Structure and Function of a G-actin Sequestering Protein with a Vital Role in Malaria Oocyst Development inside the Mosquito Vector*§

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Cyclase-associated proteins (CAPs) are evolutionary conserved G-actin-binding proteins that regulate microfilament turnover. CAPs have a modular structure consisting of an N-terminal adenylate cyclase binding domain, a central proline-rich segment, and a C-terminal actin binding domain. Protozoan parasites of the phylum Apicomplexa, such as Cryptosporidium and the malaria parasite Plasmodium, express small CAP orthologs with homology to the C-terminal actin binding domain (C-CAP). Here, we demonstrate by reverse genetics that C-CAP is dispensable for the pathogenic Plasmodium blood stages. However, c-cap(-) parasites display a complete defect in oocyst development in the insect vector. By trans-species complementation we show that the Cryptosporidium parvum ortholog complements the Plasmodium gene functions. Purified recombinant C. parvum C-CAP protein binds actin monomers and prevents actin polymerization. The crystal structure of C. parvum C-CAP shows two monomers with a right-handed β-helical fold intercalated at their C termini to form the putative physiological dimer. Our results reveal a specific vital role for an apicomplexan G-actin-binding protein during sporogony, the parasite replication phase that precedes formation of malaria transmission stages. This study also exemplifies how Plasmodium reverse genetics combined with biochemical and structural analyses of orthologous proteins can offer a fast track toward systematic gene characterization in apicomplexan parasites.

Single cell eukaryotes of the phylum of Apicomplexa are obligate intracellular parasites in human and in a wide range of domestic and wild animals. They include pathogens of enormous medical and veterinary importance, such as Plasmodium, the causative agent of malaria, Toxoplasma and Cryptosporidium, two opportunistic and potentially life-threatening infections, and Theileria, which inflicts huge economic losses to cattle herders in the tropics. Toward drug target validation availability of near-complete apicomplexan genomes (1, 2), in combination with experimental genetics in the model rodent malaria parasites Plasmodium berghei and P. yoelii, comprehensive in vivo characterization of parasite genes throughout the complex life cycle in the vertebrate and invertebrate hosts is feasible. However, complementary biochemical and structural analyses are often hindered by difficulties to express and purify the corresponding proteins, partly due to the high genomic A/T content and the abundance of low complexity regions in Plasmodium proteins. Thus, combining the versatile model rodent malaria system with high throughput expression, purification, crystallization, and structural refinement of orthologous proteins from related apicomplexan parasites may offer an attractive path to prioritize targets for anti-infectives development.

This approach was taken here to characterize the cyclase-associated protein (CAP)5 homology protein of apicomplexan parasites (3). CAPs are ubiquitous regulators of the dynamic turnover of actin cytoskeletal structures in part through their capacity to bind monomeric actin (G-actin). We previously demonstrated that a parasite G-actin-binding protein, the unconventional actin depolymerizing factor-1 (ADF1) performs vital roles during pathogenic asexual blood stage development (4). Therefore it was important to examine whether apicomplexan CAPs behave similarly and qualify as potential drug targets.

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† The atomic coordinates and structure factors (code 2B0R) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

‡ The nucleotide sequences reported in this paper have been submitted to the GenBank with accession number(s) FJ222721.

§ The on-line version of this article (available at http://www.jbc.org/) contains supplemental Figs. S1 and S2 and Table S1.

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The available data from unicellular and higher eukaryotes suggest that CAPs are important factors that link changing cellular environments to microfilament dynamics. The founding member of the CAP family, yeast CAP/SRV2, was identified as a suppressor of the activated RAS2Val19 allele and as a component of the adenyl cyclase complex, a binding partner and Ras-mediated activator for adenyl cyclase in Saccharomyces cerevisiae (5, 6). The adenylate cyclase binding site was mapped to the N-terminal portion (7). By contrast, metazoan CAP orthologs do not regulate adenylate cyclase, and their N termini may mediate subcellular localization (8) or oligomerization (9). Importantly, overexpression of the G-actin-binding protein profilin suppressed the cytoskeletal phenotype of the svr2 mutant suggesting a close functional relationship between the two proteins (10). In good agreement, CAP associates directly with G-actin (11) through its C-terminal domain (12, 13). CAP binds preferentially to ADP-actin monomers (14) and also participates, together with actin and other actin-binding proteins, in high molecular mass complexes the functions of which are not yet fully understood (15, 16).

Crystal structures of the N- and C-terminal domains of yeast and human CAP are consistent with the general topology of the protein determined by domain mapping. The N-terminal domain is an elongated a-helical bundle consisting of 6 antiparallel helices (17, 18). The C-terminal dimerization and G-actin binding domain, termed C-CAP, is a right-handed b-helix forming dimers by interlocking two b-strands at the C-terminal base of each monomer (19). In this study, we combined experimental genetics in P. berghei with biochemical and structural analyses of the purified recombinant Cryptosporidium parvum protein to study the role of apicomplexan C-CAP proteins.

**EXPERIMENTAL PROCEDURES**

**Plasmodium C-CAP Expression Analysis**—To study C-CAP expression during the Plasmodium life cycle total RNAs were isolated from late stage schizonts from three infected NMRI mice (~10⁹ parasites), 4 × 10⁵ purified ookinetes, 6 × 10⁵ immature oocyst sporozoites, and 4 × 10⁵ infectious salivary gland sporozoites used the RNeasy kit (Qiagen). Reverse transcription for first-strand cDNA synthesis was performed using the RETROscript kit (Ambion) followed by subsequent standard PCR amplification using P. berghei gene-specific primers for C-CAP (C-CAPfor and C-CAPrev) and for the 614-bp fragment primers CAP5-for and CAP5-rev, and for the 614-bp fragment primers CAP3-for and CAP3-rev were used, respectively. Both fragments were introduced into the P. berghei targeting vector B3D+, which contains the 3'-UTR of P. berghei dihydrofolate reductase/thymidylate synthase (DHFR/TS) and a mutant Toxoplasma gondii dhfr/tsr as a selectable marker for pyrimethamine resistance (20). Transfection was done with 10 µg of the Kpnl/SacI-linearized B3D+C-CAP replacement plasmid and gradient purified schizonts of the pyrimethamine-sensitive, green fluorescent P. berghei strain ANKA (20), using the Nucleofactor(R) device (Amaza). Positive selection for stable integration was done by pyrimethamine treatment in the drinking water. Resistant parasite populations were transferred to naïve animals for propagation and genotyping. Clonal parasite populations were obtained by limiting dilution series and intravenous injection of one parasite into 10 recipient NMRI mice. Genotyping was performed by integration-specific PCRs using primers CAPtest5'-for and CAPtest5'-rev, and CAPtest3'-for and CAPtest3'-rev. The PbC-CAP wild-type signal was detected using primers CAPtest5'-for and CAPtest3'-rev.

**Trans-species Complementation of P. berghei C-CAP with the C. parvum Ortholog—**C. parvum C-CAP cDNA (cgd5_440) was subcloned by ligase-independent cloning into a vector derived from pET28a (Novagen). The C. parvum C-CAP coding sequence was amplified from the expression vector using primers CpCAPfor and CpCAPrev. The resulting fragment was cloned into the B3D+C-CAP replacement plasmid. Transfection and genotyping was done as described above. Genotyping was performed by integration-specific PCR using primers for 5'-UTR; CAPtest5'-for, CAPtest5'-rev, and for 3'-UTR; CAPtest3'-for, CAPtest3'-rev. To test the specific abundance of CcCAP-CAP in the P. berghei locus the amplification primers CcCAPfor and CcCAPrev were used.

**Phenotypic Analysis of Parasite Populations in Vivo**—To determine blood stage development of c-cap(-) parasites, seven NMRI mice each were injected intravenously with 1,000 mixed blood stage parasites of P. berghei c-cap(-) or WT parasites. Parasitemia was determined by daily microscopic examination of Giemsa-stained blood smears. Ookinetes were purified using standard conditions using parasite-infected NMRI mice for 18–20 h of culture. Mature ookinetes were pure with p28 antibodies coupled to magnetic beads. To investigate the development of c-cap(-) parasites in the mosquito host, A. stephensi mosquitoes were fed on anesthetized NMRI mice infected with mutant or WT parasites. Starting at day 4 after infection, mosquito midguts (n = 12) were monitored daily for infection. Oocyst numbers were determined by counting ten fields under ×40 magnification through the midguts randomly. Developmental growth of parasite oocysts was investigated using a Zeiss LSM510 confocal laser scanning microscope (Zeiss, Germany). Size measurement of oocysts (average n = 55) was done with LSM510 Zeiss system software. Images were processed with LSM image observer and ImageJ software. Hoechst 33342 (Molecular Probes) was used to stain nuclei. Sporozoites were harvested and counted at days 14 and 17 after mosquito infection from midgut and salivary glands,
CAP Ortholog from Apicomplexa

respectively. To check for infectivity to animals, naive C57BL/6 mice were fed on infected mosquitoes at day 17. Infection was monitored by daily examination of Giemsa-stained blood smears.

Cloning and Protein Purification—Recombinant expression and purification of CpC-CAP protein was as described before (21), involving expression in Escherichia coli BL21-CodonPlus(DE3)-RIPL cells (Stratagene), cell lysis using a cell disruptor (Microfluidizer Processor M-110EH at 18,000 psi), and purification on HisTrap HP columns (GE Healthcare). Eluted CpC-CAP protein intended for biochemical characterization was liberated from the hexahistidine affinity tag by thrombin cleavage (GE Healthcare) before gel filtration on Sephadex S-200 (GE Healthcare), and the major protein peak concentrated using Amicon Ultra cartridges (Millipore). Concentrated protein was flash-frozen in liquid N2 and stored as aliquots at −80 °C.

The C-terminal actin binding segment of ScCAP/Srv2 (residues 253–526; designated ScC-CAP) was expressed from vector pGAT2 (14, 22). Expression vector encoding ScCAP371–526 was constructed by PCR amplification using primer pair ScCBPfor and ScCBPBrev, and subcloning into pET28a. After protein production in E. coli strain BL21(DE3) (Stratagene) under standard conditions, Sc-CAP was purified on glutathione-Sepharose and either eluted or liberated from the beads by overnight cleavage with thrombin (GE Healthcare). ScCAP371–526 protein was produced as described above for CpC-CAP protein.

Lyophilized human non-muscle β-actin (cytoskeleton) was dissolved and diluted in 10 mM Tris-HCl, pH 7.6, 0.5 mM ATP, 0.1 mM CaCl2, 1 mM dithiothreitol (G-buffer). Protein aggregates were removed by centrifugation, and the protein solution was used within 1 week. Monomeric ADP-actin was obtained by dissolving lyophilized actin in G-buffer containing 0.5 mM ADP instead of ATP, adding 0.2 mM EGTA, and 50 μM MgCl2, and incubating on ice for 2 days.

Biochemical Analysis of C-CAP—Analytical size exclusion chromatography was carried out using a 16/10 Superdex-200 column attached to an Ettan FPLC system (GE Healthcare). The column was calibrated using molecular mass standards (GE Healthcare); log(MW) was plotted against (V0 − Vt)/(V0 − Vu) for each protein, where V0 is the total column volume, Vu is the exclusion volume, and Vt is the elution volume of each protein. The apparent molecular masses for the C-CAP proteins were then calculated from their elution volumes and the regression line of the above plot. Glutaraldehyde cross-linking was carried out with 5 μM C-CAP proteins as described (23).

The actin binding domains of CAP proteins characterized so far bind preferentially to monomeric ADP-actin (14). C-CAP-G-actin interaction was studied using native PAGE assays: C-CAP proteins were preincubated with ADP-actin for 30 min at room temperature and separated on 7.5% acrylamide gels containing 80 mM Tris, pH 8.4, 0.5 mM ADP, 0.1 mM CaCl2, and 20 μM MgCl2. Gels were run in 50 mM Tris pH 8.4, 30 mM Bicine, 0.1 mM GDP, 0.1 mM CaCl2, 20 μM MgCl2, and 1 mM dithiothreitol. For co-sedimentation and sequestering assays, samples of 4 μM ATP-actin were induced to polymerize by addition of 1 mM MgCl2 and 0.1 M KCl in the presence of 0–20 μM of the C-CAP proteins, and the reactions were allowed to reach steady state (2 h at room temperature). Following centrifugation at 150,000 × g for 15 min at 20 °C in a Sorvall Discovery-90 ultracentrifuge (Thermo Scientific), equal amounts of supernatants and pellets were analyzed by SDS-PAGE and Coomassie Blue staining. Steady-state actin polymerization assays were carried out using non-muscle actin supplemented with 5% pyrenyl-labeled rabbit muscle actin (cytoskeleton). Assay conditions and data analysis were as previously described (4).

Briefly, apparent Kd values were determined from the critical concentration of actin polymerization (Apc) and the concentrations of unpolymerized actin when different concentrations of CpC-CAP were present. Data from independent experiments were analyzed, and means and sample standard deviations (n-1 weighting) are given.

Crystallization—Using the hanging drop vapor diffusion method, crystals for C. parvum C-CAP were obtained by mixing 1.5 μl of protein (concentration 16.9 mg/ml) with 1.5 μl of buffer solution (0.2 m diammonium tartrate, 17% (w/v) PEG 3350) at 18 °C in a Linbro plate. A single crystal was dragged through a drop of paratone oil (Hampton Research) before being frozen in a cryo loop by placement in the cold nitrogen stream of a crystal cooling device (Oxford Cryosystems). Under continued cooling, a continuous set of 360 × 0.5° oscillation images was collected on a FR-E rotating copper anode x-ray generator paired with a R-Axis IV++ detector (Rigaku). Data were reduced with the HKL2000 software suite (24). The structure was solved by molecular replacement with the program PHASER (25) using the coordinates of PDB entry 1K8F as a search model. The amplitudes of 14,232 unique reflections between 20 and 2.6 Å were used in refinement. 5% of the reflections were selected in thin resolution shells using the program DATAMAN6 (26) and excluded from refinement for cross-validation purposes (27). After several cycles of model rebuilding in XFIT (28), restrained refinement in REFMAC (29), and validation on the MOLPROBITY server (30), coordinates and structure factor amplitudes were deposited in the PDB as entry 2B0R.

Statistical Analysis—The two-sided unpaired t test (GraphPad Prism 5.0b, GraphPad Software, La Jolla, CA) were used to determine statistical differences between parasite samples.

RESULTS

CAP Homology Protein in Apicomplexan Parasites—We previously identified sequences with homology to the C-terminal portions of ScCAP/Srv2 using BLAST searches of the genomes of apicomplexan parasites (3). These sequences, hereafter referred to as C-CAP, consist essentially of the highly conserved actin binding domain, and lack the adenylate cyclase binding domain, the WH2 domain, and the two proline-rich motifs flanking the WH2 domain, all of which are shared by the CAP proteins from other organisms (Fig. 1A). Proteins with this single domain structure are classified as PFAM family PF08603, and seem restricted to protozoa; genes have been identified in genera such as Plasmodium, Babesia, Cryptosporidium, Leishmania, Tetrahymena, and Theileria (supplemental Fig. S1). Alignment of the primary structures revealed overall limited
sequence similarity but identified a number of strictly conserved residues (Fig. 1).

**Recombinant CpC-CAP Is a Dimer-forming Protein**—We expressed the C-CAP proteins from *P. falciparum* and *C. parvum*, as well as an actin binding domain fragment of *S. cerevisiae* CAP (Srv2p253–526; Ref. 14), as hexahistidine and GST fusion proteins in *E. coli*. The *C. parvum* ortholog, CpC-CAP, and the previously described yeast protein, here designated ScC-CAP, were readily produced (5 mg of protein/liter of bacterial culture) and purified using either Ni2+ - or glutathione-affinity chromatography. By contrast, despite protein expression in several strains, from different expression vectors, and under various cell growth and lysis conditions, we were unable to produce soluble recombinant *P. falciparum* C-CAP.

CAP proteins and their actin binding domains are known to be dimeric (19), while in vivo they may form larger complexes together with actin monomers (15). To assess the molecular state of CpC-CAP, we performed size exclusion chromatography of purified recombinant proteins. For CpC-CAP (chain mass 20.1 kDa), we determined an apparent molecular mass of 38.7 kDa, which is in good agreement with a dimer (Fig. 2A). By contrast, ScC-CAP (Srv2p253–526; chain mass 29.2 kDa) eluted in a single peak with an apparent molecular mass of 165 kDa, suggestive of a hexamer. This indicated that the N-terminal section of ScC-CAP, which is missing in apicomplexan C-CAP proteins (Fig. 1 and supplemental Fig. S1), may be responsible for the formation of higher molecular mass complexes. To test this hypothesis, we produced a shorter ScC-CAP variant (Srv2p371–526) with the WH2 homol-
ogy and proline-rich regions missing as in the parasite C-CAPs (Fig. 1B). Srv2p\(^{371-526}\) was readily produced as soluble protein. In size exclusion chromatography, this protein (chain mass 17.2 kDa) migrated as a dimer of approximately the size of the \(CpC\)-CAP dimer (Fig. 2A). In addition, glutaraldehyde cross-linking experiments showed that \(CpC\)-CAP as well as both \(Sc\)-CAP proteins were readily cross-linked into a dimeric species (Fig. 2B). Together, these results suggest that apicomplexan C-CAP proteins and presumably other C-CAP orthologs that lack the N-terminal WH2 homology and proline-rich regions form dimers rather than higher molecular mass complexes.

\(CpC\)-CAP Binds Actin Monomers and Sequesters Them from Polymerization—We analyzed the interaction of our recombinant C-CAP proteins with actin using non-denaturing polyacrylamide gel electrophoresis. Both \(CpC\)-CAP and \(Sc\)-CAP (Srv2p\(^{253-526}\)) formed complexes with MgADP-actin (Fig. 3, A and B). In addition, we performed competition experiments with DNaseI using the same assay system. In samples that contained equimolar amounts of MgADP-actin, DNaseI, and either \(CpC\)-CAP or \(Sc\)-CAP, significant amounts of DNaseI-actin complex formed whereas C-CAP-actin complex was no longer observed (Fig. 3A). These results show that \(CpC\)-CAP binds to monomeric actin in a fashion that is mutually exclusive with actin binding to DNaseI. As monomeric actin-binding proteins participate in regulation of the F/G-actin balance, we assessed the effect of \(CpC\)-CAP on the extent of actin polymerization. Mixtures of C-CAP proteins and actin polymers in the presence of MgATP were subjected to ultracentrifugation. The levels of unpolymerized actin remaining in the supernatants were analyzed by SDS-PAGE and Coomassie Blue staining (Fig. 3C). The parasite C-CAP protein kept substantial amounts of actin in an un-
polymerized state, and the yeast ortholog displayed a similar activity.

Actin sequestering activity of parasite C-CAP protein was confirmed using a steady-state F-actin dilution assay (Fig. 3D). Actin polymerized with a critical polymerization concentration of 0.41 μM, in good agreement with literature values for the non-muscle isofrom (e.g. Ref. 4). In the presence of CpC-CAP, the concentrations of unpolymerized actin were shifted to higher total actin concentrations, whereas slopes were unaffected, indicating monomer sequestration by CpC-CAP with no effect on actin polymer ends. From these and similar experiments we calculated an apparent affinity of the protein-crystal structure of C. parvum C-CAP—We determined the crystal structure of C. parvum C-CAP using a structural genomics platform (21). The crystals contained four chains in the unit cell, and the structure was refined to 2.6 Å (Table 1). The CpC-CAP protein monomer displays a right-handed β-helical fold consisting of 6 individual β-helical turns, and the protein crystallized as the putative physiological homodimer (Fig. 4A). This is similar to the structures of the C-terminal segments of yeast CAP/Srv2p and human CAP (19). Supersposition of individual β-helices gives an overall R.M.S. difference of 1.74 Å for the Ca atom positions of the C. parvum and yeast, and of 2.3 Å for the C. parvum and human protein structures. However, the human and yeast proteins align with an R.M.S. of only 0.6 Å (19). The CpC-CAP β-helix is stabilized by a hydrophobic core of side chains that arrange in register in the inner cavity (supplemental Fig. S2). In all three C-CAP crystal structures determined to date, the β-helix features five stacks of hydrophobic (almost exclusively aliphatic) side chains and one stack of Cys/Ser side chains (19). The residues contributing to them are well conserved across species (supplemental Fig. S1).

The first three N-terminal rungs feature Cys/Ser side chains that bend back to hydrogen bond with main chain amides in the preceding rung, while the C-terminal end continues as the usual hydrophobic stack (supplemental Fig. S2).

Dimerization of CpC-CAP involves domain swapping of the C-terminal segment of dimeric proteins. Sequences show that these residues stabilize the Gln162-Ser179 motif by hydrogen bonds between the Ser163 backbone amide and the Glu161 carboxyl, and between the

![Figure 4. Crystal structure of C. parvum C-CAP.](image)

One of the CpC-CAP hydrophobic ladders is a dipartite ladder: The first three N-terminal rungs feature Cys/Ser side chains that bend back to hydrogen bond with main chain amides in the preceding rung, while the C-terminal end continues as the usual hydrophobic stack (supplemental Fig. S2).

The motif is also linked to the lower end of the sister β-helix through the hydrophobic environment formed by the Thr163 and Leu174 side chains of one chain and the Leu144, Leu157, and Ile159 side chains of the other. Sequence alignment suggested the importance of residues that are specifically conserved among C-CAP proteins, but not to related monomeric proteins (Fig. 4B). Inspection of the structure shows that these residues stabilize the Gln162-Ser179 motif by hydrogen bonds between the Ser140 backbone amide and the Glu161 carboxyl, and between the

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Gln\textsuperscript{163} backbone amide and the Ser\textsuperscript{179} hydroxyl, within each chain (Fig. 4C). The C terminus is stabilized by folding back to form hydrophobic interactions between Val\textsuperscript{178} and Pro\textsuperscript{180} of the same monomer. Other important symmetrical interactions across the dimer interface are π-stacking interactions of the two Phe\textsuperscript{162} side chains (Fig. 4C) as well as the Phe\textsuperscript{146} and Tyr\textsuperscript{167} side chains of each monomer. In conclusion, the C\textsubscript{PCP}-CAP crystal structure highlights many features that are typically found in β-helix proteins, and highly conserved dimer interactions that distinguish C\textsubscript{CAP} from related monomeric proteins.

C\textsubscript{CAP} Is Non-essential for Plasmodium Asexual Blood Stages—*C. parvum* is presently not tractable by experimental genetics. To study the in vivo function of C\textsubscript{CAP} for parasite life cycle progression, we employed reverse genetics in the model rodent malaria parasite *P. berghei*, where all life cycle stages, including pathogenic merozoites that infect red blood cells, ookinetes that penetrate the mosquito midgut epithelium, and sporozoites that are eventually transmitted back to the mammalian host, are readily obtained.

We first studied the expression of C\textsubscript{CAP} throughout the *Plasmodium* life cycle and profiled *PbC-CAP* transcripts. Reverse transcriptase PCR (Fig. 5A) and quantitative RT-PCR (Fig. 5B) revealed that C\textsubscript{CAP} transcripts are most abundant in merozoites.

FIGURE 5. **Expression of C\textsubscript{CAP} in extracellular *Plasmodium* life cycle stages.** A. *PbC-CAP* and *PbHSP70* transcript expression was monitored in four parasite stages: Merozoites (*mer*) that invade erythrocytes, ookinetes (*ook*), that penetrate the mosquito midgut epithelium, and midgut (*mg*) and salivary gland (*sg*) sporozoites (*spz*). Transcript amplification was detected in the presence (+) but not in the absence (−) of reverse transcriptase. WT genomic DNA (*gDNA*) was loaded as a control to show proper splicing of the C\textsubscript{CAP} transcripts. B. Quantitative real-time RT-PCR experiments using the same cDNA templates as in A show the relative C\textsubscript{CAP} transcript levels in merozoites and ookinetes are an order of magnitude higher in midgut and salivary gland sporozoites. Transcript levels were normalized to *GFP*, which is expressed as a transgene under the control of the constitutive EF\textsubscript{1α} promoter.

To study the role of C\textsubscript{CAP} for parasite life cycle progression and to validate C\textsubscript{CAP} as a potential drug target against protozoan parasites, we performed a reverse genetics analysis in the model rodent malaria parasite *P. berghei*. The endogenous gene locus was targeted by a gene replacement construct (Fig. 6A). Upon transfection this targeting plasmid is predicted to replace the C\textsubscript{CAP} open reading frame with a positive selection marker via a double homologous recombination event. Drug selection, parasite cloning by a limited in vivo dilution series, and subsequent genotyping of recombinant clonal parasite lines revealed the successful C\textsubscript{CAP} gene replacement in two independent clonal lines (Fig. 6B). Successful gene replacement was corroborated by transcript analysis in asexual blood stage parasites (Fig. 6C). The processed C\textsubscript{CAP} mRNA was no longer detectable in the c\textsubscript{cap}-(-) clones.

Stable gene deletion already indicated a non-vital role for C\textsubscript{CAP} during asexual parasite replication in the blood of infected mice. To test if c\textsubscript{cap}-(-) parasites displayed a growth defect we inoculated recipient mice with defined parasite numbers and monitored blood stage parasite development by daily examination of Giemsa-stained blood smears (Fig. 7). Replication of c\textsubscript{cap}-(-) parasites during the exponential growth phase in vivo was indistinguishable from WT parasites. Together, these results establish that C\textsubscript{CAP} is not essential for the pathogenic phase of the *Plasmodium* life cycle.

C\textsubscript{CAP} Is Essential for *Plasmodium* Oocyst Development in the Mosquito Midgut—To study the fate of c\textsubscript{cap}-(-) parasites during life cycle progression, we then fed mosquitoes on mice infected with either mutant or wild-type parasites and monitored parasite development in mosquito midguts over a period of 2 weeks after infection. Fluorescence microscopy revealed that c\textsubscript{cap}-(-) parasite oocysts were markedly reduced in size and numbers as compared with the WT parasite.
mosquito as compared with an average of 213 (vivo) gland-associated sporozoites in mosquitoes confirmed the complete absence of oocyst- and salivary systematic quantification of sporozoite numbers in infected mos-
parasites occurs after onset of the first mitotic divisions. Sys-

tematic quantification of sporozoite numbers in infected mos-

cap(-) infected mosquitoes we never detected a single sporo-
zoite in mosquitoes inoculated with c-cap(-) ookinetes. As expected, exposure of naive mice to mosquitoes naturally infected with high numbers of c-cap(-) parasites did not induce a malaria infection (data not shown). In conclusion, C-CAP is a vital gene for Plasmodium oocyst maturation inside the insect vector.

Cryptosporidium C-CAP Complements the Defects of the Plasmodium C-CAP Knock-out—To integrate our biochemical and structural studies of the C. parvum protein with the gene knock-out experiments in the malaria in vivo model we finally performed a trans-species complementation of the P. berghei knock-out line with the CpC-CAP gene under the control of the endogenous PbC-CAP promoter (Fig. 9A). Transfection of this construct yielded viable parasites that contained the predicted allelic replacement in the first attempt (Fig. 9B). Similar to the non-vital role of PbC-CAP in blood stage these ΔPbC-CAP::CpC-CAP parasites showed normal blood stage development (data not shown). Importantly, when tested in sporogony the ΔPbC-CAP::CpC-CAP parasite line developed normally, including formation of large oocysts (Fig. 9C) and normal growth rates (Fig. 9D), indicating essentially complete complementation of the c-cap(-) growth defects.

We next tested whether this parasite line produced sporo-
zoites (Fig. 10). Quantification of midgut- and salivary gland-associated sporozoites revealed complete parasite development inside the mosquito vector. ΔPbC-CAP::

CpC-CAP-infected mosquitoes typically yielded fewer sporozoites, indicating imperfect complementation. Differences were, however, non-significant (Fig. 10). Similarly, when transmitted to naive animals, ΔPbC-CAP::CpC-CAP sporozoites consistently induced patent blood stage infections 3 days after inoculations (data not shown). This pre-
patent period is indistinguishable from WT sporozoites. These data show successful trans-species complementation of the c-cap(-) mutant by the C. parvum ortholog.

DISCUSSION

Toward systematic functional gene analysis and target vali-
dation in apicomplexan parasites new strategies that reliably inform preclinical anti-infectives development are urgently needed. In the present study we combined biochemical and structural insights from a purified C. parvum protein with experimental genetics in the model rodent parasite P. berghei. A crucial link between the two complementary approaches was the trans-species complementation experiment, which proved that the biochemically active G-actin sequestering C-CAP protein has the capacity to complement the corresponding Plasmodium loss-of-function mutant. This strategy permits simultaneous target validation, in our case a vital role in the insect vector, with biochemical assay development and high-resolu-
tion structure determination, both of which are instrumental for targeted drug design. We propose that systematic testing of conserved apicomplexan target proteins by this combined genetics and recombinant production approach could substan-
tially expedite drug target validation in some of the most prev-
alent infectious diseases.

In this study we demonstrate the first stage-specific vital role for a small G-actin binding protein in apicomplexan parasites.
Plasmodium parasites that lack the minimal cyclase associated protein, C-CAP, are deficient in oocyst maturation inside the insect vector. Components of the actin cytoskeleton of Apicomplexa have been described and analyzed by reverse genetics and biochemistry (4, 32–34). Despite these recent advances, the regulation of the parasite motor machinery and its potential implementation for drug development is not understood. We show that parasite C-CAP is a dimer-forming, actin monomer-sequestering protein with the canonical C-terminal CAP domain fold. Plasmodium C-CAP protein appears to fulfill redundant roles in asexual and sexual blood stage development in the mammalian host. In marked contrast, depletion of C-CAP protein results in complete developmental arrest during oocyst maturation inside the mosquito vector. Two other Plasmodium G-actin-binding proteins, profilin and actin depolymerizing factor-1 (ADF1), both exert vital, non-redundant roles during asexual blood stage development (4, 33). In good agreement with a conserved essential function, conditional gene depletion of profilin in T. gondii revealed a fundamental role in tachyzoite locomotion and cell-to-cell propagation (34). The vital role of Plasmodium C-CAP, on the other hand, is restricted to parasite maturation inside the invertebrate vector, the only extracellular expansion phase of the otherwise obligate intracellular parasite. Plasmodium shares this mode of maturation with other coccidian parasites, where the infectious transmission stages, termed sporozoites, are formed inside sturdy oocysts. Therefore we propose that the apicomplexan C-CAP proteins exert their essential roles during this extracellular replication phase, which is preserved from an ancestral extracellular lifestyle. Roles for C-CAP in the developing oocyst may be the regulation of actin during microfilament dependent processes such as arrangement of mitotic spindles and vesicular trafficking.

Work in yeast established that cap- mutants lacking the C-terminal actin binding domain are growth deficient in rich medium, most likely due to sensitivity to excess amino acids (7). In Drosophila, CAP loss-of-function mutants are associated with aberrant F-actin levels suggesting a cellular role in temporal and spatial orchestration of actin polymerization (35). By analogy, the observed central role of Plasmodium C-CAP in sporogony may directly connect its biochemical activity, i.e. G-actin sequestration, to parasite development. We attempted to detect F-actin by phalloidin staining in the c-cap(-) mutant and wild-type parasites, but it remained elusive (data not shown), a feature typically seen in apicomplexan parasites under physiological conditions.

Our biochemical data support a direct role of C-CAP in sequestration of actin monomers. However, the atomic details of the actin-C-CAP interaction remain elusive. Conventional CAPs were initially regarded as actin monomer binders with a

**FIGURE 8.** Disruption of C-CAP leads to complete attenuation of oocyst development in the mosquito midgut. A, c-cap(-) oocysts fail to mature. Shown are representative micrographs of GFP expressing WT and c-cap(-) mutant parasites. The time after the infectious blood meal is indicated on the left. Note also the reduced density of the mutant oocysts as compared with WT. Scale bars, 20 μm. B, quantification of oocyst maturation. Shown are the oocyst diameters of WT (green) and c-cap(-) (red) oocysts as a function of time. Note that WT oocysts display a continuous size increase whereas c-cap(-) oocysts arrest at an early developmental stage. The data points represent the oocyst diameters from randomly selected oocysts of parasite-infected midguts (n = 12) from two and six independent feeding experiments for WT and c-cap(-), respectively. Horizontal lines indicate the median oocyst size. p < 0.0001. C, morphology of oocysts at day 14. Shown are representative micrographs of WT (top) and c-cap(-) (bottom) oocysts. Parasites and nuclei are labeled with endogenously expressed GFP and Hoechst 33342, respectively. Note that c-cap(-) oocysts do not contain any sporozoites. Scale bars, 20 μm.
partial functional redundancy with profilin (10). After the discovery of more complex actin-associated CAP functions the emerging cellular role of conventional CAPs is that of a scaffold that regulates diverse F- and G-actin-related activities. Given the several distinct modes of CAP-actin interactions proposed (15, 16), it is currently unclear which residues in each C-CAP monomer contribute to actin binding, and at what stoichiometry. Parasite C-CAPs lack the N-terminal proline-rich extensions that, in conventional CAPs, recruit SH3-domain-containing proteins (36) and profilin (14, 37). In pull-down experiments and native gel assays, binding of \( \text{CpC-CAP} \) to either \( P. \text{falciparum} \) profilin or human profilin-1 or -2a could not be detected.6 Parasite C-CAPs also lack the N-terminal WH2 homology domain (38) of conventional CAPs, which may be involved in the formation of higher molecular mass complexes with actin (39). The mobility of actin-CpC-CAP complexes on native gels is in agreement with a small complex, with a major contribution by the net charge of actin, rather than by size (Fig. 3A). The N-terminal H9251/helical bundle domain of conventional CAPs, which has recently been demonstrated to contribute significantly to ADF-mediated F-actin turnover (16), is absent in parasite C-CAPs. In line with this evidence, our data suggest that the consensus sequence of C-CAP proteins of apicomplexan parasites supports only dimerization. This is also consistent with the absence of F-actin binding activity in the constitutive \( \text{P. berghei} \) ADF1 (4) and supports the theory that parasite actin polymers need stabilizers, rather than turnover enhancers, for their proper functioning (3). Although we cannot formally exclude that parasite C-CAPs can form larger complexes in conjunction with actin, our data suggest that C-CAPs are \textit{bona fide} monomer binders that regulate a pool of unpolymerized actin.

The \textit{Cryptosporidium} C-CAP crystal structure illustrates the typical features of right-handed \( \beta \)-helical proteins. Our structure confirms dimerization by \( \beta \)-strand exchange across monomers, and provides insights into the side chains needed to stabilize the exchanged strands (19). The importance of these residues becomes apparent also when compar-

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6 H. Schüler, unpublished results.
CAP Ortholog from Apicomplexa

...ing the sequences of the dimeric C-CAPs with related monomeric proteins. Examples here are the RP2 (retinitis pigmentosa-2) protein (40) and tubulin specific chaperone-C, both of which had been predicted to be β-helical structural homologs of C-CAP (19). We show that the sequence PEQ(F/Y) before the penultimate β-strand of the C-CAP fold is indicative for dimer formation (Fig. 4B). Incidentally, the absence in yeast CAP/Srv2p of a small aromatic side chain (Phe or Tyr), which in our structure stack to each other, at the dimer interface, may be compensated for by other dimer interactions, such as those of the yeast N-terminal CAP domain (9, 16). The internal Cys/Ser side chain stack is shared with other β-helical proteins (41), but a prominent difference between the parasite and yeast C-CAP structures is the bipartite ladder observed in our CpC-CAP structure, with an N-terminal Cys/Ser stack and a C-terminal hydrophobic stack (supplemental Fig. S2). However, here the serine and cysteine side chains do not arrange as an internal ladder but hydrogen bond with main chain amides in the preceding rung thereby stabilizing the N-terminal part of the fold. We speculate that the N-terminal segment (adenylate cyclase binding and/or WH2- and proline-rich segments) stabilize the N terminus of the β-barrel in human and yeast CAP. In the C-CAP proteins their absence might be compensated for by the interactions of the cysteine and serine side chains of the bipartite ladder.

To date it is unclear how C-CAP sequesters actin monomers. Mutagenesis studies can now be employed to establish the critical residues that mediate actin binding, and subsequently, to pinpoint the functions of C-CAP during parasite oocyst development. Such an analysis requires the combination of robust biochemical and in vivo assays, such as G-actin sequestration (Fig. 3) and trans-species complementation of the essential in vivo role in Plasmodium sporogony (Fig. 9). Apicomplexan parasites, as unicellular eukaryotes with complex developmental programs, may offer important insights into cell biological processes in addition to their exquisite medical importance.

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