The malarial “apicoplast” derived from an algal plastid, has stimulated interest for its novel evolutionary biology and potential as a drug target. An endoplasmic reticulum-type signal sequence followed by a plastid targeting sequence are required to target proteins to the apicoplast but the pathway by which proteins are transported to the organelle is unknown. By stage regulating the expression of transgenes we show that early (0–12 h) in the parasite’s development in red cells, newly synthesized green fluorescent protein that contains the plastid targeting sequence (plastid targeting sequence-green fluorescent protein (PTS-GFP)) is recruited into the parasite’s secretory pathway. PTS-GFP in 0–12-h parasites is found released into the parasitophorous vacuole (PV) and in apposition with the Golgi. However, import into the apicoplast and processing to GFP does not occur until 18–36 h in development. In intermediate, 18-h parasites PTS-GFP resides in the PV. Quantitative exit of PTS-GFP from the PV and its conversion to GFP is seen at 36 h. The data suggest that: (i) import into the apicoplast is stage regulated and (ii) the PTS can signal endomembrane targeting from the PV to the apicoplast.

The protozoan Plasmodium falciparum causes the most virulent form of human malaria. It contains a novel, non-photosynthetic plastid organelle of prokaryotic origin called the apicoplast (1, 2). Its functions are not well understood, but the organelle appears to be critical for vacuolar development of blood stage parasites and possesses biosynthetic processes (such as lipid synthetic enzymes) found in plants as well as prokaryotes (3–5) that are not prominent in mammalian cells. These features make the apicoplast a good target for chemotherapy and inhibitors.

Nonphotosynthetic plastids are also present in a variety of other parasitic and non-parasitic, single cell eukaryotes (1, 6). Many plastid genes have invaded the host’s nuclear genome. Thus resident plastid enzymes that are nuclear-encoded proteins must be targeted to the organelle, as is seen in chloroplasts and mitochondria. Three to four membranes surround secretory plastids. Thus they are believed to have arisen by secondary endosymbiosis when a progenitor cell engulfed a prokaryotic alga (7). In a four-membrane structure, the outer two membranes are expected to correspond to the endovacuolar membrane of the host and the plasma membrane of the engulfed cell. Loss of either membrane or their fusion may result in a three-membrane plastid. However, even in a three-membrane organelle, nuclear-encoded proteins targeted to this compartment have to cross one additional membrane compared with a primary endosymbiont.

Studies in Euglena (which contains a three-membrane plastid), indicate that the LHCP II protein and ribulose-1,5-biphosphate carboxylase/oxygenase small subunit precursor destined for the plastid, contain at their N termini an ER1-type signal immediately followed by a chloroplast-like targeting sequence (8, 9). In the apicomplexan parasites, Toxoplasmagondii and P. falciparum, a bipartite N-terminal signal comprised of an ER secretory signal sequence (SS) and a plastid targeting sequence (PTS) drives quantitative targeting of a reporter-like green fluorescent protein (GFP) to the plastid (4, 10–12). Thus the model for plastid targeting proposes that recruitment to the secretory pathway followed by cleavage of the signal sequence leads to exposure of the PTS, which then mediates transport into the plastid. Yet it has not been possible to reliably detect PTS intermediates associated with secretory structures such as the ER-Golgi membranes. Pulse-chase and immunoelectronmicroscopy studies in Euglena, suggest that there is transport from the Golgi to the plastid (8, 13). In P. falciparum, a detailed electron microscopy study shows membrane-bound ribosomes in close apposition to the plastid (14), suggesting that there may be direct membrane connections between the ER and the plastid. Ribosomes have also been shown to decorate the outer membrane surrounding the plastid in some chlorophyte algae (15). Thus it is possible SS-PTS-GFP may be recruited directly across the outer plastid membrane, rather than enter the ER. If ER recruitment is the preferred mechanism, it remains unknown if the proteins move through Golgi and/or other distal secretory structures en route to the apicoplast. Since the plastid is a target for antimalarial therapy, understanding how proteins are delivered there may present insights for drug development as well as organellar biogenesis in eukaryotes.

The asexual life cycle of the P. falciparum malaria parasite in red cells, has four, distinct morphological stages (Fig. 1A). Infection begins when the extracellular “merozoite” stage parasite enters the red cell to become an intracellular “ring” stage parasite for the first 24 h. From 24 to 36 h, parasites mature through the “trophozoite” stage. “Schizont” stage parasites (36–48 h) undergo active mitosis and cell division. At the end of schizogony, the infected red cell ruptures to release daughter merozoites that re-infect erythrocytes and thereby maintain blood stage infection. Using a promoter that is active only in the first half of the parasite’s asexual life cycle, we created a...
temporal separation between the synthesis of a transgenic form of PTS-GFP in ring stage parasites (0–12 h) and its uptake and processing to mature GFP in later ring, trophozoite (18–36 h), and schizont (36–48 h) stages. Our results show that PTS-GFP transits through the PV en route to the apicoplast, implicating a function for the PTS in endocytotic targeting to the apicoplast.

EXPERIMENTAL PROCEDURES

Culture, Plasmodia, and Transfection—P. falciparum 3D7 was grown in vitro under standard culture conditions (16, 17). Plasmodia pH: Tg23, pHPRGFP, and pHPRACGFP and transfection conditions are described (18). Steady state cultures of transformed cells were maintained in 25 ng/ml pyrimethamine.

Flow Cytometry—For detection by flow cytometry, live infected red cells were washed with RPMI 1640 without phenol red and subjected to flow cytometry on a BD PharMingen FACScalibur and analyzed by CellQuest version 3.1.

Fluorescence Microscopy and Quantitative Analysis—Cell preparations were washed three times in RPMI 1640 and then resuspended at 1 × 10⁶ cells/ml, and allowed to adhere to poly-l-lysine-coated coverslips (for 30 min at room temperature). For standard indirect immunofluorescence assays, cells were then fixed in 1% formaldehyde, permeabilized with 0.05% saponin, blocked with 0.2% fish skin gelatin (fsg), and probed with the relevant primary antibodies (diluted 1:100 to 1:500), secondary antibodies or Fab fragment in phosphate-buffered saline containing 0.05% fsk. For all cells, parasite nuclei (blue) were stained with 10 g/ml Hoechst for 30 min. Where indicated, live cells attached to coverslips were incubated with 0.01% saponin to permeabilize the red cell membrane, TVN and PVM, then subsequently fixed and processed under standard conditions for immunofluorescence assays.

Fluorescence microscopy and digital image collection were performed on a Olympus IX inverted fluorescence microscope and a Photomicrox cooled CCD camera (CH5300/CCCD) driven by DeltaVision software from Applied Precision Inc. (Seattle, WA). Twenty-60 nm optical sections were taken through the depth of the cell, and DeltaVision software (softWoRx) was used to deconvolve these images and construct three-dimensional volume views. DeltaVision softWoRx uses a Constrained Iterative Deconvolution algorithm to remove out of focus blur in fluorescence optical sections and was set for a minimum of 15 iterative cycles. The optical transfer function used in deconvolution was computed from a measured point spread function which in turn was obtained by optically sectioning a fluorescent bead, for all of the available objective lenses (×40, 60, and 100). For quantitative projections, the “Additive” method of data collection and analysis was used to delineate the sites of interest in the infected red cell. Background (or nonspecific) signal was subtracted by imaging areas that had no cells. Autofluorescence and standard curves were established using 0.05 units of commercially available glutamate dehydrogenase (Sigma) and were shown not to be inhibited by the presence of either 0.01% saponin or 0.01% Triton X-100, 0.005% sodium deoxycholate, and 0.01% SDS, introduced by adding the indicated supernatant or pellet extracts. Trophozoite stage parasites contain 2-fold higher levels of GDH compared with rings.

Extracts for immunoprecipitations were prepared by adjusting lysates at a final concentration of 1% Triton X-100, 0.05% sodium deoxycholate, and 0.1% SDS in phosphate-buffered saline and protease inhibitors (1 Roche Molecular Biochemicals “complete mini” tablet per 50 ml). Lysates were cleared by centrifugation at 100,000 × g for 1 h, and supernatants were used to carry out the immunoprecipitations as follows. Lysates containing parasite cell equivalents of 5 × 10⁶ were diluted 1:10 in 20 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 2 mg/ml BSA, anti-GFP, (2 µl of CLONTECH number 8372-2 for each 500-µl incubation) and incubated at room temperature (22 °C) for 60 min with shaking. Protein G-Sepharose (Upstate Biotechnologies; 50 µl of a 50% suspension) was subsequently added and the incubation was maintained for a subsequent 60 min at room temperature. Samples were centrifuged at 5000 × g, washed five times with 20 mM Tris, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% Triton X-100, 2 mg/ml BSA, anti-GFP, (2 µl of CLONTECH number 8372-2 for each 500-µl incubation) and incubated at room temperature (22 °C) for 60 min with shaking. To permeabilize the red cell membrane, TVN and PVM, cells were attached to coverslips were incubated with 0.01% saponin to permeabilize the red cell membrane, TVN and PVM, then subsequently fixed and processed under standard conditions for immunofluorescence assays.

Immunofluorescent data were collected and analyzed with Adobe Photoshop and the image analysis software Image J. For quantitation, fluorescence data were collected as mean fluorescence intensity (MFI) and error bars at any given stage. Metabolic Labeling of the ER—Pulse-Chase, Pulse-chase and immunoprecipitation, and Western Blots—These procedures were carried out essentially as described by Haldar et al. (17). Briefly, highly synchronized early ring-infected red cells (at 15–20% parasitemia) were incubated (at 5 × 10⁶ parasites/ml) with [35S]methionine and [35S]cysteine (EasyTag EXPRE 35S 35S Protein Labelling Mix, from PerkinElmer Life Science containing 75% methionine and 25% cysteine) at a final concentration of 50 µCi/ml, in methionine-free RPMI in the absence of human serum, for 1 h. Metabolic labeling of trophozoite stage parasites was conducted at 350 µCi/ml as indicated in the text. Cells were chilled to 4 °C on ice, washed free of excess label in complete RPMI containing 5 mg/ml cold methionine. Where indicated, cells were chased for 24 h in complete RPMI 1640 containing 10% human serum. Western blots were probed with specific antibodies (diluted 1:2000) and probed in appropriate dilutions of secondary antibody. Bands were quantitated using IMAGE QUANT MAC version 1.2 (Molecular Dynamics). An average of three, scanned data sets were used for each quantitation.

Immunoelectron Microscopy—Synchronized P. falciparum (3D7 and ACPGFP) infected erythrocytes were fixed for 30 min at 0 °C with 2% formaldehyde, 0.1% glutaraldehyde in 0.1 m phosphate buffer, pH 7.4. Fixed samples were washed, dehydrated, and embedded in LR White resin (Polysciences, Inc., Warrington, PA) as described (22). Sections were cut to 0.5 µm and mounted on 4% gelatin-coated nickel grids. Sections were then incubated with Living Colours A.v. monoclonal antibody (JL-8, CLONTECH, Inc., Palo Alto, CA) diluted 1:40 in PBS for 2 h at room temperature. Negative controls included normal mouse serum and PBS applied as the primary antibody. After washing, grids were incubated for 1 h in 15-nm gold-conjugated goat anti-mouse IgG (Amersham Biosciences, Arlington Heights, IL) diluted 1:20 in PBS.
phosphate-buffered saline containing 1% (w/v) BSA and 0.01% (v/v) Tween 20 (PBTB), rinsed with PBTB, and fixed with glutaraldehyde to stabilize the gold particles. Samples were stained with uranyl acetate and lead citrate, and then examined in a Zeiss CEM902 electron microscope.

RESULTS

Expression of SS-PTS-GFP Using 5’hrp3, Its Processing and Localization to the P. falciparum Plastid—We have recently shown that co-transfection of a P. falciparum line with two plasmids, one expressing a green fluorescent protein (gfp) reporter and the other expressing a drug resistance marker (Tgdhfr-ts M23), allowed selection of a population in which about ~30% of the parasites produce GFP (23), due to recombination of transfected plasmids into chimeric episomes that can be maintained under drug pressure. We placed the SS-PTS of Pfapc at the N terminus of GFP in the pHRPFGP plasmid under control of the 5′hrp3 untranslated region generating the plasmid pHHRPACPGFP (23). This was then used with pDT.Tg23 at a ratio of 1:1 in a standard co-transfection assay (Ref. 23 and Fig. 1B). As for cytosolic GFP, within 7–14 days, ~25% of pyrimethamine-resistant parasites were found to express GFP in continuous culture (for over 16 weeks). GFP-expressing cells were sorted by flow cytometry and cultures containing 90% fluorescent cells were maintained for long-term in vitro growth (23). Episomal copy numbers of 1–2 per infected red cell was maintained by using the minimum drug concentration required to retain the exogenous plastid (23). There was no detectable growth defect relative to transformants expressing cytosolic GFP (data not shown).

As shown in Fig. 1C, i, in early trophozoites (~24 h), co-transfectants with pHHRPGFP show a cytosolic fluorescence, while those with pHHRPACPGFP display green fluorescence (Fig. 1C, iii and iv) at a single perinuclear region (Fig. 1C, iii and iv). This co-localized with extrachromosomal DNA (Fig. 1C, ii and iv), consistent with GFP being delivered to the plastid (24). Development of transformed parasites through the schizont stage results in segmenters where every nucleus in a daughter merozoite has an associated green plastid (Fig. 1D). Western blots of the transformed parasites confirmed the presence of 32- and 27-kDa bands that co-migrate with PTS-GFP and GFP, respectively (Fig. 1E). This suggests that the chimeric precursor is correctly delivered to the apicoplast and proteolytically processed there, consistent with a requirement for the PTS to target a protein to the apicoplast. Immunoelectron microscopy (Fig. 1F) localized apicoplast-targeted GFP to a perinuclear structure surrounding three membrane (indicated by red arrows), in P. falciparum-infected red cells. Hopkins et al. (14) have shown by transmission electron microscopy that the P. falciparum plastid has three membranes.

Stage-dependent Synthesis and Processing of PTS-GFP—The primary difficulty in detecting the movement of secretory precursors in subcellular organelles en route to the apicoplast was thought to be due to the fact that biosynthetic transport was rapid and thus the intermediates of transport were difficult to capture (11). We were therefore interested in attempting to uncouple the synthesis of the precursors from apicoplast import. In previous studies we have shown that transgenes returned to the chromosome and regulated by 5′hrp3 are actively transcribed only in the early ring stages (18). Since transcription and translation appear to be linked in Plasmodium, the use of 5′hrp3 to drive SS-PTS-GFP expression offered the possibility of restricting the synthesis of the precursor form to ring stage parasites. This would effectively uncouple the synthesis of the precursor from its active import at the trophozoite stage.

To determine the activity of the hrp3 promoter in the ACPeripheral co-transformed line during blood stage development, we synchronized the parent and transformed cell lines and probed Northern blots containing RNA isolated from young ring (6–12 h) and trophozoite (24–36 h) parasites for transcripts of the transgene. As shown in Fig. 2A, only rings from the transformed line contained significant amounts of RNA. We estimate that the rings contain at least 100-fold more SS-PTS-GFP transcript relative to trophozoite stages, confirming that 5′hrp3 expression is ring stage specific.

Immunoprecipitation of [35S]methionine and [35S]cysteine-labeled protein (see “Experimental Procedures”) synthesized over 60 min confirmed that a 32-kDa protein that migrated as PTS-GFP was actively produced in ring stages, but was not processed to mature GFP at this stage (Fig. 2B). In contrast, low levels of both newly synthesized precursor and product (marked by asterisks) were detected at the trophozoite stage. Estimates based on densitometric analyses (see “Experimental Procedures”) suggested that PTS-GFP synthesis in rings is at least 10-fold greater than in trophozoites. When metabolically labeled 6–12 h ring stage parasites were chased into the (36 h) trophozoite stage (Fig. 2C), PTS-GFP was processed and a band corresponding to mature GFP was detected. The lack of processing of PTS-GFP to GFP in early ring stage parasites suggested that either the precursor was not imported into the plastid or that the protease responsible for PTS cleavage within the apicoplast was inactive at this stage of growth. However, PTS-GFP made in ring stage parasites can be processed to mature GFP in trophozoites, suggesting that the deficient, plastid-linked function is gained at a later stage of asexual development.

This apparent stage-specific processing of PTS-GFP is not due to the absence of the apicoplast in early asexual stages of P. falciparum. The apicoplast 35-kb circular genome is an endogenous marker of the organelle and is visible by staining with Hoechst, in early or late ring and trophozoite stages of Plasmodium (Fig. 2D) (24), as well as in Toxoplasma (4, 10, 11, 25, 26). Electron micrographs of P. falciparum also confirm the presence of the apicoplast in early rings (Fig. 2E) (14) as well as later stages of growth (Ref. 14, and data not shown).

Stage-specific Localization of Apicoplast-targeted GFP: Effects of Brefeldin A—We were interested in determining whether the stage-dependent processing of PTS-GFP to GFP, seen in Fig. 2, reflected stage-dependent localization of green fluorescence to the plastid. As shown in Fig. 3A, i–iii, PTS-GFP (green) and the ER marker PBIp (red) both displayed peripheral and internal, perinuclear fluorescence, but there was little overlap between red and green signals (shown as yellow in Fig. 3A, iii). At the trophozoite stage (Fig. 3B, i–iii) when PTS-GFP was processed to GFP, green fluorescence concentrated at a single, perinuclear apicoplast distinct from the ER (shown in red). Control experiments indicated that the expression of the transgene did not alter the organization of PBIp or other secretory markers at any stage of asexual parasite development (not shown). These data suggested that the stage-specific, biosynthetic processing of PTS-GFP to GFP shown in Fig. 2, correlates with the delivery of green fluorescence to the apicoplast.

As indicated in Fig. 3A, iii, PTS-GFP in rings did not show significant overlap with the resident ER marker PBIp. Nonetheless, its tubular distribution suggested that it resided in one or more membranous compartments, possibly of the secretory pathway. If PTS-GFP enters the secretory pathway through the ER, we reasoned that the fungal metabolite brefeldin A (Bfa) if added during ring to trophozoite development, might block its transport to the apicoplast and accumulate green

2 M. Kadekoppala and K. Haldar, unpublished data.
Detection of PTS-GFP using the co-transfection assay, its processing, and targeting of GFP to the plastid in *P. falciparum*. A, asexual life cycle of *P. falciparum*. RBC, red blood cell; PVM, parasitophorous vacuolar membrane; TVN, tubovesicular membrane network that emerges from the PVM. Although not shown, “late-trophozoites” at 33–36 h contain 2–4 nuclei. Apicoplasts are shown in green. At the end of schizogony (48 h), the infected red cell lyse to release merozoites that re-infect red cells. B, schematic of co-transfection assay. Plasmids were constructed as described under “Experimental Procedures.” Essential control regions such as *hrp3* (1.7 kb), *hrp2* (0.6 kb), and the coding regions...
fluorescence in early secretory structures. Bfa is a heterocyclic lactone that blocks vesicular secretory protein export in cells (27). In earlier studies, we demonstrated that Bfa reorganizes the PfERD2 Golgi site and effects a complete block in the export of newly synthesized proteins from ring stage parasites without affecting protein synthesis (20). Distal secretory destinations in the Golgi, as well as the PPM and PVM lie beyond the brefeldin block. This is consistent with the action of brefeldin in eukaryotic cells, and suggests that it acts within the plasmoidal Golgi complex. An unexpected feature of the action of Bfa in rings of *P. falciparum*, that is extended treatments for 24 h or longer are entirely reversible: thus washing Bfa out completely restores parasite protein export (20). This unusual feature enables examining effects of this secretory block on relatively long-term events of biosynthetic protein transport during the ring to trophozoite transition.

When rings were allowed to mature in the presence of Bfa for 24 h (Fig. 3c, i-iii), the distribution of PTS-GFP as well as PfBiP (Fig. 3c, ii) were altered compared with either control rings or trophozoites shown in Fig. 3, a and B. In the presence of Bfa, PfBiP lost its reticular staining and PTS-GFP accumulated in diffuse globular regions that show significant overlap with regions of PfBiP stain: however, PTS-GFP and BiP failed to show the identical distribution in Fig. 3c. The Golgi marker PfERD2 was also reorganized by Bfa treatment, consistent with the action of the drug on blocking transport through the Golgi (Fig. 3c, iii). If the cells were then washed free of drug and incubated for 18 h without Bfa, green fluorescence localized to the apicoplast (Fig. 3, D and E). In these cells, PfERD2 (Fig. 3E, ii and iii) was restored to its multiple sites of punctate staining that is expected for the distribution of PfERD2. The addition of Bfa to trophozoites had no significant effect on the accumulation of GFP in the apicoplast (compare Fig. 3, F, i-iii, with B, i-iii). There was some loss of the reticular stain of PfBiP (compare Fig. 3, F, i, to B, ii-iii) in Bfa-treated trophozoites. PfERD2 was reorganized from punctate perinuclear distribution to a largely tubular morphology (Fig. 3F, iii) and secretory protein export from these trophozoites was blocked (not shown), indicating that the drug was active. These data suggest that Bfa influences the organization of ER as well as Golgi membranes during *P. falciparum* ring and trophozoite development. Moreover, Bfa could block movement of PTS-GFP from ER-Golgi secretory membranes to the apicoplast, but it did not reorganize the apicoplast back to the ER.

**PTS-GFP Is Secreted into the PV and Associates with the Golgi, in the Early Ring Stage**—Our results from the previous section strongly suggested that in early rings, PTS-GFP enters the secretory pathway. However, as shown in Fig. 3a, a significant portion of the PTS-GFP-associated green fluorescence is entirely distinct from that of the ER marker, PfBiP (red). This suggests that although PTS-GFP enters the early ring-ER, it may not accumulate there to any appreciable extent. To further define the location of PTS-GFP, we examined its distribution by immunoelectron microscopy (Fig. 4A). As shown, gold particles detecting PTS-GFP are detected at the parasitophorous vacuole and vacuolar membrane (indicated by black arrows) of 6–12-h rings. Internal sites of PTS-GFP staining are also seen (indicated by arrowheads in Fig. 4A), confirming that PTS-GFP accumulates at both the PV and internal secretory sites, in these early parasites. In indirect immunofluorescence assays the peripheral PTS-GFP fluorescence showed some overlap with PfEXP1 a marker for the PVM as well as the Golgi marker PfERD2, consistent with the presence of label in the PV and internal secretory sites.\(^3\)

To further investigate the delivery of PTS-GFP to the PV, we examined whether the distribution of PTS-GFP-associated green fluorescence is altered in ring-infected cells treated with low levels of saponin (a detergent that acts by intercalation with cholesterol). Although high levels of saponin can permeate secretory membranes, low levels can be used to selectively perforate cholesterol-rich compartments. In *P. falciparum*-infected cells, we have previously shown (by electron microscopy and biochemical evidence) that 0.01% saponin perforates the red cell membrane and the PVM but not the parasite plasma membrane or secretory compartments within the parasite (20). This is consistent with the concentration of cholesterol in the PVM (28). We therefore used saponin-permeabilized cells to determine the distribution of PTS-GFP between the PV and secretory compartments within the young ring parasite.

To follow the fate of the released protein directly, the cells were metabolically labeled for 60 min (see “Experimental Procedures”), and subsequently treated with 0.01% saponin. The pellet and supernatant fractions were separated by centrifugation at 100,000 \(\times g\) for 60 min and subjected to immunoprecipitation. As shown in Fig. 4B, i, 50% of PTS-GFP (as judged by densitometry) can be immunoprecipitated from the supernatant fraction. The parasite cytosolic enzyme GDH is not released into the supernatant fraction (Fig. 4B, ii). This confirms that 0.01% saponin does not permeabilize the parasite plasma membrane and thus released PTS-GFP must come from material released into the PV. As a second measure of latency, Western blots reveal that the soluble ER protein PfBiP is entirely retained in the parasite (Fig. 4B, iii), which is to be expected if the parasite plasma membrane is intact. In the Western blots, a nonspecific reaction is seen with BSA (68 kDa) in the supernatant fraction and is marked with an asterisk. BSA minimizes membrane damage, is therefore included in the saponin-lysis step (20), and can be detected in the supernatant fraction by staining the filter with Ponceau.\(^4\)

Microscopic analysis of early ring-infected cells treated with 0.01% saponin (Fig. 4C, i-iii) revealed loss of the peripheral green fluorescence (Fig. 4C, i-iii). Instead, the saponin-insensitive PTS-GFP associated green fluorescence was largely detectable in a single, major site within the parasite. As expected this site showed no overlap with the PVM marker PfEXP1 (Fig. 4C, i). It also showed no significant overlap with the ER marker PfBiP (Fig. 4C, ii). Thus low levels of PTS-GFP overlap seen with PfBiP in Fig. 3a, iii (and Supplementary Material), probably reflect the difficulty in resolving two complex peripheral

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\(^3\) P. Cheresh, T. Harrison, H. Fujioka, and K. Haldar, unpublished data.

\(^4\) P. Cheresh and K. Haldar, unpublished data.
signals in a small cell rather than their true mixing in the ER. In addition, the saponin-insensitive PTS-GFP showed no overlap with apicoplast DNA (Fig. 4C, iii and iv; apicoplast DNA is marked with an arrow; in panel iv it is pseudo-colored cyan) as detected in >80% of cells. However, it was closely apposed to and partially overlapped with the PfERD2 Golgi site (Fig. 4C, iii), as seen in 75% of cells. These data strongly support that in early rings, PTS-GFP is not recruited to the apicoplast. Rather intracellular PTS-GFP resides at a secretory site proximal to, or in the Golgi. We occasionally detected a second, minor site of saponin-insensitive PTS-GFP accumulation that did not co-localize with any of the available plasmodial Golgi or ER markers or the apicoplast. This may reflect a second, yet unknown secretory (Golgi?) compartment traversed by PTS-GFP in ring stage parasites. Although PTS-GFP accumulation associated with the Golgi was reliably seen as a spot within the parasite, the pattern of peripheral distribution of PTS-GFP in cells can vary. This suggests that PTS-GFP may not be freely diffusible in the PV and may interact with specific components in this compartment.

Quantitative analyses of stacked 0° projections of micrographs of intact- and saponin-lysed cells (Fig. 4D, i and ii) carried out as described under “Experimental Procedures,” showed that the signal lost upon saponin treatment corresponded to a median value of 63 ± 7% of total ring-associated PTS-GFP. This was consistent with our biochemical analyses in early rings (Fig. 4B) that a significant fraction of PTS was secreted into the PV.

The Distribution of PTS-GFP and Apicoplast GFP during Asexual Maturation through the Late Ring to the Trophozoite
Stage—Our data in Fig. 4 showed that early ring stage parasites release PTS-GFP into the PV. However, these parasites did not recruit PTS-GFP to the apicoplast. In contrast, results from Figs. 2 and 3 showed that trophozoite stage parasites import PTS-GFP into the apicoplast and process it to GFP. Our interest was to determine whether PTS-GFP from the PV is returned to the apicoplast for import and processing. We therefore examined in Western blots, the distribution of total levels of precursor and product detected during ring to trophozoite development. We synchronized cells of the late ring (up to 18 h) stage and allowed them to mature to the late trophozoite stage (36 h). The 18-h time point was chosen to obtain cells predicted
PTS-GFP is detected in the parasitophorous vacuole and in apposition with the Golgi in early rings. A, immunoelectron micrograph of rings showing localization of PTS-GFP in the parasitophorous vacuole and PVM (black arrows) as well as within the parasite (P; blue arrowheads). RBC, red blood cell. Scale bar, 1 μm. B, supernatant (S) and pellet (P) fractions obtained from 6 to 12-h ring-infected cells metabolically labeled (see “Experimental Procedures”) and subsequently treated with 0.01% saponin were: i, subjected to immunoprecipitation (IP) to detect PTS-GFP; ii, assayed for parasite glutamate dehydrogenase activity (expressed as arbitrary units/min/5×10⁶ parasites); or iii, probed in Western blots (WB) to detect PfBiP. In iii, nonspecific label due to excess albumin in the supernatant fraction is marked with an asterisk. In i and iii, molecular weight markers and PTS-GFP are as shown in kDa. C, i-iv: single optical sections showing green fluorescence in young rings permeabilized with 0.01% saponin relative to secretory markers PfEXP1, PfBiP, PfERD2 (shown in red in i-iii), and apicoplast DNA (marked with an arrow in iii and iv), as detected by indirect immunofluorescence and DeltaVision Microscopy (see “Experimental Procedures”). In C, iv, the Hoechst stain is pseudo-colored cyan to facilitate visualization of apicoplast DNA. D, 0° projection of total cell-associated green fluorescence before (i) and after (ii) permeabilization with 0.01% saponin. Parasite DNA was labeled with Hoechst 33342 and is shown in blue.
to contain maximal levels of PTS-GFP (since 5'hrp3 is active throughout the ring stage). The 36-h time point was chosen to obtain cells predicted to contain predominantly mature, apicoplast-associated GFP. As shown in Fig. 5A, i and iii, PTS-GFP was the predominant signal in 18-h ring stage parasites, while processed GFP dominates at the trophozoite stage. Some (less than 20%, as judged by densitometry) mature GFP is detected in the 18-h rings. This could be due to the presence of contaminating early trophozoites at ~24 h of development (that convert PTS-GFP to GFP) in this ring preparation. Alternatively, it could be due to the initiation of import and processing of PTS-GFP to GFP, in the apicoplast at 18 h. In contrast to late rings, in 36-h trophozoites the levels of PTS-GFP decreased to ~20%, while those of GFP increased to ~80%, of the total cell associated fluorescence signal (Fig. 5A, i and iii). The ratio of precursor:product in rings was closely comparable to that of product:precursor in trophozoites (Fig. 5A, iv). The combined, total intensities of the GFP + PTS-GFP signal detected in trophozoites showed only a small (~20%) reduction compared with those seen in rings (Fig. 5A, iii). Some of this observed reduction could have been due to the loss of cells (estimated to be as much as 10%) between the 18- and 36-h time points. This suggested that the total levels of GFP seen in trophozoite stage parasites are ~90% of the PTS-GFP detected in rings. Furthermore, since trophozoite-synthesis of PTS-GFP is 10-fold lower than ring synthesis (see Fig. 2B), the relative abundance of GFP in the trophozoites (in Fig. 5A, i) must have come from precursor synthesized in the ring stage that persisted until the trophozoite stage, when it was processed to GFP (in the apicoplast) and accumulated in the saponin-insensitive fraction.

Another striking feature of the data in Fig. 5A, i and iii, was that in late rings and trophozoites, PTS-GFP quantitatively resided in the saponin-sensitive fraction and there was no precursor detected in the saponin-insensitive fraction (even upon overexposure of the blots: not shown). The parasite plasma membrane was not permeabilized by 0.01% saponin as judged by the failure to release GDH (Fig. 5A, ii; note that relative to rings, trophozoites show approximately a 2-fold increase in cell associated GDH, reflecting the increased transcription of this gene at this stage (29)). This suggested that from the late ring stage onwards, virtually all PTS-GFP was quantitatively exported to the PV and resides there. By the late trophozoite stage ~80% of the PTS-GFP is recruited from the PV and converted to GFP, indicating its transport to the apicoplast. The remaining ~20% which was not converted, continues to reside as PTS-GFP in the PV.

Microscopic analysis of 18-h rings revealed green fluorescence primarily in the periphery of the parasite. Low levels of green fluorescence were also seen in association with apicoplast DNA (Fig. 5B, i and ii). When cells were treated with 0.01% saponin, there was loss of peripheral fluorescence. However, apicoplast-associated green fluorescence could be detected in the parasite (compare Fig. 5B, i and ii with iii and iv). In contrast in 36-h trophozoites, green fluorescence was prominently associated with the apicoplast (Fig. 5C, i and ii). This association was preserved even after treatment with 0.01% saponin (Fig. 5C, iii and iv). On the basis of the Western blot shown in Fig. 5A, i, apicoplast-associated fluorescence in either late rings or trophozoites is entirely due to processed GFP. The peripheral fluorescence associated with late rings is due to PTS-GFP. We failed to detect low levels of PTS-GFP in the PV of trophozoites by microscopy (see Fig. 5C, i and ii) probably because it is dispersed and thus diluted over the vacuole. In contrast, processed GFP within the apicoplast was concentrated in a relatively small volume, resulting in high intensity of green fluorescence in the organelle. These data provide visual corroboration of the relative distribution of PTS-GFP and GFP in 18-h rings and 36-h trophozoites presented in Fig. 5A. They are summarized in Fig. 6A and strongly support that the bulk (70–80%) of PTS-GFP synthesized in the ring stages, transited through the PV prior to processing to GFP in the apicoplast at the trophozoite stage (note, previous studies (11, 14) and our unpublished data show that the apicoplast is enlarged in 36-h parasites).

To provide direct evidence that PTS-GFP newly synthesized at the trophozoite stage can be released to the PV, we followed the distribution of protein metabolically labeled at this stage. The cells were incubated at an elevated specific activity (~350 μCi/ml) of radiolabel (for 60 min) since the levels of PTS-GFP synthesis were greatly reduced at this stage. As shown in Fig. 5D, i, despite the low levels of protein synthesis, labeled PTS-GFP could be detected in the saponin-sensitive compartment. The parasite plasma membrane was not permeabilized by 0.01% saponin as judged by the failure to release GDH (Fig. 5D, ii). Thus within 60 min, a substantial portion of PTS-GFP synthesized in trophozoites could be released into the PV (Fig. 5D, i). This strongly argues that secretion of PTS-GFP to the vacuole is not a consequence of blocking import to the apicoplast or overexpression of the transgene. PTS-GFP could also be detected within the parasite. After 60 min of chase, the intensities of precursor bands in the PV and parasite decreased and there was concomitant increase in processed GFP within the parasite. The extent of decrease of the precursor form in the PV was comparable with that within the parasite, suggesting that they were both processed similarly and converted to product in the apicoplast. Biosynthetically labeled PTS-GFP seen within trophozoites in Fig. 5D, i, was not detected by Western blots in Fig. 5A, i, and must constitute a very small fraction of the total PTS-GFP pool, arguing that in trophozoites, there are no major sites of precursor accumulation within the parasite. The low level of transgene biosynthesis in trophozoites precludes a rigorous, kinetic analysis of the t1/2 of biosynthetic transport of PTS-GFP to the apicoplast. Nonetheless, in conjunction with our results in Fig. 5A, the data clearly showed that independent of whether PTS-GFP was synthesized in the ring or trophozoite stages, it transited through the PV prior to processing to GFP in the apicoplast. This suggests that the apicoplast can interact with pathways of endocytic uptake of PTS-GFP from the PV (Fig. 6B).

DISCUSSION

The targeting of a protein to the apicoplast by SS-PTS suggests that the organelle is a destination in the secretory pathway. Our results indicate that the plastid does not contain markers of the ER and Golgi in the plasmodial secretory pathway. However, stage-specific transgene expression shows that PTS-GFP synthesized in ring stage parasites is recruited into secretory compartments. The addition of Bfa to these parasites results in a high degree of overlap between PTS-GFP and a region of the ER, labeled by PfBiP, strongly supporting that PTS-GFP enters the ER. The targeting of a protein to the apicoplast by SS-PTS suggests that the plastid is a destination in the secretory pathway. Our results indicate that the plastid does not contain markers of the ER and Golgi in the plasmodial secretory pathway. However, stage-specific transgene expression shows that PTS-GFP synthesized in ring stage parasites is recruited into secretory compartments. The addition of Bfa to these parasites results in a high degree of overlap between PTS-GFP and a region of the ER, labeled by PfBiP, strongly supporting that PTS-GFP enters the ER. The reasons for the lack of uniform distribution of PTS-GFP accumulated in the ER are not known. A "secondary ER" that accumulates secretory protein in the presence of Bfa has been described in P. falciparum (30). However, the secondary ER is thought to be depleted in PfBiP, suggesting that it does not accumulate there.

Biochemical and microscopy data indicate that at the time of active synthesis in early ring stages, there are two major sites of secretory PTS-GFP accumulation. One is saponin-sensitive and the other, insensitive; neither are the ER or the apicoplast, but correspond to the PV and a second intracellular site that is not the apicoplast, but is apposed to the early Golgi. We suggest
that this Golgi (or proximal-Golgi site) indicated by the dashed box (in Fig. 6B) may be an intermediate compartment during protein export from the ER to the PV. Intracellular secretory accumulation here may be due to backup of PTS-GFP from the PV, because it cannot move forward to the apicoplast at these stages. Alternatively, it may be that the ERD2-associated site is an endocytic compartment traversed en route from the PV to the Golgi. However, endocytic pathways usually interact with distal Golgi membranes that are not susceptible to reorganization to the ER by Bfa (31). Since Bfa reorganizes PTS-GFP

**Fig. 5.** The distribution of PTS-GFP and apicoplast GFP during asexual maturation through late rings to the trophozoite stage. A, synchronized cultures were harvested at 18 and 36 h of asexual development, permeabilized with 0.01% saponin, and the supernatant and pellet fractions were separated by centrifugation. 5 x 10⁶ parasite equivalents of each fraction were subjected to Western blots with anti-GFP antibodies (A, i) and GDH assays (A, ii, activity is expressed as arbitrary units/min/5 x 10⁶ parasites). Densitometric analyses indicate the intensities of PTS-GFP and GFP detected in each fraction (A, iii), and ratios of PTS-GFP:GFP and GFP:PTS-GFP are presented (A, iv). B, 18-h rings untreated (B, i and ii), or treated with 0.01% saponin (B, iii and iv). C, 36 h trophozoites untreated (C, i and ii), or treated with 0.01% saponin (C, iii and iv). In B and C, Hoechst-stained parasite DNA is pseudocolored red, white arrow indicates apicoplast. GFP fluorescence is green. D, trophozoite-infected red cells were metabolically labeled for 60 min, and then chased in complete medium for 60 min. Both sets of samples were treated with saponin and the supernatant (S) and pellet (P) fractions were subjected to immunoprecipitation (IP) using anti-GFP antibodies (D, i), as well as assayed for glutamate dehydrogenase activity (D, ii). In A, i, and D, i, molecular weight markers, PTS-GFP and GFP are as shown.
FIG. 6. A, summary of stage-specific GFP distribution. B, proposed model for transport of PTS-GFP to the apicoplast. In B, SS-PTS-GFP enters the ER, where the signal sequence is cleaved. PTS-GFP rapidly exits the ER and may enter a Golgi (or proximal Golgi site) as indicated by the dashed square. It is subsequently released into the PV (bold arrow). The P. falciparum Golgi is “unstacked” (34) and the movement of PTS-GFP suggests a pathway for protein transport directly from an ERD2-associated site to the parasite plasma membrane. The apicoplast is shown to be a discrete organelle distinct from other secretory compartments of the ER-Golgi as well as the food vacuole containing the dense hemozoin crystal. PTS-GFP in the PV is delivered to the apicoplast which enlarges at the trophozoite stage (11, 14) (data not shown). PPM, parasite plasma membrane; EM, erythrocyte membrane; PV, food vacuole. PfEXP1 (pink) is a parasite protein marker for the PVM and vesicles of the TVN. PfBiP and PfERD2 are markers for the ER and Golgi, respectively.
accumulation in rings, the simplest explanation for the involvement of Golgi/proximal-Golgi membranes here, are as intermediates in export of PTS-GFP to the PV (Fig. 6, A and B). Newly synthesized PTS-GFP made in trophozoites is also detected in the PV and is processed to GFP in the apicoplast. Thus PTS-GFP may be secreted to the PV independent of parasite stage. The levels of PTS-GFP associated green fluorescence in individual rings and trophozoites can vary by about $\pm 2$-fold. This is likely due to the presence of plasmid chimeras that contain either a single or two acgfp genes after recombination (23). However, this variation in total cell associated fluorescence has no significant effect on the relative distribution of PTS-GFP in the Golgi or the PV.

Our Western and microscopy results following ring to trophozoite development strongly support that at steady state, PTS-GFP quantitatively accumulates in the PV and from there is delivered to the apicoplast (see Fig. 6). This suggests that the apicoplast does not lie within the lumen of the ER-Golgi complex, but is a distinct secretory destination. Studies in T. gondii suggest that mutants lacking an apicoplast (32) accumulate PTS-GFP and other reporter-like PTS-GFP, our results imply that the PTS of PfACP also interacts with secretory and vacuolar membranes and therefore mediates the "handing off" between these membranes and apicoplast. PTS-GFP can be released by low levels of saponin, suggesting that cholesterol may be important to its membrane association. We have recently shown that there are high levels of cholesterol in the PVM (28, 39–41) that interact with the PTS may influence the vacuole during intracellular growth. As indicated earlier, studies in Euglena suggest that protein transport occurs from the Golgi to the plastid (8). It is possible that there are multiple secretory routes of transport to the apicoplast, whose dynamics are regulated by recycling pathways between Golgi/proximal plastid sites and the plasma membrane.

When GFP is secreted into the PV or another secretory reporter, HRPIImyc is targeted to the red cell, they are not delivered to the apicoplast (11) 5 Rather a fraction of each protein is eventually delivered to the food vacuole, the digestive organelle of the parasite. In contrast, we show that PTS-GFP released into the PV can be retrieved and delivered to the apicoplast. This implies that in addition to mediating import across plastid membranes, the PTS functions as signal for endocytosis sorting in the parasitophorous vacuole to target proteins to the apicoplast and that the malarial organelle can be an endosomal destination. Recent studies show that segregation of the T. gondii plastid is linked to the movement of centrosomes (36). This was proposed to reflect the endosymbiotic origins of the organelle and retention of machinery that couples endosomes to the centrosomes. In malaria parasites, since PTS-GFP is released to vacuole but not delivered to the apicoplast in the ring stage, it suggests that vesicular (or endosomal) targeting to the apicoplast is stage regulated.

We have constructed a stage-specific reporter to facilitate our analysis of transport and processing of the precursor PTS-GFP. Although there is evidence that protein import into chloroplasts of Euglena and Chlamydomonas is regulated by light (reviewed in Ref. 6), at the present time little is known about the stage-specific expression of apicoplast-targeted proteins in P. falciparum and other parasitic protozoa. At time of writing, only four plasmodial proteins have been experimentally proven to be in the apicoplast (37). Our data suggest that by varying the timing of expression, the PTS may confer shared biosynthetic properties between the PV and apicoplast. However, regardless of whether PTS-GFP is synthesized in the ring or the trophozoite stage, the presence of the PTS allows delivery from the PV to the apicoplast.

PTSs have been known to underlie multiple interactions with distinct components of chloroplast membranes (38). However, since we have examined the transport of a soluble targeted reporter-like PTS-GFP, our results imply that the PTSs of PfACP also interacts with secretory and vacuolar membranes and therefore mediates the "handing off" between these membranes and apicoplast. PTS-GFP can be released by low levels of saponin, suggesting that cholesterol may be important to its membrane association. We have recently shown that there are high levels of cholesterol in the PVM (28, 39–41) that interact with the PTS may influence the vacuole during intracellular growth. As indicated earlier, studies in Euglena suggest that protein transport occurs from the Golgi to the plastid (8). It is possible that there are multiple secretory routes of transport to the apicoplast, whose dynamics are regulated by recycling pathways between Golgi/proximal plastid sites and the plasma membrane.

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