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A Role for Apical Membrane Antigen 1 during Invasion of Hepatocytes by *Plasmodium falciparum* Sporozoites*

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Plasmodium **sporozoites are transmitted through the bite of infected mosquitoes and invade hepatocytes as a first and obligatory step of the parasite life cycle in man. Hepatocyte invasion involves proteins secreted from parasite vesicles called micronemes, the most characterized being the thrombospondin-related adhesive protein (TRAP). Here we investigated the expression and function of another microneme protein recently identified in *Plasmodium falciparum* sporozoites, apical membrane antigen 1 (AMA-1). *P. falciparum* AMA-1 is expressed in sporozoites and is lost after invasion of hepatocytes, and anti-AMA-1 antibodies inhibit sporozoite invasion, suggesting that the protein is involved during invasion of hepatocytes. As observed with TRAP, AMA-1 is initially mostly sequestered within the sporozoite. Upon microneme exocytosis, AMA-1 and TRAP relocate to the sporozoite surface, where they are proteolytically cleaved, resulting in the shedding of soluble fragments. A subset of serine protease inhibitors blocks the processing and shedding of both AMA-1 and TRAP and inhibits sporozoite infectivity, suggesting that interfering with sporozoite proteolytic processing may constitute a valuable strategy to prevent hepatocyte infection.**

The apicomplexan parasite *Plasmodium falciparum* is a causative agent of malaria, one of the major human infectious diseases, responsible for more than 1 million deaths per year worldwide. *Plasmodium* sporozoites are transmitted through the bite of infected mosquitoes and invade the liver of the mammalian host, where they undergo schizogony and differentiate into merozoites that subsequently invade erythrocytes and cause disease. Blocking sporozoite invasion of hepatocytes represents an attractive anti-malarial strategy because it would prevent malaria symptoms and parasite transmission to the mosquito, which both occur at erythrocytic stages. Sporozoites migrate through tissues and invade target cells using gliding motility (1), a process involving secretory vesicles called micronemes. Upon microneme exocytosis, proteins are delivered onto the parasite surface and then redistributed from the anterior to the posterior end of the parasite, leading to its forward movement and penetration into a host cell (2). Only two sporozoite microneme proteins, namely circumsporozoite protein (CSP)[1] and thrombospondin-related adhesive protein (TRAP), have been extensively studied (3). It is quite likely, however, that other microneme proteins are involved during the invasion process. Recently, Florens *et al.* (4) reported a comprehensive view of the proteome of *P. falciparum* obtained with high resolution liquid chromatography and tandem mass spectrometry. Interestingly, some of the proteins detected in sporozoites had not been reported in this stage before, including apical membrane antigen 1 (AMA-1), a microneme protein involved in merozoite invasion of erythrocytes that is considered to be a leading candidate for inclusion in a vaccine against erythrocytic stages of *P. falciparum* (5–7). Here, we have analyzed AMA-1 expression in *P. falciparum* sporozoites and investigated its potential role during invasion of hepatocytes.

EXPERIMENTAL PROCEDURES

Antibodies and Inhibitors—We used previously characterized anti-*P. falciparum* AMA-1 (PfAMA-1) domain III monoclonal antibody (mAb) DV5 (8), anti-PfAMA-1 prodomain mAb 5G8 (9), anti-PfAMA-1 domain I mAb 1F9 (9), anti-3D7 strain PfAMA-1 polyclonal rabbit IgG (10), anti-FVO strain PfAMA-1 polyclonal rabbit IgG (11), anti-PfTRAP mAb SSP2.2 (12), and anti-PfCSP mAb E9 (13). For liver schizont counting, we used anti-*Plasmodium* HSP-70 sera obtained from mice immunized with i72 recombinant protein (gift from D. Mattei, Institut Pasteur, Paris, France). Secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-mouse IgG (GAM-FITC; Sigma), Alexa Fluor® 594 goat anti-rabbit IgG (GAR- Alexa Fluor® 594; Molecular Probes), peroxidase goat anti-mouse and peroxidase goat anti-rabbit IgG (Becton Dickinson). Cytochalasin D (500 μM stock in Me_2SO) and protease inhibitors chymostatin (2 mg/ml stock in Me_2SO), pepstatin A (1 mg/ml

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[1] The abbreviations used are: CSP, circumsporozoite protein; AMA-1, apical membrane antigen 1; DAPI, 4,6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-tosyl-L-lysine chloromethyl ketone; TRAP, thrombospondin-related adhesive protein; Z-GML-CH_2Cl, N-benzyloxycarbonyl-Gly-Met-Leu-chloromethyl ketone.

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stock in Me_2SO), leupeptin (1 mg/ml stock in water), phenylmethylsulfonyl fluoride (PMSF, 100 mM stock in ethanol), and *N*-tosyl-L-lysine chloromethyl ketone (TLCK, 20 mM stock in water) were from Sigma. The peptidyl chloromethylketone *N*-benzyloxycarbonyl-Gly-Met-Leu-CH_2Cl (Z-GML-CH_2Cl, 10 mM stock in Me_2SO) was synthesized as described (14).

Isolation of P. falciparum Sporozoites—Anopheles stephensi adult females were infected with the NF54 strain of *P. falciparum*, using a membrane-based feeder system (15). After 14–21 days, mosquitoes were killed, and their salivary glands were aseptically dissected and disrupted by trituration in a glass tissue grinder, and the sporozoites were counted in a KovaSlide® chamber.

Isolation and Culture of Human Hepatocytes—Primary human hepatocyte cultures were prepared as described (16), with minor modifications. We used either fresh or cryopreserved hepatocytes, both susceptible to infection with *P. falciparum* sporozoites (17, 18). In our hands, *P. falciparum* sporozoite infectivity is similar in fresh and cryopreserved hepatocytes.[2] Briefly, the cells were isolated by collagenase (PAA Laboratories) perfusion of human liver fragments, used in agreement with the French ethical regulations, and further purified over a 40% Percoll gradient. The hepatocytes were cryopreserved in liquid nitrogen in fetal calf serum with 10% Me_2SO; after thawing, viable hepatocytes were purified over a 40% Percoll gradient. The hepatocytes were seeded in eight-chamber plastic Lab-Tek slides or in 24-well culture plates (Nalge Nunc International) coated with rat tail collagen I (Becton Dickinson) at a density of 18×10^4 cells/cm^2 (fresh hepatocytes) or 25×10^4 cells/cm^2 (cryopreserved hepatocytes). The hepatocytes were cultured at 37 °C in 4% CO_2, in William's E medium (Invitrogen) supplemented to a final concentration of 10% fetal calf serum (Invitrogen), 2% penicillin-streptomycin (100× stock solution; Invitrogen), 1% sodium pyruvate (100× stock solution; Invitrogen), 1% L-glutamine (100× stock solution; Invitrogen), 1% insulin-transferrin-selenium (100× stock solution; Invitrogen). After complete cell adherence (12–24 h), the culture medium was supplemented with 10^{-7} M dexamethasone (Sigma) and 2% Me_2SO to maintain hepatocyte differentiation (19).

Culture of P. falciparum Liver Stages—Human hepatocytes were cultured for at least 48 h before inoculation with *P. falciparum* sporozoites (18). After the removal of medium from the culture chambers, sporozoites in culture medium were added to the Lab-Tek wells (1×10^5 sporozoites/well) or plate wells (1×10^6 sporozoites/well) and incubated with the hepatocytes for 3 h. The cultures were then washed and further incubated in complete culture medium supplemented with 0.25% Me_2SO. Culture medium was renewed every 2–3 days.

Reverse Transcriptase-PCR—Total RNA was purified from *P. falciparum* NF54 sporozoites using the QIAamp RNA Blood Mini Kit (Qiagen), including a DNase treatment step. Reverse transcription was performed using the ProSTAR First-Strand reverse transcriptase-PCR kit (Stratagene). One μl of cDNA was PCR amplified with the sense primer 5′-TTCAAATACTACTTTTTTAACACCG-3′ and the antisense primer 5′-CTCTTTCGATTTCTTTCATTATTTC-3′, by use of 40 cycles of 45 s of denaturation at 94 °C, 45 s of annealing at 55 °C, and 60 s of elongation at 70 °C. The resulting products (890-base pair amplicon) were stained with ethidium bromide and run on a 1% agarose gel in Tris acetate-EDTA buffer.

Immunofluorescence Assays—For detection of AMA-1 expression in *P. falciparum* sporozoites and liver stages, air-dried sporozoites and methanol-fixed infected hepatocyte cultures were blocked with 3% bovine serum albumin in phosphate-buffered saline for 30 min and then incubated with anti-PfAMA-1 mAb DV5 for 1 h at 37 °C, followed by incubation with GAM-FITC and 1 μg/ml DNA stain diamidino-phenylindole (DAPI) for 30 min before examination by fluorescence confocal microscopy. Surface staining was performed on nonpermeabilized sporozoites deposited on poly-L-lysine-coated multiwell glass slides, in a "wet" sporozoite assay (20); sporozoites were fixed in 2% paraformaldehyde for 20 min at room temperature, and endogenous fluorescence was quenched with 0.1 M glycine. The parasites were blocked with 3% bovine serum albumin in phosphate-buffered saline for 1 h and then incubated with primary antibodies (anti-PfAMA-1 mAbs DV5, 1F9, or 5G8 or rabbit polyclonal anti-PfAMA-1 IgG, as indicated in the figure legends) for 2 h at 37 °C, followed by incubation with secondary antibodies (GAM-FITC for mAbs or GAR- Alexa Fluor® 594 for rabbit IgG) and DAPI for 1 h before examination by fluorescence microscopy.

Western Blotting and Secretion Assays—For detection of parasite proteins in sporozoite lysates and supernatants, the parasites were dissolved in SDS-PAGE sample buffer and incubated at 70 °C for 5 min. The protein samples were subjected to 10% SDS-PAGE (1×10^5 parasites or equivalent/lane for AMA-1 and TRAP detection, 1×10^3 parasites or equivalent/lane for CSP detection) and transferred onto polyvinylidene membranes. These were probed with primary antibodies, followed by a peroxidase-conjugated secondary antibody, and antigens were revealed by enhanced chemiluminescence. To analyze proteins secreted in the parasite supernatant, sporozoites were resuspended in medium without serum and incubated for 2 h at 37 °C in the presence or absence of hepatocytes. The parasite suspension was then dissolved by adding SDS-PAGE sample buffer. In some experiments, the supernatants were separated from the sporozoites by centrifugation at $15{,}000 \times g$ for 5 min, and the parasite pellets and supernatants were dissolved in SDS-PAGE sample buffer separately.

Gliding Assays—To analyze sporozoite motility, 30,000 sporozoites were deposited on multispot glass slide wells precoated with anti-PfCSP mAb E9 (100 μg/ml 1 h at 37 °C) and incubated at 37 °C for 1 h. The slides were then washed, and the deposited CSP trails were fixed with 4% paraformaldehyde for 15 min. The trails were then labeled using the anti-PfCSP mAb E9 conjugated to Alexa Fluor® 488 and visualized under a fluorescence microscope.

Inhibition of Sporozoite Invasion Assays—To analyze sporozoite invasion, triplicate hepatocyte cultures were inoculated with *P. falciparum* sporozoites (1×10^5/Lab-Tek well). After 3 h at 37 °C, the cultures were washed, further incubated in fresh medium for 3 days, and then fixed in methanol. Liver schizonts were stained using an anti-HSP-70 mouse serum followed by goat anti-mouse FITC conjugate and counted under a fluorescence microscope. To determine the effects of anti-AMA-1 antibodies on sporozoite infectivity, sporozoites were incubated with hepatocytes in the presence of increasing concentrations of anti-PfAMA-1 rabbit IgG. The percentage of inhibition was determined in comparison with control rabbit IgG. To determine the effects of protease inhibitors and cytochalasin D on sporozoite infectivity, *P. falciparum* sporozoites were incubated with these inhibitors (each from a 100× stock solution diluted directly in the parasite suspension) for 5 min at room temperature and then washed (except for cytochalasin D, the effects of which are reversible) before inoculation onto hepatocytes. The percentage of inhibition was determined in comparison with control sporozoites treated with solvents alone. Inhibition results were analyzed for statistical significance using the one-way analysis of variance followed by the Tukey multiple comparison test.

RESULTS

AMA-1 Is Expressed in P. falciparum Sporozoites—In a recent analysis of *P. falciparum* proteome, peptides corresponding to the merozoite antigen AMA-1 were identified in sporozoites. Because AMA-1 had never been reported in sporozoites before, we were interested in further characterizing its expression at this stage. Using reverse transcriptase-PCR, we could readily detect *ama-1* transcripts in salivary gland sporozoites (Fig. 1*A*), thus confirming the expression of the gene at this stage. To test whether AMA-1 protein is also present in sporozoites, we performed immunofluorescence assays using the anti-PfAMA-1 domain III mAb DV5 (8). All permeabilized sporozoites displayed a strong fluorescence (Fig. 1*B*), with a bipolar nucleus-sparing pattern similar to the immunofluorescence pattern observed with antibodies to TRAP, also known as sporozoite surface protein 2 (12). In contrast, surface labeling with the anti-AMA-1 mAb showed no or very little surface fluorescence on sporozoites (Fig. 1*C*). When detected, AMA-1 surface fluorescence was restricted to the apical extremity of the sporozoites, on the same side of the nucleus as the apicoplast, a DNA-containing organelle visualized as a DAPI-positive spot forward of the nucleus, as described in *Toxoplasma* (21, 22). A similar surface fluorescence pattern was observed using polyclonal anti-PfAMA-1 antibodies (see Fig. 5*A*). Western blotting of protein extracts from *P. falciparum* sporozoites probed with DV5 confirmed AMA-1 expression in sporozoites. Two proteins of ~83 and ~66 kDa were detected in both sporozoites and parasitized erythrocytes and were absent from noninfected mosquito salivary glands (Fig. 1*D*). In *P. falciparum* erythrocytic stages, these two bands have been shown to correspond respectively to a precursor protein that contains a prosequence and to a mature form of AMA-1 that lacks the

[2] O. Silvie, unpublished observations.

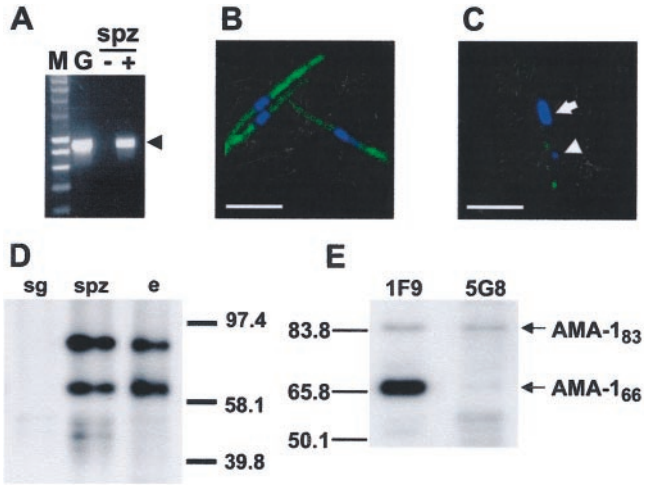

FIG. 1. **AMA-1 is expressed in *P. falciparum* sporozoites.** *A*, reverse transcriptase-PCR analysis of *ama-1* expression in *P. falciparum* NF54 sporozoites (*spz*), with (+) or without (−) reverse transcriptase; *Lane M*, size markers; *lane G*, 3D7 *P. falciparum* genomic DNA. The *arrowhead* indicates the 890-base pair amplicon. *B*, immunofluorescence microscopy of air-dried permeabilized *P. falciparum* sporozoites stained with anti-AMA-1 mAb DV5 (*green*) and DAPI (*blue*). *Bar*, 5 μm. *C*, surface immunofluorescence of *P. falciparum* sporozoites stained with anti-AMA-1 mAb DV5 (*green*) and DAPI (*blue*). The sporozoite nucleus is indicated with an *arrow*, and the apicoplast is indicated with an *arrowhead*. *Bar*, 5 μm. *D*, Western blotting of lysates from noninfected mosquito salivary glands (*lane sg*), *P. falciparum* sporozoite infected salivary glands (*lane spz*), and *P. falciparum* infected erythrocytes (*lane e*), using the anti-AMA-1 mAb DV5. *E*, Western blotting of lysates from *P. falciparum* sporozoites using the anti-AMA-1 mAbs 1F9 and 5G8.

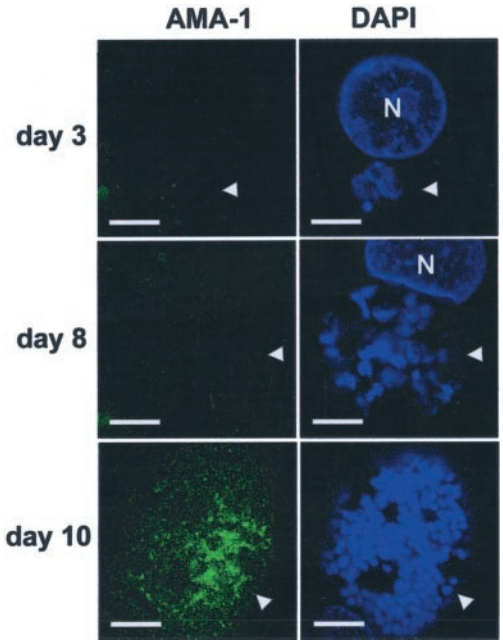

FIG. 2. **AMA-1 is lost after invasion of hepatocytes and re-expressed in liver merozoites.** *P. falciparum* liver schizonts (*arrowheads*) stained with the anti-AMA-1 mAb DV5 (*green*) and DAPI (*blue*), 3, 8, or 10 days after infection of human hepatocyte cultures with sporozoites. *N*, hepatocyte nucleus. *Bars*, 5 μm.

prosequence (23, 24). To confirm that the two bands observed in the sporozoite lysates also correspond to the precursor and mature forms, we performed Western blot analysis using the anti-AMA-1 mAbs 1F9, specific for *P. falciparum* AMA-1 domain I, and 5G8, specific for AMA-1 prosequence. The mAb 5G8 recognized only the upper ~83-kDa band, whereas 1F9 recognized both the ~83-kDa and ~66-kDa proteins (Fig. 1*E*), thus confirming that in *P. falciparum* sporozoites these two bands correspond to the precursor and the mature form of AMA-1, respectively. The relative amount of precursor and mature proteins detected by Western blot varied between the different sporozoite preparations (Fig. 1 and see Fig. 4).

AMA-1 Is Lost after Invasion of Hepatocytes and Is Re-expressed in Liver Merozoites—To assess the fate of AMA-1 after sporozoite invasion of hepatocytes, we analyzed AMA-1 expression in primary human hepatocytes infected with sporozoites (18). We consistently failed to detect the protein in early liver stages by immunofluorescence assay (Fig. 2), suggesting that it was lost after invasion of hepatocytes. AMA-1 was only re-expressed in mature *P. falciparum* liver schizonts, with a punctate pattern likely corresponding to nascent liver merozoites (Fig. 2).

Anti-AMA-1 Antibodies Inhibit the Invasion of Human Hepatocytes by P. falciparum Sporozoites—The expression of AMA-1 in *P. falciparum* sporozoites and the fact that AMA-1 was not detected in liver schizonts until the differentiation of liver merozoites suggested that AMA-1 could be involved in the process of invasion of hepatocytes by sporozoites. Therefore, we tested whether antibodies to AMA-1 could inhibit the invasion of hepatocytes by *P. falciparum* sporozoites. Polyclonal IgG raised in rabbits immunized with a recombinant AMA-1 protein from the *P. falciparum* 3D7 clone (10) inhibited invasion of human hepatocytes by NF54 (homologous strain) *P. falciparum* sporozoites in a concentration-dependent manner (Fig. 3). Relatively high concentrations were necessary to achieve significant inhibition, but equivalently high concentrations of IgG from rabbits immunized with a reduced and alkylated form of the recombinant 3D7 PfAMA-1 protein did not inhibit sporozoite invasion (Fig. 3), indicating that inhibition is mediated by antibodies directed to conformational epitopes. IgG from rabbits immunized with a recombinant AMA-1 from the *P. falciparum* heterologous strain FVO (11) also inhibited NF54 *P. falciparum* sporozoite invasion (Fig. 3).

Upon Microneme Exocytosis, AMA-1 and TRAP Are Translocated to the Sporozoite Surface and Shed as Soluble Forms after Proteolytic Processing—Only a minority of salivary gland sporozoites (~5–10%) displayed detectable amounts of AMA-1 on their apical surface (Fig. 1*C* and see Fig. 5*A*), suggesting that most of the protein is sequestered within the sporozoite micronemes, as observed with TRAP (25). Upon incubation at 37 °C, which induces microneme exocytosis (26, 27), most of the sporozoites (~60–80%) expressed AMA-1 on their surface, distributed either over the whole surface or as a posterior cap (see Fig. 5*B*). Sporozoite surface AMA-1 reacted with 1F9 but not with 5G8 mAb, indicating that only the mature 66-kDa protein translocates to the parasite surface (see Fig. 6, *C* and *D*). As expected, both 5G8 and 1F9 mAbs labeled air-dried permeabilized sporozoites (see Fig. 6, *A* and *B*) with a fluorescent pattern similar to that observed with DV5. In *P. falciparum* merozoites, AMA-1 translocates to the surface upon microneme exocytosis and is proteolytically cleaved and shed as soluble fragments (14, 24). To determine whether similar processing events also occur in *P. falciparum* sporozoites, we analyzed by Western blot lysates from sporozoites incubated at 37 °C. Indeed, an AMA-1 cleavage product of ~48 kDa, associated with a less abundant product of ~52 kDa, was found in lysates from sporozoites incubated at 37 °C but not in lysates from sporozoites kept at 4 °C upon isolation from mosquito salivary glands (Fig. 4*A*). A TRAP cleavage product was also detected in the preparations from sporozoites incubated at 37 °C, 5–10 kDa smaller than the sporozoite TRAP, consistent with the size of the TRAP ectodomain (Fig. 4*B*). Importantly, when sporozoites and supernatants were analyzed separately, the cleavage products of

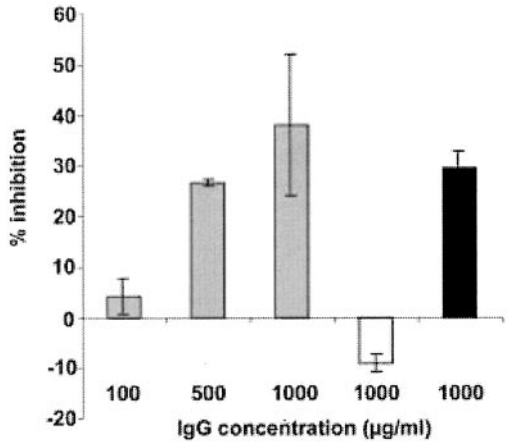

FIG. 3. **Anti-AMA-1 polyclonal antibodies inhibit *P. falciparum* sporozoite invasion of human hepatocytes *in vitro*.** Human hepatocytes were inoculated with *P. falciparum* NF54 sporozoites in the presence of IgG from rabbits immunized with a 3D7 clone PfAMA-1 recombinant protein (*gray bars*), with a reduced and alkylated form of this 3D7 PfAMA-1 recombinant protein (*white bar*), or with a FVO strain PfAMA-1 recombinant protein (*black bar*). The results are expressed as the means of the percentage of inhibition of at least two independent experiments, each done in triplicate wells (± S.D.).

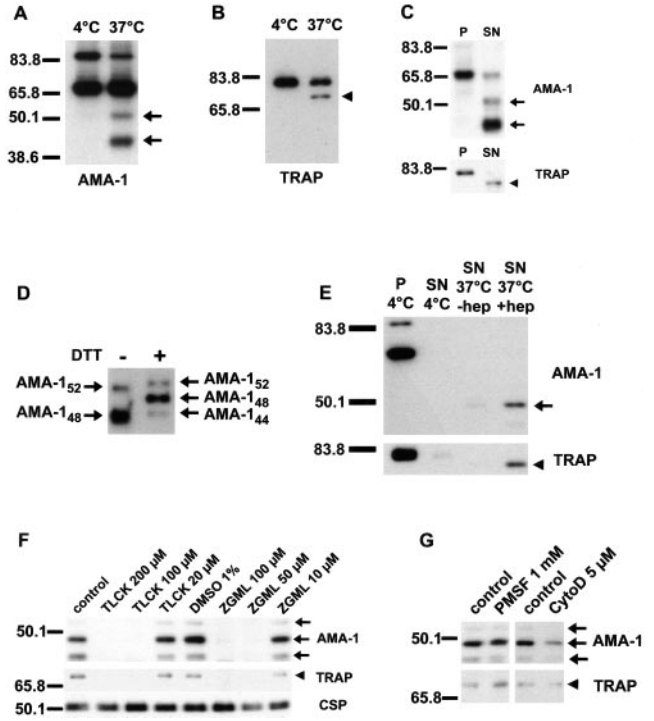

FIG. 4. **Upon incubation of sporozoites at 37 °C, *P. falciparum* AMA-1 and TRAP undergo proteolytic processing and shedding, which is sensitive to a subset of serine protease inhibitors but not to cytochalasin D.** *A*, Western blotting of sporozoite lysates probed with anti-AMA-1 polyclonal rabbit IgG. The *arrows* indicate the 48 and 52 kDa AMA-1 cleavage products, respectively. *B*, Western blotting of sporozoite lysates probed with anti-TRAP mAb SSP2.2. The *arrowhead* indicates the TRAP cleavage product. *C*, Western blotting of pellet (*P*) and supernatant (*SN*) from sporozoites incubated at 37 °C, probed with anti-AMA-1 polyclonal rabbit IgG or with anti-TRAP mAb SSP2.2. The *arrows* and the *arrowhead* indicate the cleavage products of AMA-1 and TRAP, respectively. *D*, Western blotting of supernatants from sporozoites incubated at 37 °C, run under reducing (with dithiothreitol, *DTT* +) or non reducing (without dithiothreitol, *DTT* −) conditions and probed with anti-AMA-1 polyclonal rabbit IgG. *E*, Western blotting of pellet (*P*) and supernatant (*SN*) from sporozoites kept at 4 °C or incubated at 37 °C, in the absence (−*hep*) or presence (+*hep*) of hepatocytes, run under reducing conditions, and probed with anti-AMA-1 polyclonal rabbit IgG or with anti-TRAP mAb SSP2.2. The *arrow* and the *arrowhead* indicate the 48-kDa AMA-1 and the TRAP cleavage products, respectively. *F*, Western blotting of supernatants from sporozoites treated with protease inhibitors TLCK and Z-GML-CH_2Cl (*ZGML*) before incubation at 37 °C. The samples were run under reducing conditions and probed with anti-AMA-1 polyclonal rabbit IgG, anti-TRAP mAb SSP2.2, or anti-CSP mAb E9. The *arrows* and the *arrowhead* indicate the cleavage products of AMA-1 and TRAP, respectively. *G*, Western blotting of supernatants from sporozoites treated with PMSF or cytochalasin D and incubated at 37 °C. The samples were run under reducing conditions and probed with anti-AMA-1 polyclonal rabbit IgG or anti-TRAP mAb SSP2.2. The *arrows* and the *arrowhead* indicate the cleavage products of AMA-1 and TRAP, respectively.

both AMA-1 and TRAP were found only in the supernatants not in the sporozoite pellets (Fig. 4*C*), demonstrating that these products correspond to soluble proteins shed in the supernatant. When sporozoite supernatants were run under reducing conditions, the AMA-1 48-kDa protein migrated as two distinct bands, a major ~48-kDa product and a minor ~44-kDa product (Fig. 4*D*), as observed with merozoite AMA-1 (14). The shed TRAP product migrated as a single band under both reducing and nonreducing conditions (data not shown). AMA-1 and TRAP processing and shedding was observed in sporozoites incubated at 37 °C in the absence of host cells, showing that the protease(s) involved in this processing is (are) encoded by the parasite. Nevertheless, incubation of sporozoites at 37 °C in the presence of hepatocytes consistently enhanced the shedding of AMA-1 and TRAP in the sporozoite supernatants (Fig. 4*E*).

AMA-1 and TRAP Are Processed by a Sporozoite Serine Protease—To further characterize the protease activities mediating AMA-1 and TRAP processing, we used a set of protease inhibitors. Chymostatin, leupeptin, and pepstatin A had no effect on AMA-1 and TRAP shedding (data not shown). In contrast, two serine protease inhibitors, TLCK and Z-GML-CH_2Cl, had a profound dose-dependent inhibitory effect on both AMA-1 and TRAP processing and shedding but had no effect on CSP release into the supernatant (Fig. 4*F*). CSP was detected as a doublet in both sporozoite pellets and supernatants, without any additional cleavage product detectable in parasites incubated at 37 °C (data not shown). Strikingly, PMSF, another serine protease inhibitor, had no significant activity on AMA-1 or TRAP processing in sporozoites (Fig. 4*G*). To further characterize the timing of processing events, we analyzed the effects of protease inhibitors on the surface exposure of AMA-1 and TRAP. Treatment with 200 μM TLCK or 100 μM Z-GML-CH_2Cl, although profoundly inhibiting the shedding of AMA-1 and TRAP, did not prevent the translocation of AMA-1 to the sporozoite surface at 37 °C. On the contrary, the surface exposure of AMA-1, which tended to accumulate at the posterior end of the parasites, was rather enhanced following treatment with these inhibitors (Fig. 5, *C* and *D*). These observations, together with the fact that AMA-1 and TRAP cleavage products were not found associated with the sporozoite pellets, indicate that the proteolytic processing and shedding of AMA-1 and TRAP follows the translocation of these proteins to the parasite surface upon microneme exocytosis, suggesting that the protease involved operates on the sporozoite surface.

Surface Translocation and Proteolytic Processing of AMA-1 and TRAP Are Not Sensitive to Cytochalasin D—Gliding motility and host cell invasion rely on a parasite actomyosin motor that operates beneath the parasite plasma membrane and drives the anterior-to-posterior translocation of microneme proteins once they are released onto the parasite surface (28). Because this actomyosin motor is blocked by the actin polymerization inhibitor cytochalasin D (29), we investigated the effects of cytochalasin D on AMA-1 and TRAP processing. Treatment of sporozoites with 5 μM cytochalasin D completely inhibited both sporozoite gliding and invasion of hepatocytes (data not shown) but did not prevent the translocation of

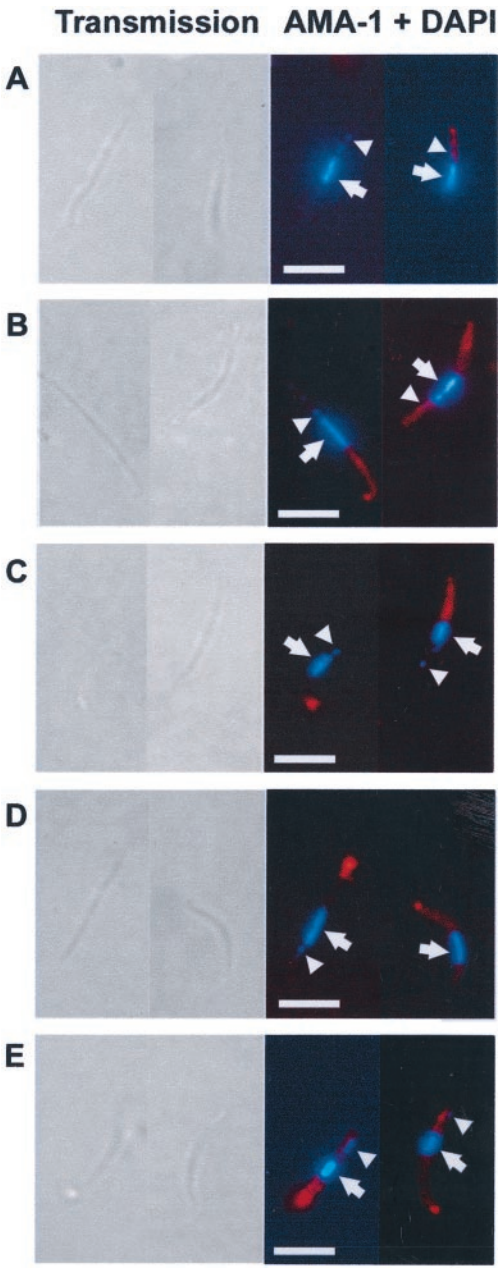

FIG. 5. **Protease inhibitors and cytochalasin D do not prevent AMA-1 translocation to the sporozoite surface upon incubation at 37 °C.** Immunofluorescence analysis of AMA-1 surface exposure using anti-AMA-1 polyclonal rabbit IgG (*red*) and DAPI (*blue*). *A*, untreated sporozoites kept at 4 °C after isolation from salivary glands. *B*, untreated sporozoites incubated at 37 °C for 1 h. *C*, sporozoites treated with 200 μM TLCK and incubated at 37 °C for 1 h. *D*, sporozoites treated with 100 μM Z-GML-CH_2Cl and incubated at 37 °C for 1 h. *E*, sporozoites treated with 5 μM cytochalasin D and incubated at 37 °C for 1h. The *arrow* and the *arrowhead* indicate the sporozoite nucleus and the apicoplast, respectively. *Bar*, 5 μm.

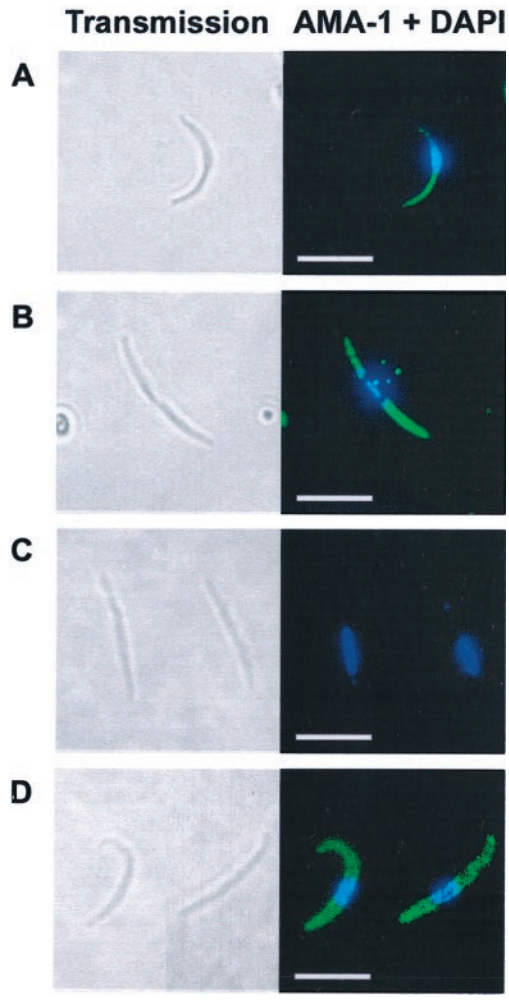

FIG. 6. **Only the mature AMA-1 protein translocates to the sporozoite surface upon incubation at 37 °C.** Immunofluorescence analysis with anti-AMA-1 mAbs 5G8 (*A* and *C*, *green*) or 1F9 (*B* and *D*, *green*) and DAPI (*blue*), in air-dried permeabilized sporozoites (*A* and *B*) or nonpermeabilized sporozoites incubated at 37 °C for 1 h (*C* and *D*). *Bar*, 5 μm.

AMA-1 and TRAP to the sporozoite surface nor their subsequent processing upon passage at 37 °C (Figs. 4*G* and 5*E*). However, a slight reduction of AMA-1 and TRAP shedding was observed when sporozoites were co-incubated with hepatocytes in the presence of cytochalasin D (Fig. 4*G*), probably because cytochalasin D, by suppressing sporozoite motility, prevents the up-regulation of microneme exocytosis induced by sporozoite migration through cells (30).

Interfering with Surface Protein Processing Inhibits P. falciparum Sporozoite Infectivity—Finally, to determine whether AMA-1 and TRAP processing is required during sporozoite invasion of hepatocytes, we analyzed the effects of protease inhibitors on sporozoite infectivity *in vitro*. Pretreatment of sporozoites with 200 μM TLCK and 100 μM Z-GML-CH_2Cl, both inhibiting AMA-1 and TRAP shedding, abrogated sporozoite gliding on glass slides (data not shown). More importantly, treatment with TLCK and Z-GML-CH_2Cl inhibited sporozoite invasion of hepatocytes in a dose-dependent manner (Fig. 7). In contrast, treatment of the parasites with chymostatin, leupeptin, pepstatin A, and PMSF, which do not prevent AMA-1 and TRAP processing, had no significant effect on sporozoite motility and infectivity, and pretreatment of hepatocytes with the different protease inhibitors had no effect on sporozoite invasion (data not shown).

DISCUSSION

Until recently, most of the studies on hepatocyte invasion by sporozoites focused on two sporozoite proteins, CSP and TRAP (3). Many other proteins are expressed at the sporozoite stage (4), with some of them being potentially involved in the invasion process. Here we show that *P. falciparum* AMA-1, a microneme protein involved in merozoite invasion of erythrocytes (7), is also expressed in sporozoites, thus confirming mass spectrometry data (4). AMA-1 is lost after sporozoite invasion and is only re-expressed in liver merozoites, suggesting that it could play a role during invasion of hepatocytes. Indeed, anti-

bodies specific for the *P. falciparum* clone 3D7 AMA-1 inhibit invasion of human hepatocytes by sporozoites of the homologous strain NF54. This inhibitory activity is directed primarily to conformational epitopes, as indicated by the absence of neutralizing activity of antibodies raised against a reduced and alkylated form of recombinant AMA-1, as previously reported for blood stages (10). Antibodies raised against AMA-1 of the heterologous FVO strain also inhibit NF54 strain sporozoite invasion, suggesting that at least part of the targeted epitopes are conserved. Relatively high concentrations of antibodies induced only partial inhibition of invasion. The neutralizing anti-AMA-1 antibodies presumably represent a relatively small proportion of the total rabbit serum IgG preparations used in these experiments. However, AMA-1 is initially mostly sequestered within the sporozoites and therefore may not be readily accessible to antibodies, as previously reported with TRAP (25). Much higher degrees of inhibition have been reported with *P. falciparum* merozoite invasion, using the same rabbit IgG preparations (10, 11). Interestingly, AMA-1 relocates to the merozoite surface upon schizont rupture, so that most of the free merozoites express AMA-1 on their surface (10, 14, 31). These differences in AMA-1 surface exposure may explain the higher level of inhibition seen with merozoite as compared with sporozoite invasion. Nevertheless, the inhibition of sporozoite invasion by anti-AMA-1 antibodies, although partial, still indicates that AMA-1 could be considered as a potential candidate to be included in a multi-stage malaria vaccine, targeting both erythrocytic and pre-erythrocytic stages.

In *P. falciparum* blood stages, the mature 66-kDa AMA-1 relocates to the merozoite surface upon schizont rupture and is proteolytically cleaved, resulting in the shedding of nearly the whole AMA-1 ectodomain as a 48-kDa protein (24), associated with a 44-kDa product resulting from an additional intradomain III cleavage (14) and with a less abundant 52-kDa protein consistent with AMA-1 intramembrane cleavage as described for *Toxoplasma* microneme proteins (32). Here we show that similar processing events occur for AMA-1 in sporozoites upon incubation at 37 °C, leading to the shedding of a major 48-kDa protein and of less abundant products of 44 and 52 kDa. Similarly, incubation of sporozoites at 37 °C induces TRAP processing and shedding of a fragment 5–10 kDa smaller than full-length TRAP, consistent with the size of the TRAP ectodomain and confirming that TRAP is released after cleavage of the protein, as suggested previously (26, 33). Both AMA-1 and TRAP processing are observed in the absence of host cells, clearly demonstrating the parasite origin of the protease(s) involved. Still, incubation of *P. falciparum* sporozoites at 37 °C in the presence of human hepatocytes up-regulates the shedding of both AMA-1 and TRAP in the culture supernatants. This effect is likely due to enhanced microneme exocytosis induced by host cells (25, 30). In both merozoites (14) and sporozoites (this study), AMA-1 translocation to the parasite surface and its subsequent processing are not sensitive to cytochalasin D, suggesting that microneme exocytosis in *Plasmodium* is independent of the actin-dependent gliding machinery.

Strikingly, using a set of protease inhibitors, we observed exactly the same profile of activity on both AMA-1 and TRAP processing. Chymostatin, leupeptin, pepstatin A, and PMSF are ineffective, whereas TLCK inhibits in a dose-dependent fashion the processing of both proteins. Another chloromethyl ketone, Z-GML-CH_2Cl, initially designed to inhibit merozoite surface protein 1 processing in *P. falciparum* merozoites (14), also inhibits both AMA-1 and TRAP processing. In *P. falciparum* merozoites, a single protease is thought to process both AMA-1 and merozoite surface protein 1 (14), this protease being extremely sensitive to PMSF but not to TLCK. In contrast, PMSF does not inhibit AMA-1 and TRAP processing in sporozoites, whereas TLCK is very effective. This finding raises the interesting hypothesis that the protease mediating AMA-1 and TRAP processing in sporozoites may not be the same as the one mediating AMA-1 and merozoite surface protein 1 processing in merozoites. Thus, stage-specific regulation of protein processing may occur in *P. falciparum* through the expression and/or activity of distinct proteases during the parasite life cycle.

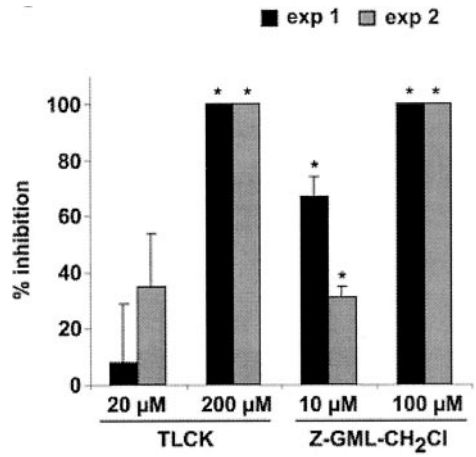

FIG. 7. **Serine protease inhibitors TLCK and Z-GML-CH_2Cl inhibit *P. falciparum* sporozoite infectivity.** Human hepatocytes were inoculated with *P. falciparum* NF54 sporozoites pretreated with the serine protease inhibitors TLCK and Z-GML-CH_2Cl. For each inhibitor, the results of two independent experiments are shown and are expressed as the mean percentages of inhibition of invasion ± S.D. *, $p < 0.05$, as determined using the one-way analysis of variance followed by the Tukey multiple comparison test.

Treatment of *P. falciparum* sporozoites with the inhibitors TLCK and Z-GML-CH_2Cl, both of which irreversibly block AMA-1 and TRAP processing, inhibits the gliding motility of sporozoites and, more importantly, their ability to invade human hepatocytes. These results suggest that proteolytic processing of sporozoite proteins is essential for sporozoite motility and infectivity but do not allow us to conclude that the loss of sporozoite motility and infectivity results solely from inhibition of TRAP and/or AMA-1 processing. It has been proposed that posterior translocation of sporozoite TRAP provides the force for gliding and host cell invasion via interaction with the parasite cytoskeleton (2). This critical role of TRAP has been demonstrated using gene knock-out (34) or amino acid substitution experiments, which have notably suggested that the TRAP cytoplasmic tail mediates anterior to posterior redistribution and posterior shedding of the protein, both functions crucial for sporozoite gliding motility and host cell invasion (33). Our inhibition results are consistent with this proposed critical role of TRAP. Unlike TRAP, which is not expressed during erythrocytic stages, AMA-1 plays a central role during *Plasmodium* erythrocytic multiplication, so that *ama-1* gene knock-out is not possible (7). Alternative strategies will be required, including conditional gene inactivation, to confirm that AMA-1 is required during sporozoite invasion of hepatocytes. Further studies will be needed to identify and characterize the sporozoite proteases involved in AMA-1 and TRAP processing. Even though other processing events may be affected by TLCK and Z-GML-CH_2Cl, our results nevertheless demonstrate that interfering with proteolytic processing inhibits sporozoite infectivity and suggest that sporozoite serine proteases may constitute potential drug targets for preventive anti-malarial strategies.

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