



Host biotin is required for liver stage development in malaria parasites

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Acetyl-CoA carboxylase (ACC) is a biotin-dependent enzyme that is the target of several classes of herbicides. Malaria parasites contain a plant-like ACC, and this is the only protein predicted to be biotinylated in the parasite. We found that ACC is expressed in the apicoplast organelle in liver- and blood-stage malaria parasites; however, it is activated through biotinylation only in the liver stages. Consistent with this observation, deletion of the biotin ligase responsible for ACC biotinylation does not impede blood-stage growth, but results in late liver-stage developmental defects. Biotin depletion increases the severity of the developmental defects, demonstrating that parasite and host biotin metabolism are required for normal liver-stage progression. This finding may link the development of liver-stage malaria parasites to the nutritional status of the host, as neither the parasite nor the human host can synthesize biotin.

acetyl-CoA carboxylase | apicoplast | holocarboxylase synthetase | biotin ligase | *Plasmodium*

Biotin is an essential metabolite in all known free-living organisms. It functions as a CO₂ carrier in carboxylation and decarboxylation reactions catalyzed by a small family of enzymes, the biotin carboxylases (1). Biotin carboxylases participate in central metabolic processes such as gluconeogenesis, lipogenesis, amino acid metabolism, and energy transduction (2). Bacteria, plants, and some fungi can synthesize biotin de novo from a pimeloyl-CoA precursor, but other organisms acquire biotin from their diet or environment (3). Regardless of how biotin is acquired, a dedicated ATP-dependent biotin ligase, or holocarboxylase synthetase (HCS), is required to catalyze the covalent attachment of biotin to a specific lysine in the target carboxylase (4).

Acetyl-CoA carboxylase (ACC) is the only protein predicted to be biotinylated in the parasite (5). ACC enzymes, the most common member of the biotin carboxylase family, are found in most living organisms and catalyze the first and rate-limiting step in fatty acid synthesis (FAS) (6). The *Plasmodium* ACC resembles the homomeric ACC found in the chloroplasts of grasses in that it contains three functional domains expressed as a single large polypeptide (7), and localizes to a plastid organelle called the apicoplast (8). The ACC orthologs that function in the plant chloroplast are common targets for herbicides (1). Some of these inhibitors have been tested against blood-stage malaria parasites (9), but it was shown that any growth inhibition observed with these compounds is the result of off-target effects, as *Plasmodium falciparum* ACC can be disrupted (8). ACC has two catalytic domains: a biotin carboxylase (BC) domain that transfers CO₂ from bicarbonate to biotin and a carboxyltransferase (CT) domain that transfers CO₂ from biotin to acetyl-CoA to generate malonyl-CoA. A third domain, known as biotin carboxyl carrier protein (BCCP), contains the conserved lysine to which biotin is attached. Biotin functions as a swinging arm to transfer the CO₂ moiety between the active sites of the BC and CT domains (1). The enzymatic domains of *Plasmodium* ACC have not yet been studied, but it has been shown that the BCCP domain of *P. falciparum* is biotinylated when expressed in *Escherichia coli* (10).

Malaria parasites contain a type II FAS (FASII) pathway in the apicoplast (11) that relies on malonyl-CoA as the two-carbon subunit for fatty acid elongation (12). Gene KO of FASII pathway enzymes in the rodent parasites *Plasmodium yoelii* and *Plasmodium berghei* demonstrated that the FASII pathway is required for normal liver-stage development, but not for blood- or mosquito-stage development (13, 14). Thus, ACC and biotin should be required in the liver stages to provide malonyl-CoA for downstream FASII pathway enzymes. Consistent with this idea, pyruvate dehydrogenase, which produces acetyl-CoA, the substrate of ACC, has also been shown to be critical for liver-stage development in *P. yoelii* (15).

Biotin metabolism may also be important for other stages of parasite development. Malaria parasites appear to encode enzymes comprising a fatty acid elongation (ELO) pathway similar to that described in the apicomplexan parasite *Toxoplasma gondii* (16). As is the case for typical ELO pathways (17), enzymes of the *T. gondii* ELO pathway are associated with the ER membrane and use malonyl-CoA as a substrate (16). *P. falciparum* parasites lacking a functional FASII pathway can still elongate fatty acids, possibly because of the activity of the ELO pathway (14). If malonyl-CoA can cross the apicoplast membranes, ELO as well as FASII could depend on ACC activity and biotin metabolism.

Perhaps the most unusual feature of biotin metabolism in malaria parasites is the presence of two HCS paralogs encoded

Significance

Malaria parasites require certain host nutrients for growth and survival. In this project, we examined the role of the human vitamin biotin in all stages of the malaria life cycle. We cultured blood- and liver-stage malaria parasites in the absence of biotin and found that, whereas blood-stage replication was unaffected, liver-stage parasites deprived of biotin were no longer capable of establishing a blood-stage infection. Interestingly, biotin depletion resulted in more severe developmental defects than the genetic disruption of parasite biotin metabolism. This finding suggests that host biotin metabolism also contributes to parasite development. Because neither the parasite nor the human host can synthesize biotin, parasite infectivity may be affected by the nutritional status of the host.

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in the genomes of *Plasmodium* species. Plant cells also contain two HCS paralogs (18), and HCS activity is partitioned among the three compartments in which biotin-dependent carboxylases are found: the mitochondria, chloroplasts, and cytosol (19, 20). In *Arabidopsis thaliana*, alternative splicing governs the production of multiple *AtHCS1* isoforms that are targeted to different compartments in the cell (21). Multiple isoforms of *AtHCS2* are also produced, but these do not appear to have HCS activity, and deletion of the *AtHCS2* gene has no observable phenotype in *A. thaliana* (21). We sought to determine the role of biotin across the malaria life cycle, including how biotin is acquired, the activity and localization of the biotin ligases, and the consequence of disrupting biotin metabolism in malaria parasites.

Results

ACC Is Located in the Apicoplast in Liver and Blood Stages but Is Not Biotinylated During the Blood Stages. ACC is the only predicted biotin-dependent enzyme in the *Plasmodium* genome. ACC has previously been localized to the apicoplast in blood-stage *P. falciparum* (8), but the localization has not been confirmed in the other stages of the parasite life cycle or in other *Plasmodium* species. We localized ACC in liver-stage *P. berghei* through immunofluorescence microscopy of infected HepG2 human liver cells by using antibodies directed against the BCCP domain of *PfACC* (10). We found that BCCP colocalizes with an apicoplast marker, the acyl carrier protein (ACP), indicating that endogenous *PbACC* resides in the apicoplast in liver-stage parasites (Fig. 1A).

ACC enzymes are only active when covalently modified by biotin. To determine whether *PbACC* could be biotinylated in the liver stages, we performed immunofluorescence microscopy of HepG2 cells infected with *P. berghei* by using antibodies against biotin. We observed that biotin colocalizes with the apicoplast marker ACP, indicating that biotinylated proteins reside in the apicoplast in liver-stage malaria parasites (Fig. 1B).

We next looked at ACC expression and biotinylation in blood-stage *P. berghei* by using immunofluorescence analysis with antibodies specific to BCCP and biotin. We found that *PbACC* is expressed in the apicoplast in blood-stage parasites (Fig. 1C), but we were unable to detect any biotinylated proteins in the blood stages (Fig. 1D). Thus, ACC is expressed in the apicoplast in blood- and liver-stage malaria parasites, but appears to be biotinylated only in the liver stages.

To determine whether endogenous ACC is biotinylated in blood-stage *P. falciparum*, we performed immunofluorescence analysis of Dd2^{attB} parasites by using antibodies specific to BCCP and biotin. We could detect ACC in the apicoplast, and, in some experiments, ACC protein appeared to be proximal to the apicoplast as well (Fig. 1E). This phenomenon was also observed in *P. berghei* and may reflect slow kinetics of trafficking such a large protein (~3,000 aa). We could not detect any biotinylated protein in *P. falciparum* because there was no significant signal above the background autofluorescence observed for uninfected red blood cells (Fig. 1F). These results indicate that, as in *P. berghei*, *PfACC* is expressed but not biotinylated in the blood stages, and raise the possibility that ACC activity is regulated through stage-specific biotinylation.

To investigate the requirements for ACC trafficking to the apicoplast, we expressed the first 115 residues of *PfACC* (containing the putative targeting domain) fused to a C-terminal GFP tag in Dd2^{attB} parasites. We found that ACC-GFP localizes to an elongated, branched organelle distinct from the mitochondrion, which is consistent with apicoplast morphology (SI Appendix, Fig. S1A), and colocalizes with the apicoplast marker ACP (SI Appendix, Fig. S1B). We also observed that the fusion protein was processed normally after import into the apicoplast (SI Appendix, Fig. S1C). Taken together, these results demonstrate that the ACC leader peptide is sufficient to direct the protein to the apicoplast in blood-stage *P. falciparum*.

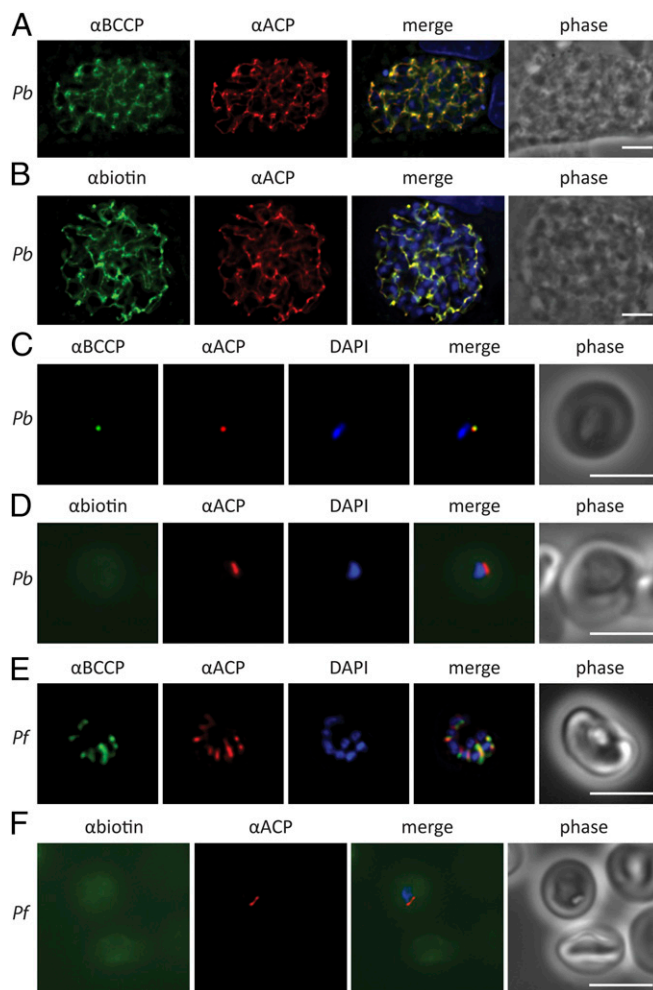


Fig. 1. ACC is expressed in the apicoplast and is biotinylated only in liver-stage parasites. (A) Immunofluorescence analysis of HepG2 cells at 48 hpi with *P. berghei* showing colocalization of *PbACC*, labeled with antibodies specific to the BCCP domain of ACC (α BCCP), with the apicoplast marker α ACP. The nuclei are labeled with DAPI. (B) Immunofluorescence analysis similar to A showing colocalization of biotinylated proteins (α biotin) with the apicoplast marker α ACP. (C) Immunofluorescence analysis of blood-stage *P. berghei* showing colocalization of *PbACC* (α BCCP) with the apicoplast marker α ACP. (D) Immunofluorescence analysis of blood-stage *P. berghei* showing no detectable biotinylated protein (α biotin). (E) Immunofluorescence analysis of blood-stage *P. falciparum* showing colocalization of *PfACC* (α BCCP) with the apicoplast marker α ACP. (F) Immunofluorescence analysis of blood-stage *P. falciparum* showing no detectable biotinylated protein (α biotin) compared with an uninfected control. (Scale bars: 5 μ m.)

HCS1 and HCS2 Localize Primarily to the Cytosol. We identified two biotin ligase paralogs (HCS1 and HCS2) in the *Plasmodium* genome. At least one of these enzymes should be responsible for catalyzing the biotinylation of *PbACC*; however, neither was predicted to localize to the apicoplast by targeting algorithms. To determine the subcellular location of the two biotin ligases in *P. berghei*, we tagged both genes with GFP through single cross-over recombination (SI Appendix, Fig. S2). Immunofluorescence analysis demonstrated that the GFP-tagged proteins are not predominantly located in the apicoplast in liver-stage or blood-stage parasites (SI Appendix, Fig. S3). Because of its size, the GFP tag may alter protein trafficking and subcellular location. To address this possibility, we generated HA-tagged parasite lines through double cross-over recombination (SI Appendix, Fig. S4). Consistent with the results from the GFP-tagged lines, the

HA-tagged proteins do not have obvious apicoplast colocalization (Fig. 2). This was true at different time points during liver-stage development (24, 48, and 56 h) and during blood-stage development (Fig. 2C).

We also localized the two biotin ligase paralogs in blood-stage *P. falciparum*. We overexpressed a second copy of each ligase as a GFP fusion protein in Dd2^{attB} parasites and characterized these lines by using fluorescence microscopy of live cells labeled with DAPI. We found that both fusion proteins primarily localize to the cytosol (Fig. 3A and B). We also performed Western blot analysis on whole-cell lysate from these parasites by using antibodies against GFP and detected a band of the correct mass for the full-length fusion protein in both biotin ligase overexpression lines (Fig. 3C and D). Interestingly, *PfHCS2*-GFP was more difficult to detect than *PfHCS1*-GFP despite the fact that the expression of both proteins was driven by the same regulatory elements. This may be a result of a difference in protein stability between the two ligases. Together, these results demonstrate that the two biotin ligases are primarily cytosolic in the liver and

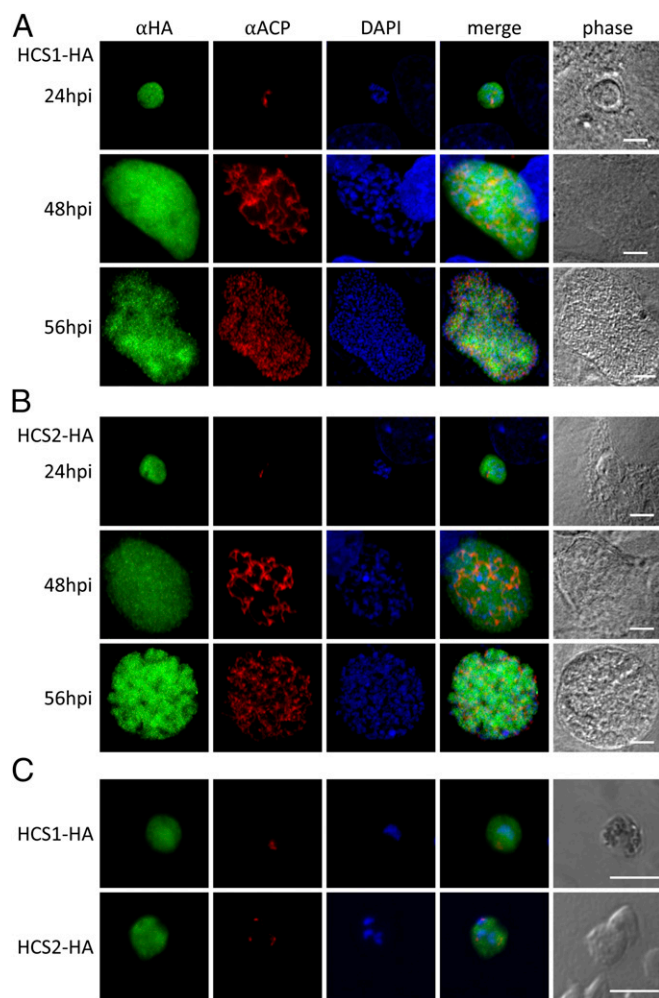


Fig. 2. HCS1 and HCS2 localize primarily to the cytosol in *P. berghei*. (A) Immunofluorescence analysis of HepG2 cells 24, 48, and 56 hpi with *P. berghei* parasites in which *PbHCS1* was tagged with an HA tag through double cross-over recombination. The tagged proteins are labeled with specific antibodies (α HA), the apicoplast is labeled with α ACP, and the nuclei are labeled with DAPI. (B) Immunofluorescence analysis similar to that shown for A using *P. berghei* parasites in which *PbHCS2* was tagged with an HA tag. (C) Immunofluorescence analysis of blood-stage parasites from the transgenic lines shown in A and B. (Scale bars: 5 μ m.)

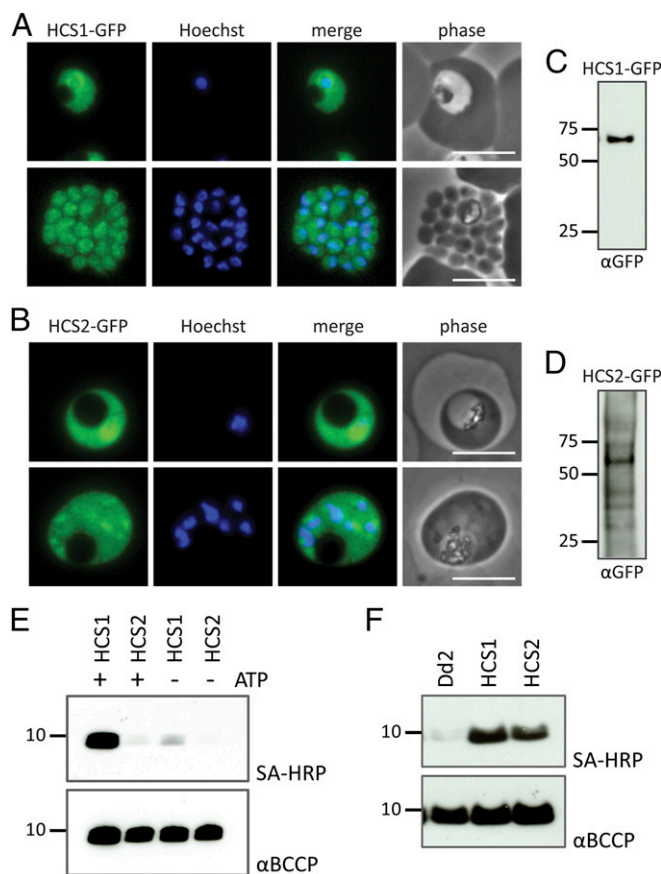


Fig. 3. *PfHCS1* and *PfHCS2* are active biotin ligases. (A and B) Epifluorescence images of live *P. falciparum* blood-stage parasites expressing full-length *PfHCS1* fused to GFP (HCS1-GFP) or *PfHCS2* fused to GFP (HCS2-GFP). The parasites were stained with Hoechst to identify nuclei. GFP fluorescence localizes to the cytosol in trophozoite (Top) and schizont (Bottom) parasites. (Scale bars: 5 μ m.) (C and D) Western blot analysis of lysate from the transgenic parasites shown in A and B using antibodies against GFP (α GFP) showing a dominant band that corresponds to the expected size of the full-length fusion protein. (E) Recombinant *PfHCS1* and *PfHCS2* were expressed in *E. coli* and purified by affinity chromatography, and their activity was tested by using an in vitro biotin ligase assay. *PfHCS1*, but not *PfHCS2*, biotinylated *PfBCCP* in vitro in an ATP-dependent reaction. (F) The activities of *PfHCS1* and *PfHCS2* were also tested in a cell-free assay. Lysates from parasites overexpressing the two ligases (shown in A and B), and a parental control, were incubated with purified BCCP under reaction conditions similar to *E. PfHCS1* and *PfHCS2* exhibit biotin ligase activity in this assay.

blood stages of the parasite life cycle. As biotinylated proteins are located in the apicoplast of liver-stage parasites, this raises the question of whether the biotin ligase paralogs are active ligases and are responsible for protein biotinylation in the parasite.

***PfHCS1* and *PfHCS2* Are Active Biotin Ligases.** To assess biotin ligase activity for *PfHCS1* and *PfHCS2*, we first tested the activity of the two ligases in vitro. Recombinant *PfHCS1* and *PfHCS2* were expressed in *E. coli* and purified by affinity chromatography. The BCCP domain of *PfACC* was also expressed and purified as a biotinylation substrate. We found that overexpression of *PfBCCP* in *E. coli* results almost exclusively in apoprotein with less than 1% of the purified *PfBCCP* biotinylated. We tested the reactivity of each ligase toward *PfBCCP* in vitro and assessed *PfBCCP* biotinylation through affinity blotting with streptavidin-HRP followed by Western blotting with antibodies specific to *PfBCCP*. We found that *PfHCS1*, but not *PfHCS2*, biotinylated *PfBCCP* in an ATP-dependent reaction (Fig. 3E). The very low signal in the absence

of ATP is probably the result of a small amount of biotinyl-AMP copurified with *PfHCS1*.

In addition, we performed streptavidin affinity-blot analysis of whole parasite lysate and observed a dramatic increase in total protein biotinylation in the *PfHCS1* overexpression line (generated as described here earlier), which was not present in the *PfHCS2* line or a parental control (*SI Appendix, Fig. S5*). This indicates that *PfHCS1* has unusually lax substrate specificity, likely because of the release of the biotinyl-AMP conjugate, which reacts spontaneously with proteins when it is free in solution.

In a final approach, we performed cell-free assays with parasite lysate. Whole-cell extract was generated from parasites overexpressing *PfHCS1* or *PfHCS2*, as well as the parental control, and the reactivity of these extracts toward recombinant *PfBCCP* was tested by using conditions similar to the *in vitro* biotinylation assay. In this assay, both ligases were active against *PfBCCP* (Fig. 3*F*), although *PfHCS2* activity was slightly, but consistently, weaker than *PfHCS1* in each repetition of this assay. The low level of biotinylation observed in the parental control is likely a result of endogenous biotin ligase activity. Taken together, these results show that malaria parasites express two functional biotin ligases, which represents an unusual example of an organism that contains two active biotin ligases. The fact that we observed activity for *PfHCS2* only in the cell-free assay may be because *PfHCS2* folds correctly only when expressed in *P. falciparum* or because this enzyme requires a binding partner not present in *E. coli*.

Robust Biotinylation Activity Can Be Detected in the Cytosol of Blood-Stage Parasites. To determine whether blood-stage parasites are capable of biotinylating apicoplast proteins, we used the BCCP domain of *PfACC* as a genetic probe for biotinylation activity in *P. falciparum*. We expressed *PfBCCP* with a C-terminal GFP tag in Dd2^{attB} parasites. In the absence of any targeting sequence, the BCCP-GFP fusion protein would be expressed in the cytosol. We also expressed BCCP-GFP with an N-terminal apicoplast-targeting peptide, the first 55 residues of *PfACP*. We generated two constructs for each protein, one in which expression was driven by the strong calmodulin (CaM) promoter, and one in which expression was driven by the moderate ribosomal protein L2 (RL2) promoter (22). We were able to generate transgenic parasites from three of the constructs: the two cytosolic constructs and the apicoplast-localized protein with the RL2 promoter. The final construct failed perhaps because overexpression of apicoplast proteins by the CaM promoter is poorly tolerated—a phenomenon we have observed before (23). We first verified that the GFP fusion proteins were correctly localized in each line by using live fluorescence microscopy (Fig. 4 *A–C*) and immunofluorescence analysis (*SI Appendix, Fig. S6*). We then analyzed whole-cell lysate from the transgenic parasites by using affinity blotting with streptavidin-HRP and Western blotting with antibodies specific to GFP. We observed robust biotinylation of *PfBCCP* when it was targeted to the cytosol, but could not detect any biotinylation activity in the apicoplast in blood-stage parasites (Fig. 4 *D–F*). The lack of biotinylation activity in the apicoplast agrees with the biotin ligase localization data, and eliminates the possibility of a cryptic or noncanonical biotinylation mechanism in the apicoplast. This also supports the conclusion that ACC is not biotinylated, and thus inactive, in parasite blood stages.

Biotin Is Not Essential for the Survival of Blood-Stage Parasites. It has been previously demonstrated that the FASII pathway, which should be dependent on biotin, is important for the liver stages of the parasite lifecycle (13, 14). However, it is not known whether biotin is essential for parasite survival in the blood or mosquito stages. Geary et al. (24) treated blood-stage parasites with 0.2 mg/mL avidin and found that it had no effect on parasite growth in culture, but these experiments were inconclusive because they did not test whether avidin was able to permeate erythrocytes or measure

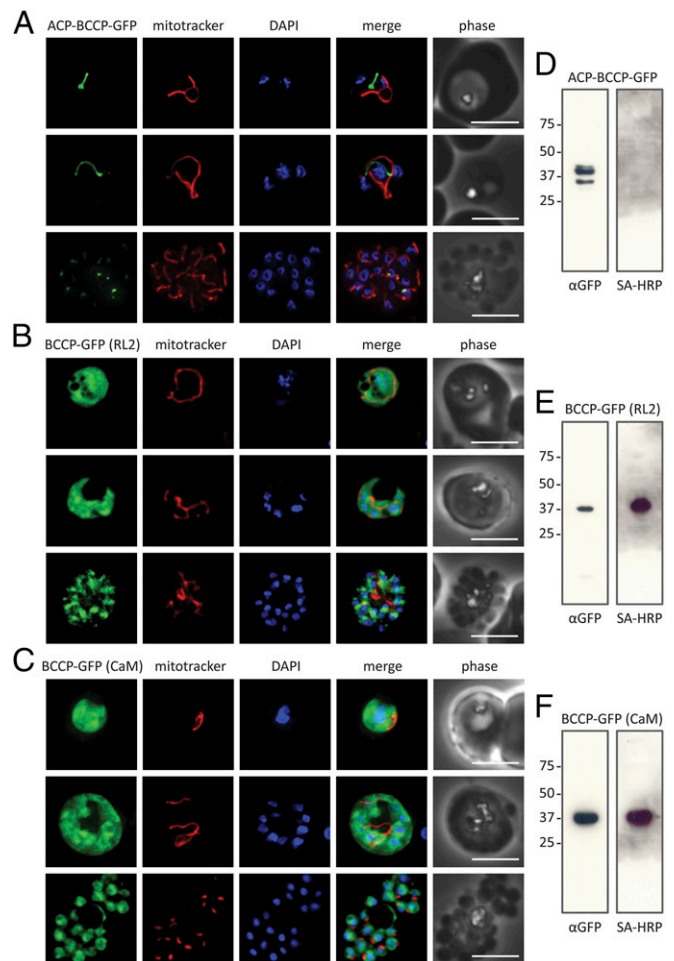


Fig. 4. Robust biotinylation activity can be detected in the cytosol but not the apicoplast of blood-stage *P. falciparum*. (*A*) The biotinylation domain (BCCP) from *PfACC* was expressed as a GFP fusion protein in the apicoplast of blood-stage *P. falciparum* by using the apicoplast targeting domain from *PfACP* (ACP-BCCP-GFP) under control of the ribosomal L2 (RL2) promoter. Localization was verified by epifluorescence microscopy in late ring (*Top*), trophozoite (*Middle*), and schizont (*Bottom*) parasites. Parasites were stained with MitoTracker to identify the mitochondrion and DAPI to identify nuclei. (*B*) BCCP was expressed as a GFP fusion protein in the cytosol (BCCP-GFP) under control of the ribosomal L2 (RL2) promoter, and localization was verified by epifluorescence microscopy as in *A*. (*C*) BCCP was expressed as a GFP fusion protein in the cytosol (BCCP-GFP) under control of the stronger CaM promoter, and localization was verified by epifluorescence microscopy as in *A*. (*D–F*) Whole-cell lysates from the parasite lines shown in *A–C* were analyzed by Western blotting with antibodies against GFP and affinity blotting with streptavidin-HRP. BCCP is biotinylated in the cytosol but is not biotinylated when expressed in the apicoplast. (Scale bars: 5 μm.)

the concentration of intraerythrocytic biotin stores. To determine whether blood-stage *P. falciparum* is auxotrophic for biotin, we measured parasite growth in biotin-depleted medium. The medium was prepared from biotin-free RPMI supplemented with the serum substitute AlbuMAX II. We tested the growth of Dd2^{attB} parasites as well as the three lines overexpressing the BCCP domain of *PfACC* (generated as described here earlier). To confirm that we had completely removed biotin from the cultures, we used *PfBCCP* as a sentinel protein to detect intracellular biotin. It should also function as a biotin sink to further sensitize parasites to biotin deprivation because it would compete with endogenous substrates for any trace amounts of biotin that remained. We found that the presence or absence of biotin in the media made no significant difference in parasite growth (Fig. 5*A*). On the eighth

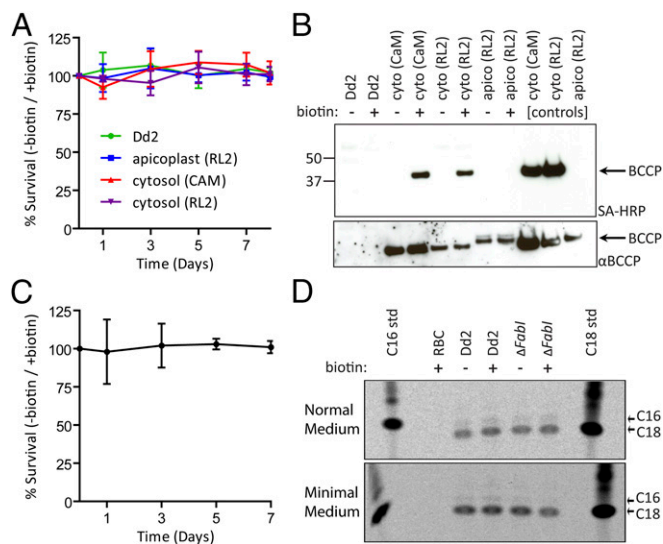


Fig. 5. Biotin is not essential for survival of blood-stage *P. falciparum*. (A) Blood-stage *P. falciparum* cultures were maintained for 8 d in media with or without biotin. Four parasite lines were used: the parental control (Dd2), parasites overexpressing BCCP in the apicoplast under the ribosomal L2 (RL2) promoter, and parasites overexpressing BCCP in the cytosol under the RL2 promoter or the stronger CaM promoter. Error bars represent SEM of three biological replicates. (B) On day 8 of the growth assay shown in A, parasites were harvested and biotinylation levels were assessed by streptavidin-HRP affinity blotting, followed by Western blotting with α BCCP antibodies. There was no detectable biotinylated BCCP under biotin-free conditions, indicating that biotin was completely depleted in these cultures. Controls were parasites cultured in complete medium. (C) Blood-stage parasites (Dd2) were cultured with or without biotin for 7 d in media containing lipid-free BSA reconstituted with 30 μ M palmitic acid and 30 μ M oleic acid. Error bars represent SEM of three biological replicates. (D) TLC of parasites labeled with 14 C-acetate showing incorporation into C16 and C18 fatty acids. This activity was seen in WT and FASII KO parasites, and was independent of biotin. Similar results were obtained when this experiment was repeated (Bottom) using the minimal fatty acid medium described in C. No activity was seen with the negative control, uninfected red blood cells (RBC).

day of the growth assay, parasites were isolated, and whole-cell extract was analyzed by affinity blotting with streptavidin-HRP followed by Western blotting with antibodies specific to *Pf*BCCP. There was no detectable biotinylated *Pf*BCCP in the cultures grown under biotin-free conditions, indicating that biotin was completely depleted from the medium (Fig. 5B). We therefore concluded that biotin is not essential for blood-stage replication in *P. falciparum*. In addition, these data show that parasites scavenge biotin rather than synthesize it, as cytosolic *Pf*BCCP is biotinylated only when biotin is added to the medium.

It has previously been shown that blood-stage malaria parasites have the ability to elongate fatty acids taken up from the medium (25). In addition, it has been demonstrated that parasites lacking a functional FASII pathway can still incorporate radiolabeled acetate into fatty acid species, most likely because of the activity of the ELO pathway (14). Based on these results, we tested whether parasites can survive without biotin under limited fatty acid culture conditions. We cultured parasites in serum-free medium supplemented with lipid-free BSA reconstituted with only palmitic acid (C_{16:0}) and oleic acid (C_{18:1}), which are the minimal requirements for parasite growth (25). Parasites cultured with or without biotin were maintained in parallel, and biotin made no difference in the growth rates of the parasites (Fig. 5C). These results demonstrate that biotin is not required for blood-stage parasite growth, even under conditions in which the FASII and ELO pathways would be needed the most.

Biotinylated Proteins Cannot Be Detected in Blood-Stage Parasites.

Thus far, we have been unable to show any biotin-dependent phenotype in blood-stage parasites. This raises the question of whether there are any biotinylated proteins in the blood stages. We were unable to detect any signal above background in immunofluorescence analysis of blood-stage *P. falciparum* (Fig. 1F) or *P. berghei* (Fig. 1D) by using antibodies specific to biotin. Similarly, we were unable to detect biotinylated proteins in the blood stages by using streptavidin-FITC (SI Appendix, Fig. S7A). Because these data do not rule out the possibility that there are biotinylated proteins present at a level below the limit of detection by microscopy, we used more sensitive techniques to probe this question further. In radiolabel uptake experiments with blood-stage *P. falciparum*, we did not detect incorporation of [3 H]biotin or [35 S]biotin into cellular proteins (SI Appendix, Fig. S7B), even though we have shown that the parasites are capable of scavenging and incorporating exogenous biotin (Fig. 5B). In a final approach, we performed affinity-blot analysis of parasite lysate by using streptavidin-HRP. Although we detected biotinylated proteins in some of the blots, these bands can often be detected in samples of uninfected red blood cells (SI Appendix, Fig. S7C). These three proteins correspond in mass to the human biotin carboxylases methyl-crotonyl-CoA carboxylase (73.7 kDa), pyruvate carboxylase (127.2 kDa), and the ACC isoforms (~268.5 kDa). Although there are no biotin carboxylases expressed in mature red blood cells, they are expressed in reticulocytes and leukocytes, which can be present in small amounts as contaminants in our culture system. Thus, if blood-stage malaria parasites contain any biotinylated proteins, these proteins are much less abundant than the trace levels of human proteins in our culture system.

Biotin- and FASII-Independent Fatty Acid Elongation Activity Is Present in Blood-Stage *P. falciparum*.

The growth of blood-stage *P. falciparum* parasites does not require biotin or ACC (8), leading us to the hypothesis that the ELO pathway may not rely on malonyl-CoA produced by ACC. We therefore tested whether ELO activity could be detected under biotin-free conditions. Synchronized ring-stage parasites were incubated with [14 C]-acetate with or without biotin, after which free fatty acids were extracted and analyzed by thin-layer chromatography (TLC). To ensure we were assessing ELO activity, we compared WT (i.e., Dd2) parasites with FASII-KO (Δ FabI) parasites (14). We detected radiolabeled C₁₆ and C₁₈ fatty acids in both parasite lines cultured with or without biotin (Fig. 5D). Similar results were obtained in minimal fatty acid culture medium (Fig. 5D), conditions previously shown to elevate FASII activity (26). Thus, blood-stage parasites are capable of elongating fatty acids independent of FASII and biotin.

Neither *Pb*HCS1 Nor *Pb*HCS2 Is Required for Blood-Stage Replication or Mosquito-Stage Development.

To determine which biotin ligase is responsible for ACC biotinylation, both enzymes were knocked out in *P. berghei* ANKA parasites. The KO constructs were designed by using the pDEF-hDHFR plasmid, which facilitates double cross-over homologous recombination, resulting in excision of the entire ORF of the target gene (SI Appendix, Fig. S8A and C). Correct integration of the hDHFR cassette and loss of *HCS1* and *HCS2* were verified in independent clones by diagnostic PCR (SI Appendix, Fig. S8B and D). The viability of both KO strains demonstrated that neither gene is essential for survival in the blood stages. To characterize development through the mosquito stages, WT and Δ HCS1 or Δ HCS2 gametocytes were fed to *Anopheles stephensi* mosquitoes, and infection levels were measured at different time points. Mosquito midgut oocysts were visualized 10 d after feeding, midgut sporozoites were counted 12 and 14 d after feeding, and salivary-gland sporozoites were counted between 16 and 20 d after feeding. Throughout mosquito stage development, Δ HCS1 and Δ HCS2 parasites exhibited no defects in oocyst formation, sporozoite development, or sporozoite

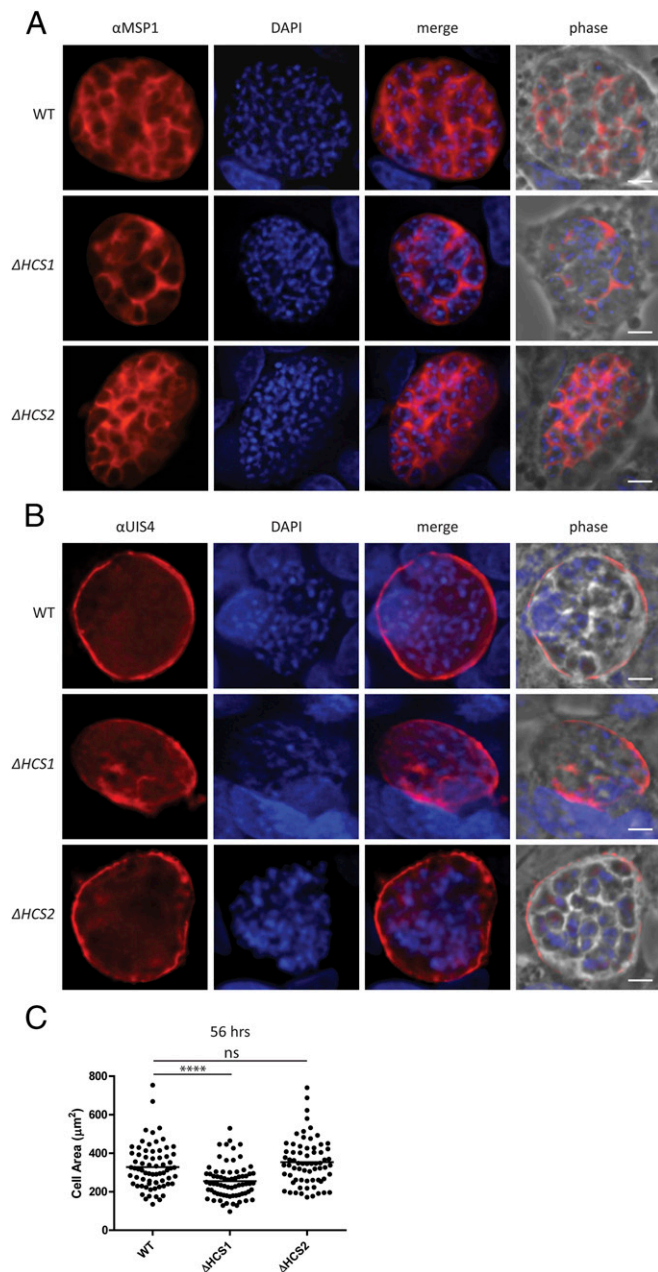


Fig. 7. Liver-stage development is abnormal in $\Delta HCS1$ parasites. (A) Immunofluorescence analysis of infected HepG2 cells 56 hpi with WT, $\Delta HCS1$, or $\Delta HCS2$ *P. berghei*. The parasite plasma membrane is illuminated with the late liver-stage marker α MSP1, and the nuclei are labeled with DAPI. (B) Immunofluorescence analysis similar to A at 56 hpi by using antibodies specific for the parasitophorous vacuole protein UIS4. (C) Quantification of cell area at 56 hpi from three biological replicates with at least 20 measurements per parasite strain. (Scale bars: 5 μ m.) ns, not significant; **** $P < 0.0001$.

WT or $\Delta HCS1$ sporozoites, and merosomes were collected and counted 65 h post invasion. At least five replicates were used for each condition. Under normal biotin-replete conditions, we found that there was an approximately fourfold reduction in the number of merosomes produced by $\Delta HCS1$ parasites compared with WT (Table 1); however, the size of the $\Delta HCS1$ merosomes appeared consistently smaller than those of WT parasites. We therefore quantified total merozoite numbers by using quantitative PCR and found that $\Delta HCS1$ merosomes contain fewer merozoites. We observed that the number of merozoites per merosome was ~ 10 -fold

less in the $\Delta HCS1$ parasites, which, when combined with the lower number of merosomes produced by this mutant, results in a 40-fold overall decrease in the number of merozoites produced. This could account for the significant difference in the prepatency assays (SI Appendix, Table S1). Under biotin-depleted conditions, fewer merozoites were produced by WT and $\Delta HCS1$ parasites than were produced by the mutant when grown in the presence of biotin (Table 1). We assessed the effect of biotin depletion on infected HepG2 cells with immunofluorescence analysis and confirmed that host and parasite biotinylation was significantly reduced (SI Appendix, Fig. S12). Together, these data indicate that liver-stage parasites rely on biotin scavenged from the host, and that biotin is required for normal liver-stage development. These data also suggest that perturbation of biotin metabolism in the host cell may further impede the development of liver-stage parasites.

Biotin Is Required for Merosome Infectivity. We assessed the ability of merosomes produced in vitro to initiate blood-stage infections in mice. Merosomes were produced as described earlier in HepG2 cells cultured in biotin-depleted media or normal media. Across three independent experiments, merosomes produced in normal medium were usually infectious (14 of 14 mice for WT and 9 of 13 mice for $\Delta HCS1$) with a delay to patency for $\Delta HCS1$ similar to that observed in SI Appendix, Table S1. However, WT merosomes produced under biotin-deficient conditions were infectious in only 1 of 14 mice. Quantification of total merozoites indicates that the reduced infectivity was not caused by lower numbers of merosomes produced (Table 2). Mutant parasites cultured in biotin-deficient medium were never infectious. When combined with the data presented in Table 1, these experiments show that biotin deficiency affects the infectivity of merosomes as well as reducing the total number of merosomes produced.

Discussion

We identified one biotinylation substrate (ACC) in malaria parasites, but two biotin ligases (HCS1 and HCS2). This unusual arrangement seems to be conserved in all species of malaria parasites; however, the organization of biotin metabolism is quite divergent among different apicomplexan genera. The apicomplexan *Cryptosporidium parvum* does not contain an apicoplast and encodes a cytosolic ACC and cytosolic biotin ligase. By contrast, *Theileria annulata* lacks both ACC and biotin ligases. In *T. gondii*, two biotinylated proteins (ACC and pyruvate carboxylase) were

Table 1. Host biotin is required for normal liver stage development

Expt	Line	Biotin	Mrsm per well (SD)	Mzt/Mrsm	Mzt per well $\times 10^3$
1	WT	+	6,900 (1,924)	—	—
	$\Delta HCS1$	+	1,550 (788)	—	—
2	WT	+	17,390 (5,663)	3,307	58,000
	WT	—	670 (303)	216	140
	$\Delta HCS1$	+	3,270 (434)	304	990
	$\Delta HCS1$	—	2,330 (1,492)	60	140
3	WT	+	1,500 (418)	4,867	7,300
	WT	—	313 (314)	319	100
	$\Delta HCS1$	+	646 (267)	480	310
	$\Delta HCS1$	—	250 (194)	148	37
4	WT	+	3,000 (576)	3,667	11,000
	WT	—	438 (304)	685	300
	$\Delta HCS1$	+	958 (359)	501	480
	$\Delta HCS1$	—	250 (188)	220	55

Expt, experiment number; Mrsm, merosome; Mzt, merozoite.

Table 2. Biotin is required for merosome infectivity

Expt	Line	Biotin	Mrsrm injected	Mice positive	P0, d	Mzt injected $\times 10^3$
1	WT	+	1,000	4/4	3.5	—
	WT	—	1,000	0/4	*	—
	$\Delta HCS1$	+	1,000	3/3	5.7	—
	$\Delta HCS1$	—	1,000	0/1	*	—
2	WT	+	2,500	5/5	3.6	27,000
	WT	—	2,500	0/5	*	520
	$\Delta HCS1$	+	2,500	2/5	5.5	170
	$\Delta HCS1$	—	2,500	0/5	*	130
3	WT	+	2,000	5/5	5.6	6,700
	WT	—	2,000	1/5	7	350
	$\Delta HCS1$	+	2,000	4/5	7.5	320
	$\Delta HCS1$	—	2,000	0/5	*	160

Expt, experiment number; Mice positive, fraction of mice infected with blood stage parasites; Mrsrm, merosome; Mzt, merozoite; P0, prepatent period.

*Parasites were not detected 1 mo after merosome injection.

identified (29), but there is only one predicted biotin ligase (TGME49_089760).

The duplication of biotin ligases in malaria parasites is similar to the situation found in the plant *A. thaliana*. The two HCS genes in *A. thaliana* share 71% sequence identity and likely resulted from a gene duplication event. Similarly, HCS1 and HCS2 from *P. falciparum* share 47% sequence identity and are likely paralogs created by gene duplication. *AtHCS1* is essential for viability, whereas disruption of the *AtHCS2* gene does not lead to any observable phenotype (21). Similarly, deletion of *P. falciparum* HCS1 results in a growth defect whereas deletion of HCS2 does not (SI Appendix, Table S1). It is possible that HCS2 and *AtHCS2* share a common role that has yet to be determined.

Both biotin ligase paralogs are predominantly located in the cytosol in the blood stages of *P. berghei* (Fig. 2C) and *P. falciparum* (Fig. 3A and B). It was anticipated that at least one of the ligases would be located in the apicoplast because this subcellular compartment was thought to contain ACC (30, 31). Indeed, ACC is located in the apicoplast in *P. berghei* and *P. falciparum* (Fig. 1C and E), consistent with apicoplast localization of ACC in the related apicomplexan parasite *T. gondii* (29). Although ACC is biotinylated in *T. gondii* (29), it is not biotinylated in blood-stage malaria parasites (Fig. 1D and F). We found this result to be surprising and used a genetic probe to determine whether any biotin ligase activity is located in the apicoplast. When we expressed the BCCP domain of ACC in blood-stage *P. falciparum* parasites, we found that the probe was biotinylated when expressed in the cytosol, but that there was no detectable biotinylation when it was expressed in the apicoplast (Fig. 4). This result is consistent with the cytosolic localization of both biotin ligases and indicates that the apicoplast of blood-stage parasites does not contain any enzymes with biotin ligase activity. Taken together, these results show that blood-stage malaria parasites express ACC and two active biotin ligases, but ACC is not biotinylated because all biotin ligase activity is confined to the cytosol whereas ACC is exclusively located in the apicoplast.

The fact that ACC is not biotinylated in blood-stage malaria parasites suggests that biotin is not needed during this stage of parasite development. We cultured *P. falciparum* parasites expressing the BCCP domain in biotin-depleted growth medium. These parasite lines grew equally well regardless of whether biotin was added back to the medium (Fig. 5A). Subsequent analysis of parasites expressing BCCP in the cytosol demonstrated that BCCP was biotinylated only when biotin was added back to the depleted medium (Fig. 5B). These experiments show that blood-stage parasites do not synthesize biotin and do not rely on this vitamin for growth, but can import external biotin

when it is supplied in the medium. Even when parasites were cultured in growth medium designed to increase the reliance on fatty acid metabolism, there was still no growth phenotype associated with biotin depletion (Fig. 5C).

The ability of blood-stage malaria parasites to grow without biotin has interesting consequences for fatty acid metabolism. All known FAS and ELO pathways use malonyl-CoA for chain elongation. ACC is the only predicted source of malonyl-CoA in malaria parasites, and thus should be required for FASII and ELO activity. Key enzymes of the FASII pathway, such as FabI, have been knocked out in human and rodent malaria species, demonstrating that FASII is dispensable for blood-stage replication (13, 14). The lack of ACC biotinylation in blood-stage parasites further suggests that FASII cannot function during this stage of parasite development. Fatty acid elongation, however, can still be observed in $\Delta FabI$ parasites, implying that the ELO pathway is active in the blood stages of *P. falciparum* and *P. berghei* (14). We used the *P. falciparum* $\Delta FabI$ line to show that radiolabeled acetate is still incorporated into parasite fatty acids regardless of whether biotin is present in the growth medium, and, furthermore, the incorporation of acetate was the same between $\Delta FabI$ parasites and the parental line (Fig. 5D). Taken together, these data suggest that FASII is inactive in blood-stage *P. falciparum* parasites whereas these parasites have ELO activity that does not depend on biotin or ACC. The ELO pathway could rely on another source of malonyl-CoA, such as a malonyl-CoA synthetase (32).

In liver-stage malaria parasites, the arrangement of ACC and the biotin ligases appears to be the same as it is in blood stages. The ACC is located in the apicoplast (Fig. 1) whereas the biotin ligases are predominantly located in the cytosol (Fig. 2 and SI Appendix, Fig. S3). The obvious difference is that apicoplast proteins (presumably ACC, although there may be others) are biotinylated, and this raised the question of which ligase is responsible for this activity. To answer this question, we deleted the genes encoding HCS1 and HCS2 in *P. berghei* and found that HCS1 is exclusively responsible for protein biotinylation whereas deletion of HCS2 has no detectable liver-stage phenotype (Fig. 6). This result suggests that HCS1 is partially located in the apicoplast of liver-stage parasites, albeit at low levels compared with the majority of HCS1 located in the cytosol (Fig. 24). There are examples in other organisms of dual targeting of biotin ligases to different cellular compartments, which is regulated by alternative splicing of a single mRNA or alternative translation initiation sites. This has been shown in some detail in *A. thaliana* (21) and has been suggested in *Pisum sativum* (20) as well as humans (33). In addition, there are examples of dual targeting of proteins to the apicoplast, mitochondrion, and cytosol in malaria parasites (34–36), although the mechanisms are not well understood. One suggested mechanism is the use of an ambiguous targeting sequence that is inefficiently recognized by the protein sorting machinery, so that a fraction of the total protein is imported into an organelle and the remainder is retained in the cytosol (37). Regardless of the mechanisms involved, our results show that biotinylation by HCS1 is stage-specific, occurring in liver-stage but not blood-stage malaria parasites.

P. berghei parasites lacking HCS1 have a defect in liver-stage development that begins early in parasite development (SI Appendix, Fig. S10) and becomes more obvious by 56 h post invasion (Fig. 7). $\Delta HCS1$ parasites produce fewer merosomes than WT parasites, and these merosomes contain significantly fewer merozoites (Table 1). In general, late liver-stage developmental defects and severely curtailed formation of merosomes are hallmarks of KO parasites with FASII gene deletions (13, 14) or deletions of genes required for the FASII pathway (15, 38). Deletion of FASII-related genes in *P. berghei* [$\Delta FabI$ (14) and $\Delta LipB$ (38)] results in a 4-d delay to patency, with few parasites able to complete liver-stage development and almost no merosome formation. By contrast, $\Delta HCS1$ parasites display a 2.1-d delay to patency and

quantifiable merosomes. If HCS1 is responsible for activating ACC to supply malonyl-CoA for FASII, why is the Δ HCS1 phenotype different from that of Δ FabI or Δ LipB? One explanation for the less severe Δ HCS1 phenotype is that the parasites have another source of malonyl-CoA, allowing them to partially bypass the need for HCS1. Hepatocyte ACC produces malonyl-CoA, and it is possible that this metabolite can be scavenged. This explanation is consistent with the observation that WT and Δ HCS1 parasites produce fewer merozoites when cultured in biotin-depleted medium (Table 1). Presumably, the deletion of HCS1 affects only parasite ACC activity, whereas biotin depletion affects parasite and host cell ACC activity.

To assess the combined effects of host cell and parasite biotin deprivation, we conducted merosome infection experiments. We found that WT and mutant Δ HCS1 merosomes produced in vitro by host cells cultured in complete medium were usually infectious in mice, but were almost never infectious if they were produced in biotin-deficient conditions (Table 2). The dramatic decrease in infectivity could result from the merozoites failing to egress from the membrane-bound merosomes, or it could be that the merozoites are defective. It is difficult to distinguish between these possibilities in our experiments; however, the fact that many merozoites are released during the injection process suggests that the merozoites themselves are less infectious.

These results raise the possibility that biotin is a host nutrient that could affect parasite development, as has been suggested for other nutrients (39). In humans, biotin deficiency is almost invariably caused by mutations in an essential enzyme called biotinidase, which is required to make use of biotin obtained from dietary sources (40). More than 165 biotinidase mutations have been described in humans (41). In some cases, these mutations are severe enough to cause disease, but it is more common for these mutations to decrease biotin utilization without causing disease. Although the data currently available are not sufficient to implicate biotin deficiency in protection from malaria infection, there is some evidence that biotinidase mutations are more prevalent in certain African populations (42).

In conclusion, we found that ACC is expressed in the apicoplast organelle in liver- and blood-stage malaria parasites, but it is biotinylated only in the liver stages, suggesting that ACC activity is regulated through stage-specific biotinylation. Because ACC is the first committed step in FASII pathways, protein biotinylation may serve as a molecular switch controlling fatty acid biosynthesis in malaria parasites. Gene deletion experiments showed that the biotin ligase HCS1 is solely responsible for biotinylation in liver-stage parasites. Parasites lacking HCS1 show a late liver-stage defect and produce fewer merosomes with abnormally low numbers of merozoites per merosome. Biotin depletion results in more severe developmental defects, resulting in merozoites that are no longer capable of blood-stage infection. These results imply that host and parasite biotin metabolism are required for normal liver-stage progression and merozoite infectivity. Thus, the development of liver-stage malaria parasites may be linked to the nutritional status of the host, as neither the parasite nor the human host can synthesize biotin.

Materials and Methods

Cells and Antibodies. HepG2 human liver carcinoma cells [HB-8065; American Type Culture Collection (ATCC)] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, and 1 \times penicillin-streptomycin. The following antibodies were used: mouse monoclonal α -biotin (no. 200-002-211; Jackson) at 1:250 or 1:500 dilution; rabbit BCCP antiserum (10) (raised against the *P. falciparum* antigen but recognizes *P. falciparum* and *P. berghei* ACC) at 1:100 (*Pf*) or 1:250 (*Pb*) dilution; rabbit or rat polyclonal α ACP (43, 44) (raised against the *P. falciparum* antigen but recognizes *P. falciparum* and *P. berghei* ACP) at 1:500 (*Pf*) or 1:1,000 (*Pb*) dilution; mouse monoclonal α CSP 3D11 (27) at 1:1,000 dilution; rabbit α UIS4 (28) at 1:1,000 dilution; *P. yoelii* MSP1-19 rabbit antiserum (MRA-23, MR4; ATCC) at 1:1,000 dilution; and mouse monoclonal α GFP

(Roche) at 1:200 (*Pf*) or 1:1,000 (*Pb*) dilution. The corresponding secondary antibodies conjugated to Alexa Fluor-488 or Alexa Fluor-594 (Invitrogen) were used as indicated.

Immunofluorescence Analysis. To visualize liver-stage parasites, HepG2 cells were seeded at a density of 10^5 cells per well on Permax eight-well chamber slides coated with collagen (no. 354236; BD Biosciences). *P. berghei* ANKA sporozoites were harvested from infected *Anopheles stephensi* mosquitoes 18–24 d post blood meal, quantified, and centrifuged onto the HepG2 cells at a density of 2×10^4 sporozoites per well. At the time point indicated, cells were fixed with 4% paraformaldehyde for 20 min at room temperature, washed with PBS solution, and permeabilized with methanol overnight at -20°C . Slides were blocked and probed in 1% BSA in PBS solution and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen). Average cell area was quantified by using Volocity software (PerkinElmer). Immunofluorescence analysis of *P. berghei* and *P. falciparum* blood-stage parasites was carried out as previously described (45) with a few modifications (46). The *SI Appendix* provides more details.

***P. falciparum* Transfections.** Parasite transfections were carried out by using the mycobacteriophage Bxb1 integrase system developed by Nkrumah et al. (47) and modified by Spalding et al. (48). Vector construction using the primers listed in *SI Appendix, Table S2* is described in the *SI Appendix*.

Endogenous Tagging and Localization of *P. berghei* Biotin Ligases. Double cross-over replacement constructs were made for the *P. berghei* biotin ligase genes HCS1 (PBANKA_051100) and HCS2 (PBANKA_132360). *P. berghei* ANKA genomic DNA was amplified and cloned into a vector designed to append a 3xHA tag to the 3' end of the ligase genes. A single cross-over strategy was used to generate GFP-tagged parasite lines (*SI Appendix*). Constructs were linearized and transfected into *P. berghei* ANKA according to Janse et al. (49).

Biotin Ligase Activity Assays and Affinity/Western Blot Analysis. The biotin ligase genes were subcloned into expression vector pMALCHT (50) for expression and purification as MBP fusion proteins. The truncated BCCP domain of PfACC was expressed as a GST fusion protein in BL21-Star(DE3) cells by using a modified pGEX-4T-3 expression vector (pGEXT) and purified as previously described (10). The biotin ligase in vitro assay was carried out by using pure recombinant BCCP and biotin ligase proteins in the presence of ATP and biotin. Biotinylated BCCP was detected by affinity blot using streptavidin coupled to peroxidase (ultrasensitive; Sigma), and the presence of BCCP protein was confirmed by anti-BCCP Western blot. For the cell-free assay, *P. falciparum* parasites were isolated by saponin lysis followed by sonication to release parasite proteins. BCCP, biotin, and ATP were added to the parasite lysates, and biotinylation activity was assessed by affinity and Western blot.

Growth Assay and Radiolabel Incorporation. Biotin-depleted medium was prepared using RPMI medium 1640 modified with L-glutamine without phenol red and biotin (R9002-01; US Biological), which was supplemented with 25 mM Hepes, 0.375% sodium bicarbonate, 12.5 $\mu\text{g}/\text{mL}$ hypoxanthine, 5 g/L AlbuMAX II, and 25 $\mu\text{g}/\text{mL}$ gentamicin. For each parasite line, the cultures and red blood cells were washed twice in 10 mL biotin-free RPMI to remove biotin and then divided into parallel cultures for growth assays conducted with or without biotin supplementation. Lipid-free BSA (Sigma) was reconstituted with palmitic acid (Sigma) and potassium oleate (Fluka) and used in lieu of AlbuMAX for growth assays under minimal lipid conditions. For radioactive biotin incorporation experiments, *P. falciparum* cultures were maintained with daily feedings of biotin-free medium. Parasite cultures were labeled for 3 d with 0.48 nmol ^3H -biotin (American Radiolabeled Chemicals) or 29.2 nmol of freshly prepared ^{35}S -biotin (10) each day for 3 d. Label incorporation was assessed by autoradiography. For acetate incorporation, synchronized ring-stage parasites were incubated for 24 h with 10 $\mu\text{Ci}/\text{mL}$ ^{14}C -acetate, after which parasites were isolated by saponin lysis. Fatty acid methyl esters were extracted and resolved by TLC as described in *SI Appendix*.

***P. berghei* Biotin Ligase KOs.** To generate the double cross-over KO construct for *PbHCS1* and *PbHCS2*, homology arms composed of the 5' and 3' UTR of *PbHCS1* and *PbHCS2* were cloned into the pDEF-hDHFR plasmid (51). Constructs were linearized and transfected into *P. berghei* as described by Janse et al. (49) with a few modifications (*SI Appendix*).

***P. berghei* Liver-Stage Development and Infectivity Assays.** For prepatency assays, salivary gland sporozoites were harvested from infected *A. stephensi* mosquitoes and injected into mice i.v. followed by daily monitoring of

parasitemia by blood smear. Merosome production was assessed by collecting the supernatant from HepG2 cells infected with salivary gland sporozoites at 65 hpi. Merosomes were quantified in a hemocytometer, and the DNA content of the merosome samples was determined with quantitative real-time PCR. For infectivity assays, harvested merosomes were quantified and prepared for tail-vein injection into mice. Blood-stage parasitemia was subsequently monitored by microscopy.

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