the presence of appropriate buffer and LR clonase plus and then grown in cells sensitive to CcdB on LB agar plates supplemented with 100 μg ml^{-1} of ampicillin.

Parasite culture and transfection

Plasmodium falciparum strains D10 and 3D7 were cultured in standard conditions (Trager and Jensen, 1976). Transfection of prepared expression constructs was carried out by electroporating 100 μg of DNA into ring-stage parasites (Wu *et al*., 1995; Crabb *et al*., 1997b) and selecting on 5 nM of WR99210 48 h after transfection.

Western blot analysis

Parasite samples were harvested from erythrocytes by treatment with 1.5 vols of 0.15% saponin (Sigma) and then mixed with Laemeli sample buffer. Proteins were separated in a 12% polyacrylamide gel and transferred to a nitrocellulose membrane, and GFP proteins detected using an anti-GFP antibody (Roche 1:1000) (all antibodies for western blot were diluted in 5% non-fat milk in TBS) followed by an HRP-conjugated secondary antibody (1:5000; Pierce).

Indirect immunofluorescence assays

Indirect immunofluorescence assays were performed on several cell lines to investigate the relationship that secretory organelles have with the apicoplast in the presence and absence of BFA. Cells were fixed using a previously described method (Tonkin *et al*., 2004). Briefly, *P. falciparum* cultures were harvested by centrifugation and washed once in PBS. Cells were then resuspended in 4% paraformaldehyde/0.0075% glutaldehyde (ProSciTech) and left for 30 min to fix at room temperature. Fixed cells were permeabilized with 0.1% Triton X-100 for 10 min. Cells were blocked in 3% BSA for a minimum of 1 h. Primary antibody was allowed to bind to the samples in the presence of 3% BSA for 1 h. Antibodies were used at the following concentrations: rabbit anti-ACP: 1:500 (Tonkin *et al*., 2004), rabbit anti-BiP (MR-19, MR4, ATCC Manassas Virginia): 1:1000, rabbit anti-ERD2 (MRA-1, MR4, ATCC Manassas Virginia): 1:500. Samples were then washed with PBS and secondary antibody applied at 1:1000 dilution for both Alexa Fluor 488 and 594 (Molecular Probes) while settling on a previously 0.1% PEI (polyethylenimine, Sigma) coated coverslip. Samples were then mounted in 50% glycerol with DABCO (Sigma) and sealed.

Microscopy

To assess the relationship of the secretory pathway structures to the apicoplast parasites were fixed and stained using markers for the ER (anti-BiP) and *cis*-Golgi (anti-ERD2). Late ring and early trophozoites parasites were chosen to view and compare these structures. At these early erythrocytic stages they are relatively simple compared with later stages, which show a much more complicated network of interconnected apicoplast, ER and *cis*-Golgi (data not shown). Cells were chosen for imaging based on one-colour fluorescence. Images in desired channels were then captured either on the Leica TCS 4D confocal equipped with a Krypton/Argon laser or a Carl Ziess axioskop with a PCO sensiCam with Axiovision 2 software. Green fluorescence of live transgenic parasites was captured on the Leica TCS 4D confocal microscope. 3D reconstructions and rotations of parasites were built up from multiple serial sections taken on a Leica TCS4 SP2 equipped with a multi-line Argon UV laser, a multi-line Argon laser, a green Helium/Neon laser and a red Helium/Neon laser.

Brefeldin A treatment

Brefeldin A – an inhibitor of ER to Golgi anterograde transport was employed as an agent to help understand the relationship between the apicoplast and the secretory pathway. ACP(s)-GFP, ACP(l)K18E_K22E-GFP (Waller *et al*., 2000; Foth *et al*., 2003) were all tightly synchronized by treating twice with 5% sorbitol 4 h apart. Cells were allowed to progress through the rest of the cell cycle and then to reinvade new erythrocytes. At this point cells were treated with 5 μg ml^{-1} of BFA (Sigma) in 100% ethanol and a control sample with 100% ethanol alone. Sixteen hours post drug cells were viewed under the microscope.

ACP(l)-GFP expressing parasites at high parasitaemia were split into two dishes one of which was treated with 5 μg ml^{-1} of BFA from a 10 mg ml^{-1} stock dissolved in 100% ethanol while the other was treated with equivalent concentration of ethanol alone for 3 h. After this time, still in the presence or absence of the BFA cells were incubated in methionine-free media for 30 min at 37°C before the addition of 100 μCi ml^{-1} Tran[^{35}S] label (ICN). Parasites were then returned to culture to take up the pulse. After 20 min pulse, label was washed out and parasites were returned to culture under constant drug pressure. At time points 0 h, 1 h, 2 h and 4 h, parasites were harvested using saponin to release them from erythrocytes. GFP fusion protein was immunoprecipi-

tated as described earlier (van Dooren *et al*., 2002). Proteins were separated by SDS-PAGE and detected by fluorography after treating with Amplify (Amersham) before drying and exposure to Kodak Biomax film.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. The apicoplast and the ER are closely associated in *P. falciparum.* ACP(I)-GFP transgenic parasites were fixed and labelled with anti-BiP antibodies. Parasites were chosen based on one-colour fluorescence and then each image was acquired with a single optical section. In every case (A–F) the apicoplast (GFP – green) can be seen closely juxtaposed with the ER (anti-BiP – red).

Movie S1. Rotation R1.

Movie S2. Rotation R2.

Movie S3. Rotation R3.

Movie S4. Rotation R4.

Movie S5. Rotation R5.

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