

The *Plasmodium* circumsporozoite protein is proteolytically processed during cell invasion

Alida Coppi, Consuelo Pinzon-Ortiz, Christina Hutter, and Photini Sinnis

Department of Medical and Molecular Parasitology, New York University School of Medicine, New York, NY 10010

The circumsporozoite protein (CSP) is the major surface protein of *Plasmodium* sporozoites, the infective stage of malaria. Although CSP has been extensively studied as a malaria vaccine candidate, little is known about its structure. Here, we show that CSP is proteolytically cleaved by a papain family cysteine protease of parasite origin. Our data suggest that the highly conserved region I, found just before the repeat region, contains the cleavage site. Cleavage occurs on the sporozoite surface when parasites contact target cells. Inhibitors of CSP processing inhibit cell invasion in vitro, and treatment of mice with E-64, a highly specific cysteine protease inhibitor, completely inhibits sporozoite infectivity in vivo.

CORRESPONDENCE

Photini Sinnis:
photini.sinnis@med.nyu.edu

Malaria infection is initiated when an infected Anopheline mosquito injects sporozoites during a blood meal. After injection, sporozoites enter the bloodstream and go to the liver, where they invade hepatocytes and develop into exoerythrocytic forms. The circumsporozoite protein (CSP) is the major surface protein of the sporozoite and forms a dense coat on the parasite's surface. Studies have shown that CSP mediates sporozoite adhesion to target cells (for review see reference 1) and that it is required for sporozoite development in the mosquito (2). In addition, CSP has been extensively studied as a vaccine candidate and, thus far, is the only *Plasmodium* protein shown to confer protection to immunized individuals (for review see reference 1).

Comparison of the deduced amino acid sequences of CS proteins from all species of *Plasmodium* shows that they have a similar overall structure (see Fig. 1 A and reference 1). They all contain a central repeat region whose amino acid sequence is species specific and two conserved regions: a five amino acid sequence called region I, immediately before the repeats, and a known cell-adhesive sequence with similarity to the type I thrombospondin repeat (TSR; reference 3). CSP has a canonical glycosylphosphatidylinositol (GPI) anchor addition sequence in its COOH terminus; however, the presence of a GPI anchor has not been demonstrated.

It was noted 20 yr ago that CSP immunoprecipitated from sporozoite lysates consists of one to two high MW bands (that differ by ~1 kD) and a low MW band that is 8–10 kD

smaller (4, 5). Biosynthetic studies showed that the initial label is incorporated into the top bands and the lower MW band appears later as a processed product (4, 5). The precise nature of this processing, as well as its functional significance, have remained unknown. In this report, we have determined the structural basis for this conserved feature of CSPs and have explored its role during sporozoite invasion of hepatocytes.

RESULTS AND DISCUSSION

The NH₂-terminal portion of CSP is proteolytically cleaved by a cysteine protease

To study the structure of the high and low MW CSP forms, we made polyclonal antisera to peptides representing the entire NH₂-terminal and COOH-terminal thirds of CSP from *Plasmodium berghei*, a rodent malaria parasite. These antisera recognized the appropriate full-length peptides (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20040989/DC1>) and did not recognize peptides representing the central repeat domain (Fig. S1 B). In addition, the NH₂-terminal antiserum did not recognize the COOH-terminal peptide and the COOH-terminal antiserum did not recognize the NH₂-terminal peptide (unpublished data). Western blot analysis of a *P. berghei* sporozoite lysate showed that the NH₂-terminal antiserum recognized only the high MW CSP form, indicating that all or part of the NH₂ terminus is proteolytically cleaved to generate the low MW CSP form (Fig. 1 B). In contrast, mAb 3D11 (which recognizes the repeat region) and the COOH-terminal antiserum recognized both CSP forms.

The online version of this article contains supplemental material.

To determine what class of protease was responsible for cleavage, we performed pulse-chase metabolic labeling experiments in the presence of different protease inhibitors. We labeled sporozoites with [³⁵S]Cys/Met and chased with cold medium containing the indicated inhibitor (Fig. 1 C). In the absence of protease inhibitors, ~80% of labeled CSP was cleaved after 2 h. In the presence of the metalloprotease inhibitor 1,10 phenanthroline or the aspartyl-protease inhibitor pepstatin, there was no effect on CSP processing. In addition, EDTA had no effect on CSP processing, indicating that divalent cations are not required. L-transepoxy succinyl-leucyl-amide-[4-guanido]-butane (E-64), a highly specific cysteine protease inhibitor, and PMSF, a serine protease inhibitor, inhibited CSP processing. Leupeptin and TLCK, inhibitors of both cysteine and serine proteases, also inhibited processing. Although PMSF has been reported to have inhibitory activity against some papain family cysteine proteases (6), it is a proto-

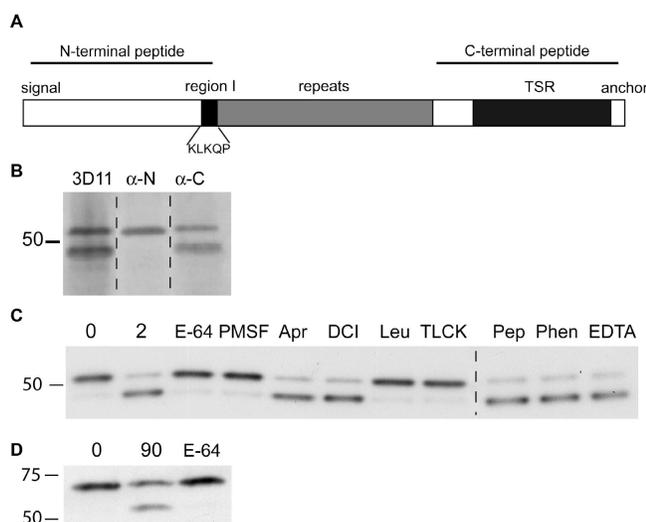


Figure 1. CSP is proteolytically cleaved by a cysteine protease. (A) CS proteins from all species of *Plasmodium* have several conserved features: a central repeat region (gray box) and two conserved regions (black boxes, region I and the cell-adhesive TSR). The first 20 residues of CSP have the features of a eukaryotic signal sequence (reference 28) and the COOH-terminal sequence contains a canonical GPI-anchor addition site. Bars show the location of peptides used for the generation of antisera. (B) Western blot of a *P. berghei* sporozoite lysate probed with polyclonal antisera generated against the NH₂- and COOH-terminal peptides shown in A; mAb 3D11 was used as a control. The membrane was cut where indicated so that it could be probed with the three different antisera, and the membrane strips were developed together. (C) *P. berghei* sporozoites were metabolically labeled and kept on ice (lane 1) or chased for 2 h in the absence (lane 2) or presence of the indicated protease inhibitors (lanes 3–11). CSP was immunoprecipitated from lysates of sporozoites and analyzed by SDS-PAGE and autoradiography. The following inhibitors were used: 10 μ M E-64, 1 mM PMSF, 0.3 μ M aprotinin, 100 μ M 3,4 DCI, 75 μ M leupeptin, 100 μ M TLCK, 1 μ M pepstatin, 1 mM 1,10 phenanthroline, and 5 mM EDTA. All samples are from the same experiment but were run on two gels and grouped as indicated. (D) *P. falciparum* sporozoites were metabolically labeled and kept on ice (lane 1) or chased with cold medium for 90 min in the absence (lane 2) or presence of E-64 (lane 3). Samples were processed as outlined before.

typical serine protease inhibitor. To further examine the role of serine proteases, we assayed two other serine protease inhibitors, aprotinin and 3,4 dichloroisocoumarin (3,4 DCI). Aprotinin inhibits most classes of serine proteases and would be predicted to inhibit the serine proteases of *Plasmodium*, which are subtilisin-like (7). 3,4 DCI is a serine protease inhibitor that has some activity against cysteine proteases but does not react with papain-like cysteine proteases (8). Neither compound had an effect on CSP processing.

We also performed pulse-chase metabolic labeling experiments with the human malaria parasite, *Plasmodium falciparum*, and found that E-64 inhibited CSP processing in this species (Fig. 1 D). These data suggest that CSP cleavage occurs by a similar mechanism in both rodent and human *Plasmodium* species.

To ensure that the protease inhibitors were not toxic to sporozoites, we incubated sporozoites with the different inhibitors and added propidium iodide, a dye that is excluded by viable cells but penetrates membranes of dying cells. The percentage of sporozoites that took up the dye in the presence of any of the protease inhibitors was no different from controls (unpublished data). In addition, we tested whether sporozoites incubated with protease inhibitors were less metabolically active. Analysis of CSP synthesis after sporozoites had been incubated with individual inhibitors for 2 h showed that it was not affected by E-64, leupeptin, or PMSF (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20040989/DC1>).

Our data suggest that the processing enzyme is a cysteine protease. The cysteine proteases found in parasites are members of two clans, CA (papain-like) and CD (legumin-like) (for review see reference 9), which can be distinguished by their sensitivity to E-64. The protease that cleaves CSP is inhibited by E-64 and, therefore, is a Clan CA, papain family cysteine protease. However, we found that PMSF, a serine protease inhibitor, also inhibited processing. As stated before, PMSF has been reported to have activity against papain family cysteine proteases and this could explain its inhibitory activity in our processing assay. Nonetheless, it is also possible that CSP cleavage is a complex multistep process involving distinct proteases.

Region I likely contains the cleavage site

To determine where CSP is cleaved, we mapped the epitopes recognized by the NH₂-terminal antiserum using overlapping peptides. As shown in Fig. 2 A, the NH₂-terminal antiserum recognized peptides interspersed throughout the NH₂-terminal third of the protein, suggesting that the processed form lacks this entire region. These data raised the intriguing possibility that region I, found at the end of the NH₂ terminus, contained the cleavage site. To test this, we used a recombinant *P. berghei* parasite in which the last 21 amino acids of the NH₂ terminus and the entire repeat region had been replaced by the orthologous region from *P. falciparum* CSP [Pf/Pb sporozoites; Fig. 2 B and reference 10]. A Western blot of Pf/Pb sporozoites shows that both

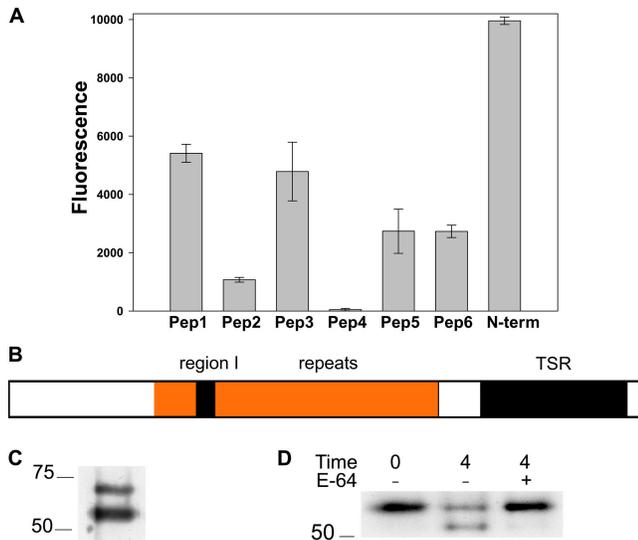


Figure 2. The conserved region I likely contains the proteolytic cleavage site. (A) The epitopes recognized by the NH₂-terminal antiserum were determined by ELISA by testing its reactivity to overlapping peptides encompassing the NH₂-terminal third of CSP. Pep 1, GYGQNKSIQAQRNLNE; Pep 2, RNLNELCYNEGNDNKL; Pep 3, NDNKLYHVLNSKNGKI; Pep 4, KNGKIYIRNTVNRLLA; Pep 5, NRLLADAPGKKNK; Pep 6, KNEKKNKIERNK; and N-term, full-length NH₂-terminal peptide. (B–D) Proteolytic cleavage of hybrid CSP. (B) Structure of CSP from Pf/Pb sporozoites. The portion of *P. berghei* CSP that has been replaced with the orthologous sequence of *P. falciparum* CSP is shown in orange and includes region I, which is shown in black. (C) Western blot of Pf/Pb sporozoites probed with mAb 2A10, directed against the *P. falciparum* CSP repeat region. (D) Pf/Pb sporozoites were metabolically labeled and kept on ice (lane 1) or chased with cold medium for 4 h, in the absence or presence of E-64. Samples were processed as outlined in Fig. 1.

CSP forms are present, suggesting it is processed (Fig. 2 C). We performed pulse-chase metabolic labeling experiments with Pf/Pb sporozoites and found that after a 4-h chase, 50–80% of the high MW CSP is processed to the low MW form (unpublished data). When we tested whether E-64 could inhibit processing of the hybrid CSP, we found that it did (Fig. 2 D), indicating that the same protease cleaves both the native and hybrid CS proteins.

These data suggest that the cleavage site is found within region I because this sequence remains unchanged in the hybrid protein. Although it is possible that the cleavage site is outside of the swapped region, this is unlikely because the NH₂-terminal antiserum, which recognized peptides throughout the NH₂-terminal third of CSP, did not recognize the low MW CSP form. Previous studies have shown that the difference in size, by SDS-PAGE, between the high and low MW forms is ~8–10 kD (4, 5, 11–13). The NH₂-terminal portion of CSP, beginning after the signal sequence and ending just before the repeat region, is predicted to be this size.

CSP cleavage occurs extracellularly by a sporozoite protease

We investigated the cellular location of CSP processing. Immunofluorescence experiments with live sporozoites showed that they were recognized by the NH₂-terminal antiserum,

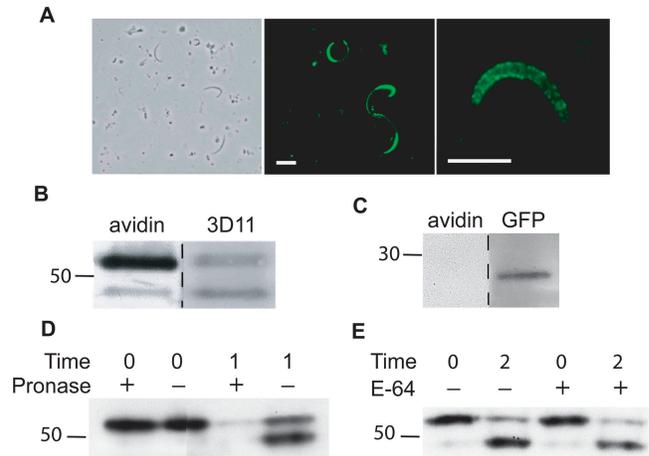


Figure 3. CSP is processed extracellularly by a parasite protease. (A) Staining of live sporozoites with the NH₂-terminal antiserum. Phase contrast and fluorescence views are shown. Bar, 10 μ m. (B and C) *P. berghei* sporozoites expressing GFP were biotinylated, lysed, and CSP (B) and GFP (C) were immunoprecipitated. A Western blot of the immunoprecipitated material was probed with streptavidin (B and C, lane 1), mAb 3D11 (B, lane 2), or polyclonal antisera to GFP (C, lane 2). The membrane was cut where indicated so that it could be probed with individual antisera and, the membrane strips were developed together. (D) Metabolically labeled *P. berghei* sporozoites were kept on ice (time = 0) or chased for 1 h, and incubated in medium containing pronase (+) or pronase plus pronase inhibitor cocktail (–). CSP was immunoprecipitated from sporozoite lysates and analyzed by SDS-PAGE and autoradiography. (E) *P. berghei* sporozoites were dissected and purified in the absence (–) or presence (+) of E-64, washed, metabolically labeled, and either kept on ice (time = 0) or chased for 2 h. Sporozoites were lysed, and CSP was immunoprecipitated and analyzed by SDS-PAGE and autoradiography.

demonstrating that full-length CSP was on the surface (Fig. 3 A). To confirm this, we biotinylated sporozoites expressing GFP with a reagent that does not enter cells. As shown in Fig. 3 B, the high MW CSP form is biotinylated, indicating that it is on the surface. As a control, we immunoprecipitated GFP, an intracellular protein, and found that it was not labeled (Fig. 3 C). These findings are in agreement with a previous paper that showed that high MW CSP was on the surface of *Plasmodium vivax* sporozoites (12) and suggest that processing occurs on the sporozoite surface.

In contrast with our findings, other investigators found that the majority of CSP on the surface was the low MW form, and concluded that processing occurred intracellularly (4, 5). In these studies, CSP was immunoprecipitated from sporozoites that were metabolically labeled and trypsinized. When compared with controls, trypsin-treated sporozoites were primarily missing the low MW CS band, indicating that the high MW CSP form was intracellular. However, in these experiments, trypsin was added immediately after labeling, which may not have allowed sufficient time for export of all the labeled CSP to the sporozoite surface. To investigate whether this was the case, we repeated this experiment and incorporated a chase into the experimental design. Sporozoites were metabolically labeled and kept on ice or chased in the presence of cyclohexamide to prevent further protein

synthesis. Next, they were treated with pronase or pronase plus an inhibitor cocktail. As shown in Fig. 3 D, if the parasites were kept on ice after labeling, the high MW CSP was not digested by pronase. However, if sporozoites were chased before pronase treatment, both CSP forms were digested, indicating that both forms were found on the sporozoite's surface, making this the likely location of processing.

Sporozoites isolated from salivary glands of infected mosquitoes are invariably contaminated with mosquito debris, raising the possibility that the protease that cleaves CSP is of mosquito origin. To address this question, we dissected and purified sporozoites in the presence of E-64, and then metabolically labeled them in medium without E-64. Cysteine proteases of mosquito origin would be extracellular and, therefore, irreversibly inhibited by the E-64 present during sporozoite isolation. However, we found that CSP was processed with the same kinetics regardless of whether sporozoites were purified in the presence or absence of E-64. These data suggest that the protease was synthesized (or secreted) after the removal of E-64 and, therefore, was of sporozoite origin (Fig. 3 E).

CSP cleavage is required for cell invasion

Proteolytic cleavage of cell surface and secreted proteins occurs during invasion of erythrocytes by the merozoite stage of *Plasmodium* (for review see reference 14). To determine whether CSP cleavage was required for sporozoite entry into cells, a variety of protease inhibitors were tested for their ability to inhibit sporozoite invasion of a hepatocyte cell line. As shown in Fig. 4 A, E-64 inhibited invasion by 90% and PMSF and leupeptin also had inhibitory activity. Pepstatin had no effect on invasion and the serine protease inhibitors aprotinin and DCI, which do not have activity against the papain family cysteine proteases, also did not have inhibitory activity on invasion. Importantly, pretreatment of target cells with E-64 had no inhibitory effect on sporozoite invasion. The ability of E-64 to inhibit invasion was not restricted to *P. berghei* sporozoites, as invasion by both *P. yoelii* and *P. falciparum* sporozoites was also inhibited by E-64. Notably, the number of extracellular sporozoites was always enhanced in the presence of E-64, suggesting that there was an accumulation of attached sporozoites that were prevented from entering (Fig. 4 B). Because attachment to cells is a distinct stage

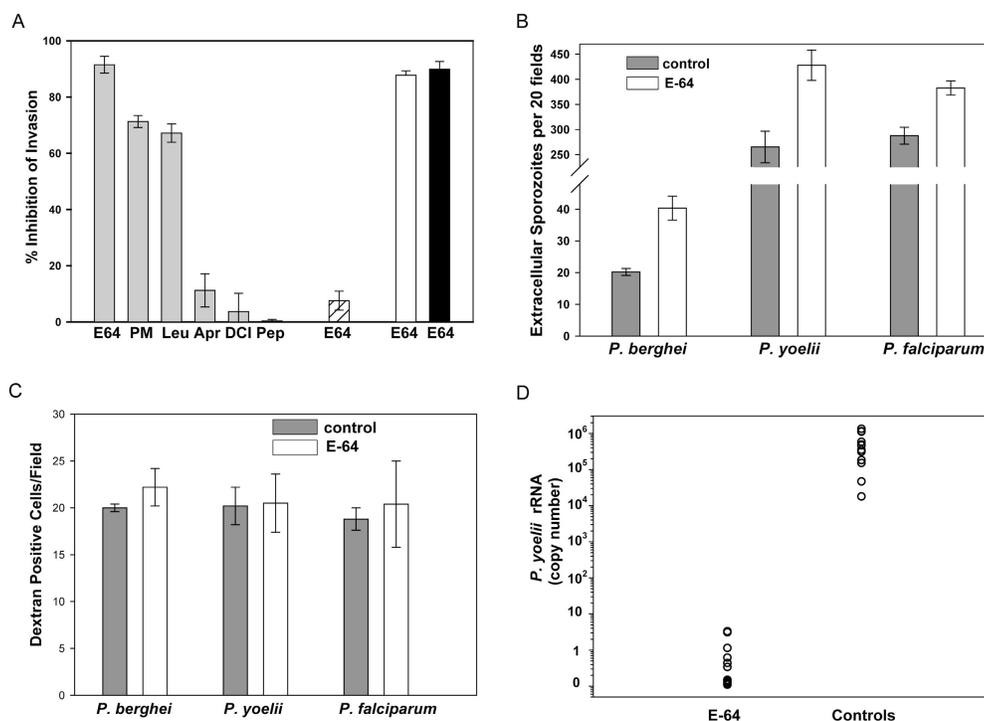


Figure 4. E-64 inhibits sporozoite infectivity in vitro and in vivo but not sporozoite migration through cells. (A) The effect of protease inhibitors on cell invasion by *Plasmodium* sporozoites. *P. berghei* (gray bars), *P. yoelii* (white bar), or *P. falciparum* (black bar) sporozoites were preincubated with the indicated protease inhibitors before being added to cells. A control (diagonally striped bar) was performed in which the target cells were preincubated with E-64, the medium was removed, and untreated *P. berghei* sporozoites were added. The following inhibitors were used: 10 μ M E-64, 1 mM PMSF, 75 μ M leupeptin, 0.3 μ M aprotinin, 100 μ M 3,4 DCI, and 1 μ M pepstatin. Each point was performed in triplicate, 50 fields/well were counted, and the means \pm SD are shown. Inhibition of invasion was calculated based on the invasion rate for sporozoites pretreated with buffer alone, which was 54% for *P. berghei*, 26% for *P. yoelii*, and 52% for *P. falciparum*. (B) Shown are the numbers of extracellular sporozoites when sporozoites are preincubated in the absence (gray bars) or presence (white bars) of E-64. Data are from the invasion assay shown. (C) Shown are the numbers of dextran-positive cells when sporozoites were preincubated \pm E-64 and added to cells in the presence of rhodamine-dextran. Each point was performed in triplicate, 50 fields/cover slip were counted, and shown are the means \pm SD. (D) E-64 inhibits sporozoite infectivity in vivo. Mice were injected with E-64 or buffer alone before inoculation of *P. yoelii* sporozoites. 40 h later, the mice were killed, total liver RNA was extracted, and malaria infection was measured by quantitative PCR. Infection is expressed as the number of copies of *P. yoelii* 18S rRNA. Results from two experiments are shown. There were six mice per group per experiment.

Table I. Contact with hepatocytes triggers cleavage of CSP

Experiment	Cells	Condition ^a	Method for sporozoite visualization	Number of sporozoites visualized ^b
1	Hepa 1-6	CD	3D11	244 ± 3
	Hepa 1-6	CD + E-64	3D11	230 ± 4
	Hepa 1-6	CD	α-N	41 ± 1
	Hepa 1-6	CD + E-64	α-N	237 ± 5
	no cells	control	α-N	80% ± 0.4
	no cells	CD	α-N	85% ± 1.1
	no cells	E-64	α-N	90% ± 4.1
	no cells	CD + E-64	α-N	84% ± 0.5
no cells	CD + 10% serum	α-N	80% ± 3.7	
2	Hepa 1-6	CD	GFP	452 ± 8
	Hepa 1-6	CD	α-N	98 ± 2
	Hepa 1-6	CD + E-64	GFP	444 ± 6
	Hepa 1-6	CD + E-64	α-N	436 ± 8

^a*P. berghei* sporozoites (wild type in experiment 1; GFP in experiment 2) were preincubated ± E-64, and before addition to coverslips, CD was added to the indicated samples. Sporozoites were spun onto coverslips, with or without cells as indicated, brought to 37°C for 2 min, fixed, and stained with the indicated antisera.

^bEach point was plated in duplicate, 50 fields/coverslip were counted, and the means ± SD are shown. When sporozoites were plated without cells, 100–200 sporozoites/coverslip were counted, and the percentage staining with the NH₂-terminal antiserum is shown.

of sporozoite invasion (15), these results suggest that E-64 specifically blocks invasion and that attachment to cells does not require proteolytic cleavage of CSP.

These data suggest that CSP is cleaved during cell invasion. Therefore, we predicted that intracellular sporozoites would lose their reactivity to the NH₂-terminal antiserum, which recognizes only full-length CSP. However, we found that the majority of sporozoites associated with cells lost their reactivity to the NH₂-terminal antiserum regardless of whether they were intracellular or extracellular (unpublished data). In the absence of cells, 80–90% of sporozoites stained with this antiserum (unpublished data), suggesting that cell contact was the trigger for CSP cleavage. To test this, sporozoites were preincubated with cytochalasin D (CD), an inhibitor of sporozoite invasion but not attachment to cells (15), in the presence or absence of E-64 and added to cells. As shown in Table I, sporozoites incubated with CD plus E-64 stained with the NH₂-terminal antiserum, whereas those incubated with CD alone did not. mAb 3D11, directed against the repeat region of CSP, bound to both E-64-treated and untreated sporozoites. Controls in which sporozoites were incubated without cells showed that neither elevated temperature nor serum alone had a significant effect on CSP cleavage (Table I).

In this assay, sporozoites were incubated with cells for only 2 min before being fixed and stained. The rapid loss of reactivity to the NH₂-terminal antiserum indicates that there is a dramatic increase in the kinetics of CSP cleavage when parasites are added to cells. In the absence of cells, the half life of newly synthesized CSP is ~1 h (Fig. 1 and references

4, 5). These data indicate that the secretion of the protease that cleaves CSP is regulated. It is likely that the low level cleavage observed in the absence of cells is due to leaky secretion from apical organelles, whereas exocytosis of larger amounts of protease is mediated by specific signals that are transduced upon contact with target cells.

CSP cleavage is not required for migration through cells

It has been shown that sporozoites interact with cells in two distinct ways: they either rupture the plasma membrane and migrate through a cell or they enter with a vacuole and productively invade the cell (16). To study whether CSP processing was preferentially associated with one of these processes, we tested whether E-64 inhibited sporozoite migration through cells. Migration can be quantified by including a high MW fluorescent tracer in the medium because it will enter cells that are wounded by sporozoites as they pass through. As shown in Fig. 4 C, E-64 had no effect on sporozoite migration through cells.

These data indicate that CSP cleavage is associated with productive invasion of cells and suggests that sporozoites differentially recognize cells that they will invade; a finding that makes sense given that, in vivo, they travel through several cell barriers to reach their target, the hepatocyte. One question raised by these findings is how do sporozoites recognize hepatocytes? Previous work has shown that CSP binds to heparan sulfate proteoglycans (HSPGs) found on hepatocytes, making these molecules likely candidates for target cell recognition (for review see reference 1). We are currently investigating whether binding of CSP to HSPGs triggers cleavage and initiates the cascade of events leading to productive invasion of cells.

Inhibition of cysteine proteases prevents malaria infection

Lastly, we tested E-64 as an inhibitor of malaria infection in vivo using a rodent model of the disease. Using a quantitative PCR assay, we compared the amounts of parasite rRNA in the livers of mice pretreated with E-64 or buffer and infected with *Plasmodium* sporozoites. We found that mice injected with E-64 were completely protected from malaria infection (Fig. 4 D). Although inhibitors of cysteine and serine proteases have not yet been used for the treatment of human disease, animal studies have shown the feasibility of using these inhibitors as drugs in the treatment of parasitic infections (for review see references 17, 18). Our finding that we can completely prevent malaria infection by targeting the cysteine proteases of the sporozoite stage could lead to the development of new prophylactic agents for malaria.

In conclusion, we have shown that the high MW CSP form is proteolytically cleaved by a papain family cysteine protease of parasite origin. Several lines of evidence support a role for CSP cleavage during cell invasion. First, under conditions in which CSP cleavage is inhibited, cell invasion is similarly inhibited. Second, rapid and complete CSP cleavage occurs when sporozoites contact target cells, indicating that cleavage is temporally associated with invasion.

And lastly, the conservation of this process across the genus indicates that it is of importance to the parasite.

These data are part of a growing body of work demonstrating that proteolytic processing of secreted and surface proteins is required for cell invasion by *Plasmodium* and other Apicomplexan parasites such as *Toxoplasma* (14, 19, 20). One of the most well-studied examples is MSP-1, the major surface protein of *Plasmodium* merozoites, the infective form of the erythrocytic stage (for review see reference 14). Interestingly, both CSP and MSP-1 have known cell-adhesive domains in their COOH termini, raising the possibility that cleavage controls the exposure of these domains. In CSP, the COOH terminus contains the TSR, a known cell-adhesive sequence that has been shown to bind with high affinity to HSPGs (for review see reference 1). Previous studies have shown that the NH₂-terminal portion of CSP also binds to HSPGs (21). Our data suggest a model for CSP cleavage that explains why this protein has two heparin-binding domains. Our hypothesis is that an initial interaction between cell surface HSPGs and the NH₂-terminal portion of CSP cross-links the protein and provides the signal for cleavage. In turn, cleavage exposes the cell-adhesive TSR, which binds with high affinity to HSPGs, initiating a cascade of events that ultimately lead to cell entry.

MATERIALS AND METHODS

Antibodies and peptides. mAb 3D11 is directed against the repeat region of *P. berghei* CSP (22); mAb NYS1 is directed against the repeat region of *P. yoelii* CSP (23); and mAb 2A10 is directed against the repeat region of *P. falciparum* CSP (11). For immunoprecipitations, mAbs 3D11 and 2A10 were conjugated to sepharose as outlined previously (24). Antisera to the NH₂- and COOH-terminal thirds of *P. berghei* CSP were generated in rabbits using peptides that were provided by G. Corradin and M. Roggero (Institute of Biochemistry, Lausanne, Switzerland). The sequences of the NH₂- and COOH-terminal peptides were GYGQNKSIQAQRNLNELCYNEGNDNKLYHVLNSKNGKIYIRNTVNRLADAPGKKNKKNKIERNKLNK and NDDSYIPSAEKILEFVKQIRDSITEEWSQC-NVTCGSGIRVRKRKGSNKKAEDLTLEDIDTEICKMDKCS, respectively. Overlapping peptides and repeat peptides were synthesized and purified by Midwest Bio-Tech.

Sporozoites. *P. yoelii*, *P. berghei*, *P. berghei*-expressing GFP (25), and recombinant *P. berghei* sporozoites expressing a hybrid *P. berghei*-*P. falciparum* CSP (Pf/Pb sporozoites; reference 10) were grown in *Anopheles stephensi* mosquitoes. *P. falciparum*-infected mosquitoes were obtained from D. Carucci (Naval Medical Research Center Malaria Program, Silver Spring, MD). Where indicated, sporozoites were purified by passage through two 3- μ m polycarbonate membranes (Whatman).

ELISAs. Peptides were coated onto wells of Immulon 2HB microtiter plates (ThermoLabsystems) and blocked, and antisera were added at the indicated dilutions. Binding was revealed with anti-mouse or anti-rabbit Ig-conjugated to alkaline phosphatase followed by the fluorescent substrate, 4-methylumbelliferyl phosphate and fluorescence was read in a Fluoroskan II plate reader.

Metabolic labeling. *P. berghei* or where indicated, *P. falciparum* or Pf/Pb sporozoites, were metabolically labeled in DMEM without Cys/Met, 1% BSA, and 400 μ Ci/ml L-[³⁵S]Cys/Met for 1 h at 28°C and chased in DMEM with Cys/Met and 1% BSA at 28°C in the presence or absence of the indicated protease inhibitor. For the pronase experiment, sporozoites were metabolically labeled in medium without BSA for 45 min at 28°C,

washed, and resuspended in DMEM with Cys/Met and 100 μ g/ml cycloheximide for 10 min and kept on ice or chased at 28°C for 1 h. Sporozoites were resuspended in 100 μ g/ml pronase, \pm pronase inhibitor cocktail (500 μ g/ml antipain, 30 μ g/ml aprotinin, 600 μ g/ml chymostatin, 5 mg/ml EDTA, 5 μ g/ml leupeptin, 10 mg/ml AEBSEF, 7 μ g/ml pepstatin, and 2 mM PMSF; reference 26) for 1 h at 4°C, washed, and lysed in lysis buffer with pronase inhibitor cocktail and 1% BSA; CSP was immunoprecipitated.

Immunoprecipitation and SDS-PAGE analysis. Metabolically labeled sporozoites were lysed in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0) with protease inhibitors for 1 h at 4°C, and lysates were incubated with mAb 3D11 agarose overnight at 4°C and washed with lysis buffer and lysis buffer with 500 mM NaCl and preelution buffer (0.5% Triton X-100, 10 mM Tris-HCl, pH 6.8). CSP was eluted with 1% SDS in 0.1 M glycine, pH 1.8, neutralized with Tris-HCl, pH 8.8, and run on a 7.5% SDS-polyacrylamide gel under nonreducing conditions. For experiments with *P. falciparum* or Pf/Pb sporozoites, a 10% SDS-polyacrylamide gel was used. Gels were fixed, enhanced with Amplify (Amersham Biosciences), dried, and exposed to film.

Immunoblot of sporozoite lysates. Sporozoite lysates were separated by SDS-PAGE, transferred to PVDF membrane, and incubated with either 4 μ g/ml mAb 3D11, NH₂-terminal antiserum (1:3,000), COOH-terminal antiserum (1:3,000), or 4 μ g/ml mAb 2A10 followed by anti-mouse or anti-rabbit Ig conjugated to horseradish peroxidase (HRP; 1:100,000). Bound antibodies were visualized using the enhanced chemiluminescence detection system (ECL).

Biotinylation of sporozoites. *P. berghei* transgenic for GFP was biotinylated using sulfo-succinimidyl-6'-(biotinamido) hexanoate according to the manufacturer's instructions (Pierce Chemical Co.). Lysates of biotinylated sporozoites were immunoprecipitated with either mAb 3D11 or polyclonal antibodies to GFP (1:200; Molecular Probes) followed by protein A coupled to agarose beads, loaded onto a 4–12% Tris-Glycine gel, transferred to PVDF, and incubated with either mAb 3D11 followed by anti-mouse Ig HRP, anti-GFP Ig (1:500) followed by anti-rabbit Ig HRP, or streptavidin-HRP (1:100,000). Bound antibodies were visualized using ECL.

Immunofluorescence assay. Live *P. berghei* sporozoites were incubated with NH₂-terminal antiserum (1:500 in DMEM/BSA) at 4°C for 2 h, washed at 4°C, and allowed to air dry on slides at 4°C. They were incubated with anti-rabbit Ig-FITC, washed, and mounted.

Sporozoite invasion assay. Invasion assays were performed as described previously (15), with some modifications. For assays with *P. berghei* and *P. yoelii*, Hepa 1–6 cells (CRL-1830; American Type Culture Collection) were used, and for assays with *P. falciparum*, HepG2 cells (HB-8065; American Type Culture Collection) were used. Sporozoites were preincubated with the indicated protease inhibitor for 2 h at 28°C and plated on cells in the continued presence of the inhibitor for 1 h at 37°C. In a control, Hepa 1–6 cells were incubated with 10 μ M E-64 for 2 h at 37°C, the medium was removed, and untreated *P. berghei* sporozoites were added. After incubation with sporozoites, cells were washed and fixed, and sporozoites were stained with a double-staining assay that distinguishes between extracellular and intracellular sporozoites.

Cell contact assay. *P. berghei* sporozoites were incubated in DMEM \pm 10 μ M E-64 at 4°C for 2 h and added to Hepa 1–6 cells on glass coverslips. 30 min before sporozoites were added to coverslips, CD was added to all samples (final concentration, 1 μ M). Sporozoites were centrifuged onto coverslips (1,250 g) for 5 min at 4°C. Coverslips were brought to 37°C for 2 min, fixed with 4% paraformaldehyde, and stained with either mAb 3D11 followed by anti-mouse Ig FITC or the NH₂-terminal antiserum followed by anti-rabbit Ig FITC. When *P. berghei* sporozoites expressing GFP were used, the cells were only stained with the NH₂-terminal antiserum. As a

control, sporozoites were spun onto coverslips without cells using the aforementioned protocol.

Sporozoite migration assay. Sporozoites were preincubated $\pm 10 \mu\text{M}$ E-64 for 2 h at 28°C and added to Hepa 1–6 cells in the continued presence of inhibitor with 1 mg/ml rhodamine-dextran. After 1 h at 37°C, the cells were washed and fixed, and rhodamine-positive cells were counted as outlined previously (16).

Assay for sporozoite infectivity in vivo. Swiss/Webster mice were given three i.p. injections of DMEM \pm E-64 (50 mg/kg/injection) at 16, 2.5, and 1 h before i.v. injection of 15,000 *P. yoelii* sporozoites. 40 h later, livers were harvested, total RNA was isolated, and malaria infection was quantified using reverse transcription followed by real-time PCR using primers that recognize *P. yoelii*-specific sequences within the 18S rRNA as outlined previously (27). 10-fold dilutions of a plasmid construct containing the *P. yoelii* 18S rRNA gene were used to create a standard curve.

Online supplemental material. Fig. S1 shows the specificity of the NH₂- and COOH-terminal antisera as determined by ELISA. Fig. S2 shows that the protease inhibitors that inhibited CSP processing are not toxic to sporozoites. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20040989/DC1>.

The authors would like to thank G. Corradin and M. Roggero for their generous gift of the long NH₂- and COOH-terminal peptides; D. Carucci and P. de la Vega for providing *P. falciparum* sporozoites; E. Nardin and G. Oliveira for providing recombinant Pf/Pb sporozoites; M. Blackman and K. Kim for helpful discussions; V. Nussenzweig, D. Eichinger, and M. Calvo-Calle for their critical reading of the manuscript; and D. Bernal and J. Noonan for their expert assistance with mosquito rearing and infection.

This work was supported by National Institutes of Health (NIH) grant no. R01 AI44470 (to P. Sinnis) and NIH training grant no. 5T32 AI07180 (to A. Coppi).

The authors have no conflicting financial interests.

Submitted: 19 May 2004

Accepted: 19 November 2004

REFERENCES

- Sinnis, P., and E. Nardin. 2002. Sporozoite antigens: biology and immunology of the circumsporozoite protein and thrombospondin related anonymous protein. In *Malaria Immunology*. P. Perlmann and M. Troye-Blomberg, editors. S. Karger Press, Basel, Switzerland. pp. 70–96.
- Menard, R., A.A. Sultan, C. Cortes, R. Altszuler, M.R. van Dijk, C.J. Janse, A.P. Waters, R.S. Nussenzweig, and V. Nussenzweig. 1997. Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes. *Nature*. 385:336–340.
- Goundis, D., and K.B.M. Reid. 1988. Properdin, the terminal complement components, thrombospondin and the circumsporozoite protein of malaria parasites contain similar sequence motifs. *Nature*. 335:82–85.
- Yoshida, N., P. Potocnjak, V. Nussenzweig, and R.S. Nussenzweig. 1981. Biosynthesis of Pb44, the protective antigen of sporozoites of *Plasmodium berghei*. *J. Exp. Med.* 154:1225–1236.
- Cochrane, A.H., F. Santoro, V. Nussenzweig, R.W. Gwadz, and R.S. Nussenzweig. 1982. Monoclonal antibodies identify the protective antigens of sporozoites of *Plasmodium knowlesi*. *Proc. Natl. Acad. Sci. USA*. 79:5651–5655.
- Solomon, M., B. Belenghi, M. Delledonne, E. Menachem, and A. Levine. 1999. The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. *Plant Cell*. 11:431–443.
- Wu, Y., X. Wang, X. Liu, and Y. Wang. 2003. Data-mining approaches reveal hidden families of proteases in the genome of malaria parasite. *Genome Res.* 13:601–616.
- Harper, J.W., K. Hemmi, and J.C. Powers. 1985. Reaction of serine proteases with substituted isocoumarins: discovery of 3,4-dichloroisocoumarin, a new general mechanism based serine protease inhibitor. *Biochem.* 24:1831–1841.
- Sajid, M., and J.H. McKerrow. 2002. Cysteine proteases of parasitic organisms. *Mol. Biochem. Parasitol.* 120:1–21.
- Persson, C., G.A. Oliveira, A.A. Sultan, V. Nussenzweig, and E. Nardin. 2002. A new tool to evaluate human pre-erythrocytic malaria vaccines: rodent parasites bearing a hybrid *Plasmodium falciparum* circumsporozoite protein. *J. Immunol.* 169:6681–6685.
- Nardin, E.H., V. Nussenzweig, R.S. Nussenzweig, W.E. Collins, K.T. Harinasuta, P. Tapchaisri, and Y. Chomcham. 1982. Circumsporozoite proteins of human malaria parasites *Plasmodium falciparum* and *Plasmodium vivax*. *J. Exp. Med.* 156:20–30.
- Gonzalez-Ceron, L., M.H. Rodriguez, R.A. Wirtz, B.J. Sina, O.L. Palomeque, J.A. Nettel, and V. Tsutsumi. 1998. *Plasmodium vivax*: a monoclonal antibody recognizes a circumsporozoite protein precursor on the sporozoite surface. *Exp. Parasitol.* 90:203–211.
- Krettli, A.U., E.M.M. Rocha, J.K. Lopes, C.R.W. Carneiro, K.K. Kambo, A.H. Cochrane, and R.S. Nussenzweig. 1988. Circumsporozoite protein of *Plasmodium gallinaceum* characterized by monoclonal antibodies. *Parasite Immunol.* 10:523–533.
- Blackman, M.J. 2000. Proteases involved in erythrocyte invasion by the malaria parasite: function and potential as chemotherapeutic targets. *Curr. Drug Targets.* 1:59–83.
- Pinzon-Ortiz, C., J. Friedman, J. Esko, and P. Sinnis. 2001. The binding of the circumsporozoite protein to cell surface heparan sulfate proteoglycans is required for *Plasmodium* sporozoite attachment to cells. *J. Biol. Chem.* 276:26784–26791.
- Mota, M., G. Pradel, J.P. Vanderberg, J.C.R. Hafalla, U. Frevert, R.S. Nussenzweig, V. Nussenzweig, and A. Rodriguez. 2001. Migration of *Plasmodium* sporozoites through cells before infection. *Science*. 291:141–144.
- McKerrow, J.H., J.C. Engel, and C.R. Caffrey. 1999. Cysteine protease inhibitors as chemotherapy for parasitic infections. *Bioorg. Med. Chem.* 7:639–644.
- Rosenthal, P.J. 1998. Proteases of malarial parasites: new targets for chemotherapy. *Emerg. Infect. Dis.* 4:49–57.
- Howell, S.A., C. Withers-Martinez, C.H.M. Kocken, A.W. Thomas, and M.J. Blackman. 2001. Proteolytic processing and primary structure of *Plasmodium falciparum* apical membrane antigen-1. *J. Biol. Chem.* 276:31311–31320.
- Kim, K. 2004. Role of proteases in host cell invasion by *Toxoplasma gondii* and other Apicomplexa. *Acta Trop.* 91:69–81.
- Rathore, D., J.B. Sacci, P. de la Vega, and T.F. McCutchan. 2002. Binding and invasion of liver cells by *Plasmodium falciparum* sporozoites. *J. Biol. Chem.* 277:7092–7098.
- Yoshida, N., R.S. Nussenzweig, P. Potocnjak, V. Nussenzweig, and M. Aikawa. 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science*. 207:71–73.
- Charoenvit, Y., M.F. Leef, L.F. Yuan, M. Sedegah, and R.L. Beaudoin. 1987. Characterization of *Plasmodium yoelii* monoclonal antibodies directed against stage-specific sporozoite antigens. *Infect. Immun.* 55:604–608.
- Harlow, E., and D. Lane. 1988. *Antibodies, a Laboratory Manual*. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
- Natarajan, R., V. Thathy, M.M. Mota, J.C. Hafalla, R. Menard, and K.D. Vernick. 2001. Fluorescent *Plasmodium berghei* sporozoites and pre-erythrocytic stages: a new tool to study mosquito and mammalian host interactions with malaria parasites. *Cell. Microbiol.* 3:371–379.
- Wieckowski, E.U., J.F. Kokai-Kun, and B.A. McClane. 1998. Characterization of membrane-associated *Clostridium perfringens* enterotoxin following pronase treatment. *Infect. Immun.* 66:5897–5905.
- Bruna-Romero, O., J.C. Hafalla, G. Gonzalez-Aseguinolaza, G. Sano, M. Tsuji, and F. Zavala. 2001. Detection of malaria liver-stages in mice infected through the bite of a single *Anopheles* mosquito using a highly sensitive real-time PCR. *Int. J. Parasitol.* 31:1499–1502.
- Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heigne. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* 10:1–6.