A single malaria merozoite serine protease mediates shedding of multiple surface proteins by juxtamembrane cleavage*

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Erythrocyte invasion by the malaria merozoite is accompanied by the regulated discharge of apically located secretory organelles called micronemes. Plasmodium falciparum apical membrane antigen-1 (PfAMA-1), which plays an indispensable role in invasion, translocates from micronemes onto the parasite surface and is proteolytically shed in a soluble form during invasion. We have previously proposed, on the basis of incomplete mass spectrometric mapping data, that PfAMA-1 shedding results from cleavage at two alternative positions. We now show conclusively that the PfAMA-1 ectodomain is shed from the merozoite solely as a result of cleavage at a single site, just 29 residues away from the predicted transmembrane spanning sequence. Remarkably, this cleavage is mediated by the same membrane-bound parasite serine protease as that responsible for shedding of the merozoite surface protein-1 (MSP-1) complex, an abundant, glycosyl phosphatidylinositol-anchored multiprotein complex. Processing of MSP-1 is essential for invasion. Our results indicate the presence on the merozoite surface of a multifunctional serine sheddase with a broad substrate specificity. We further demonstrate that translocation and shedding of PfAMA-1 is an actin independent process.
INTRODUCTION

Apicomplexan parasites possess specialised zoite stages primarily designed to invade host cells. Most zoites are extracellular only transiently, but this relatively vulnerable stage in the parasite life cycle is widely considered a target for drug or vaccine-based interventions aimed at controlling parasite replication. Invasion is accompanied by dramatic proteolytic modification of zoite surface proteins. These proteins broadly divide into two classes on the basis of their trafficking pathway. Resident surface proteins are sorted directly to the plasma membrane of the intracellular developing zoite and are often characterised by the presence of a glycosyl phosphatidylinositol (GPI)\(^1\) membrane anchor, or by being peripherally associated with a GPI-anchored protein. A well-studied example of this class is merozoite surface protein-1 (MSP-1). Synthesised as a large precursor protein, it exists on the free *Plasmodium falciparum* merozoite as a complex of four proteolytic fragments, additionally associated with protein products of two distinct genes (1). The entire complex is held on the parasite surface via interactions with a single, GPI-anchored polypeptide derived from the C-terminal end of the MSP-1 precursor. At erythrocyte invasion this is proteolytically cleaved in a process called secondary processing (reviewed in ref. 2) to form two products, MSP-1\(_{33}\) and MSP-1\(_{19}\). MSP-1\(_{33}\) is shed quantitatively, still bound to the rest of the complex, whilst MSP-1\(_{19}\), which consists of two epidermal growth factor (EGF)-like domains, remains on the invading parasite surface. The protease responsible is a membrane-bound calcium-dependent merozoite serine protease. MSP-1 is a conserved Plasmodium molecule, and the processing step is similarly conserved. Upon successful invasion it always goes to completion. When processing is inhibited invasion cannot occur, making it a potential target for protease inhibitor-based drug development.

Invasion by apicomplexan zoites also involves regulated release of secretory
organelles within the anterior domain of the parasite, called rhoptries, micronemes and dense granules. Several microneme proteins translocate onto the parasite surface where they form a second class of induced or transiently expressed surface proteins. They are thought to act in many cases as adhesive ligands promoting the interaction between parasite and target host cell (3,4). There is accumulating evidence that for productive invasion, these adhesins must eventually be released from the parasite surface by regulated proteolysis (e.g. 5). Apical membrane antigen-1 (AMA-1) was first identified as a target of monoclonal antibodies (mAb) which prevent erythrocyte invasion by merozoites of the simian malaria parasite *P. knowlesi* (6). Homologues of AMA-1 are present in all species of *Plasmodium* examined, as well as in other apicomplexans. Unsuccessful attempts to disrupt the gene in *Plasmodium* blood-stages (7) and *Toxoplasma gondii* asexual stages (8) indicate that it has an essential function in this part of the parasite life cycle. Replacement of the *P. falciparum ama-1* gene with the divergent *P. chabaudi* homologue produced transgenic parasites which invaded mouse erythrocytes more efficiently than wild type *P. falciparum* (7). These observations, together with the now numerous reports of *in vivo* protection or inhibition of host cell invasion by antibodies (e.g. 9) or by peptides (10) which bind to the AMA-1 ectodomain, implicate AMA-1 in invasion. All *Plasmodium* AMA-1 sequences contain sixteen completely conserved cysteine residues (Fig. 1), most of which are also positionally conserved in the *T. gondii* AMA-1 (11). The disulfide bonding pattern between these cysteines (12) and limited structural analysis (13) suggests that the AMA-1 ectodomain contains three domains, each stabilised by intradomain disulfide bonds. AMA-1 is secreted from micronemes onto the parasite surface and shed in a soluble form following parasite release from the host cell (8,11,14-17). This entire process involves at least two sequential proteolytic processing events. In the case of *P. falciparum* AMA-1 (PfAMA-1), transport to its initial apical location is quickly followed by the loss of an N-terminal pro-sequence, which
extends from the secretory signal cleavage site to residue Ser^{96} (16,17). The truncated protein (but not the full-length precursor), still in possession of its transmembrane (TM) anchoring sequence, is then translocated onto the parasite surface in the form of a 66 kDa species called PfAMA-1_{66}, and is finally quantitatively shed as a result of further proteolysis.

On the basis of incomplete mass spectrometric peptide mapping data, we previously proposed that the shed protein took two forms; one (called PfAMA-1_{48} in reference to its apparent molecular mass of 48 kDa on SDS-PAGE) was concluded to represent the bulk of the ectodomain, being the product of a membrane-proximal cleavage of PfAMA-1_{66}; and a second form called PfAMA-1_{44}, which was thought to represent a product of cleavage of PfAMA-1_{66} at some unidentified point between domains II and III (17).

We now report the results of a detailed extension of this earlier study. Our new data provide a definitive description of proteolytic processing of PfAMA-1, cast new light on the mechanism by which PfAMA-1 is translocated onto the merozoite surface, and reveal a previously unsuspected link between shedding of PfAMA-1 and secondary processing of MSP-1.
EXPERIMENTAL PROCEDURES

**Materials** - Sequencing grade proteases and calpain inhibitors N-acetyl-Leu-Leu-norleucinal (ALLN) and N-acetyl-Leu-Leu-methioninal (ALLM) were from Roche Diagnostics Ltd., U.K. [35S]methionine/cysteine (Pro-mix™) was from Amersham Biosciences, U.K. 18O water was from Isotec (CK Gas Products Ltd., U.K.). The metalloprotease inhibitor TAPI-2 was from BIOMOL (PA, USA). 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was from Pentapharm Ltd. (Basel, Switzerland). Cytochalasin D and B, phenylmethylsulfonyl fluoride (PMSF), 1,3-dichloroisocoumarin (DCI), N-tosyl-L-lysine chloromethylketone (TLCK), N-tosyl-L-phenylalanine chloromethylketone (TPCK), para-hydroxymercuribenzoate (pHMB), and all other protease inhibitors were from Sigma.

A proprietary combinatorial library of 2,672 random drug-like compounds was a kind gift of Evotec OAI (Abingdon, Oxford, U.K.). Compounds were screened for inhibition of MSP-1 secondary processing at a concentration of 100 µM, using the Western blot-based assay described previously (18). This identified two compounds, 239f and 2312f, which inhibit both MSP-1 processing and erythrocyte invasion in vitro with IC50 values in the low micromolar range (unpublished data).

Synthesis of the peptidyl chloromethylketone N-benzyloxycarbonyl-Gly-Met-Leu-CH2Cl (Z-GML-CH2Cl) was by the mixed anhydride method (19); complete details of the synthesis and characterization will be provided elsewhere. Design of this compound was based on the sequence flanking the secondary processing site within the *P. falciparum* MSP-1 (20). The compound inhibits both MSP-1 secondary processing and erythrocyte invasion with an IC50 value of approximately 30 µM. It is also a potent inhibitor of subtilisin...
BPN’ and chymotrypsin, but does not significantly inhibit elastase, trypsin or the \textit{P. falciparum} subtilisin-like protease PfSUB-1 (21) at the highest concentrations tested (500 \mu M; M. Blackman, unpublished data).

\textit{Parasites and antibodies}-Culture and synchronization of \textit{P. falciparum} (clone 3D7) was as previously described (18). Routinely, parasites were maintained in complete medium containing the serum substitute Albumax, though where indicated protein-free medium was employed. Mature schizonts were isolated by centrifugation on cushions of 63\% isotonic Percoll, and either biosynthetically radiolabelled with $[^{35}\text{S}]$methionine/cysteine (22) or used for preparation of naturally-released merozoites for use in processing assays as previously described (18). Purification of mAb 4G2dc1, which recognizes a conformational epitope within the PfAMA-1 ectodomain (23), was as described previously (17). The human mAb X509, which recognizes an epitope within the 3D7 MSP-133, has been described (20). The RAP-1-specific mAb 209.3 was a kind gift of Dr A. Holder (NIMR, U.K.). A mouse antiserum was raised against reduced, alkylated recombinant PfAMA-1 (9) using standard protocols.

Purification and immunoprecipitation of shed PfAMA-1-Shed forms of PfAMA-1 were purified from parasite culture supernatants. Briefly, Percoll-enriched mature schizonts (unlabelled or biosynthetically radiolabelled with $[^{35}\text{S}]$methionine/cysteine) were recultured for up to 11 hours in the presence or absence of fresh erythrocytes to allow merozoite release and reinvasion. Culture supernatants (referred to as schizont rupture supernatants) were then harvested and clarified by filtration through a 0.22 \mu m filter. Immunoprecipitation of radiolabelled material and analysis by SDS-PAGE and fluorography was as described previously (22). Quantitation of radioactivity associated with selected bands on dried gels
was performed with a Storm 860 PhosphorImager, using ImageQuant® version 5.0 software (Molecular Dynamics). All immunoprecipitations used mAb 4G2dc1 coupled to cyanogen bromide activated Sepharose 4B Fast Flow (Amersham Biosciences). An affinity column for larger scale purification was produced as described previously (17). Clarified culture supernatant (up to 3 litres at a time) was applied to the column at a flow rate of 1 ml min⁻¹ at 4°C. The column was washed with 20 mM Tris-HCl, 500 mM NaCl pH 7.6, 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS) in 20 mM Tris-HCl, 150 mM NaCl pH 7.6, and finally with the same buffer without CHAPS. Bound proteins were eluted with 0.2 M glycine-HCl, 0.15 M NaCl, pH 2.7, and further purified by gel filtration on a Superdex 200 HR 10/30 column equilibrated in 20 mM Tris-HCl pH 8.2, 150 mM NaCl. Alternatively, protein eluted from the affinity column was applied to a Vydac 4.6 mm × 15 cm C₄ reversed-phase HPLC (RP-HPLC) column equilibrated in 0.1% (v/v) TFA, and eluted at 1 ml min⁻¹ with a 0→54% (v/v) gradient of acetonitrile in 0.1% (v/v) TFA over 60 min, monitoring elution at 215 nm. Eluate samples were dried under vacuum for further analysis by RP-HPLC, SDS-PAGE, or mass spectrometry.

For re-application of samples to the RP-HPLC column following reduction and alkylation, dried samples from the first round of RP-HPLC purification were taken up in 100 µl 8 M urea in 5 mM Tris-HCl pH 8.2, 50 mM DTT, and incubated at room temperature for 30 min. Iodoacetamide was then added to 100 mM and incubation continued for a further 20 min at 37°C in the dark. Samples were made up to 1 ml with 0.2% (v/v) TFA in water, re-applied to the C₄ RP-HPLC column and eluted using the protocol described above.

Electrospray mass spectrometry (ESI-MS)-RP-HPLC-purified protein was dried under vacuum. Where previously reduced and alkylated, samples were simply taken up
directly into 60% (v/v) acetonitrile, 0.1% (v/v) formic acid and submitted to mass spectrometry. Where not previously reduced, protein was taken up in 6.25% (v/v) acetonitrile in 5 mM NH$_4$HCO$_3$, supplemented with DTT to 50 mM, and incubated for 2.5 h at room temperature to allow complete reduction. Samples were then acidified with 0.1% (v/v) formic acid and analyzed on a Micromass Platform single quadrupole mass spectrometer (Micromass, Altringham, U.K.) as described previously (17).

Proteolytic digestion and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry-Purified PfAMA-144 and PfAMA-148 proteins purified in the absence of prior reduction were solubilised directly into SDS-PAGE sample buffer containing 0.1 M DTT, alkylated with iodoacetamide, subjected to SDS-PAGE then submitted to in-gel proteolytic digestion as described previously (17). Alternatively, dried peaks from RP-HPLC fractionation of reduced and alkylated protein were taken up directly into protease digestion buffer. Digestion with Lys-C was performed in 5 mM NH$_4$HCO$_3$, using the enzyme at 2.5-5.0 ng µl$^{-1}$, whereas digestion with Glu-C was in 5 mM sodium phosphate pH 7.8, using the enzyme at 10 ng µl$^{-1}$. Where required, digestion buffers were prepared using 50% (v/v) $^{18}$O water. After overnight digestion at 32ºC the digestion supernatant was acidified by the addition of TFA to 0.4% (v/v) before analysis on a Reflex-III MALDI-TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) as described previously (17).

Analytical gel filtration chromatography-A Superdex 200 HR 10/30 column equilibrated in 20 mM Tris-HCl pH 8.2, 150 mM NaCl was calibrated with marker proteins ferritin (440 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa),
carbonic anhydrase (29 kDa) and cytochrome c (12.4 kDa). Blue dextran 2000 was used to determine the void volume ($V_0$) of the column. Schizont rupture supernatants were obtained from schizonts cultured for 4 h in protein-free medium. Freshly-harvested supernatants were concentrated twenty-fold by centrifugation in a 12-kDa molecular weight cut-off Vectaspin Micro ultrafiltration unit (Whatman), and 50 µl applied at once to the gel filtration column. The column was eluted at 0.4 ml min$^{-1}$. Fractions of 0.4 ml were collected and assayed for the presence of PfAMA-1-derived fragments by Western blot using the mouse anti-PfAMA-1 antiserum.

Immunofluorescence-Percoll-enriched mature schizonts were recultured for 1 h in fresh medium containing cytochalasin D (final concentration 2 µM, added from a 2 mM stock solution in DMSO) or DMSO only, to allow merozoite release. Residual intact schizonts and released merozoites were then pelleted by centrifugation (6,500 g, 1 min at room temperature). Thin films were air-dried, fixed in ice-cold acetone, and probed sequentially with mAb 4G2dc1, FITC-conjugated sheep anti-mouse IgG (Sigma), and the RAP-1-specific mAb 209.3 which had been directly conjugated to Texas Red as described previously (24). Slides were dipped in a solution of 4,6-diamidino-2-phenylindole (DAPI) in phosphate-buffered saline, washed, and mounted in Citifluor (Citifluor U.K.C Chemical Laboratories, Canterbury, U.K.). The cells were visualized on an integrated DeltaVision system (Applied Precision, WA, USA) including an Olympus IX70 inverted microscope with a 100x objective. Images were captured with a Princeton Instruments MICROMAX air-cooled CCD camera (Trenton, NJ, USA) on a Silicon Graphics Iris workstation, using Softworx image acquisition and deconvolution software.
RESULTS

*The C-terminus of PfAMA-1*$_{44}$ *lies within domain III of PfAMA-1.* In previous attempts to determine the precise site(s) at which PfAMA-1 is cleaved to release it from the merozoite, shed fragments were isolated and subjected to proteolytic digestion in the presence of 50% (v/v) $^{18}$O water. Under these conditions, peptide products of digestion incorporate both $^{18}$O and $^{16}$O at their terminal $\alpha$-carboxy group as a result of serine protease-mediated hydrolysis (25). Such peptides possess a distinctive isotope pattern in MALDI-TOF mass spectrometry and are distinguishable from any peptide derived from the extreme C-terminus of the polypeptide substrate, which should exhibit a normal $^{16}$O isotope spectrum. No peptide of the latter type was evident in $^{18}$O-containing chymotryptic or tryptic digests of PfAMA-1$_{44}$ and PfAMA-1$_{48}$ (17), suggesting that the C-terminal products of digestion by these proteases were too small or not sufficiently basic to be detected by MALDI-TOF.

We extended this approach, using an expanded range of proteolytic enzymes. When affinity-purified PfAMA-1$_{44}$ was digested with endoproteinase Glu-C in the presence of 50% (v/v) $^{18}$O water, MALDI-TOF analysis (not shown) now readily identified a single unlabelled species of $m/z$ 858.52. This can only correspond to the predicted Glu-C peptide with the sequence S$_{458}$KRIKLN$_{464}$ (calculated $m/z$ 858.55). This result was unexpected; the sequence lies just downstream of Cys$_{443}$, the first cysteine of domain III (Fig. 1), and therefore indicated that, rather than lying between domains II and III as previously concluded, the C-terminus of PfAMA-1$_{44}$ actually lies within domain III.

Hodder *et al.* (12) have demonstrated unequivocally that all sixteen cysteine residues
in recombinant *P. chabaudi* AMA-1 are engaged in disulfide bonds, and that the first
cysteine of domain III (Cys\(^{443}\) in the 3D7 PfAMA-1 sequence) is disulfide-bonded to
Cys\(^{502}\) which lies in the middle of a cluster of five cysteines within the C-terminal part of domain
III. An NMR study of recombinant domain III (13) has confirmed this assignment. Assuming
that this disulfide-bond architecture holds true for authentic PfAMA-1, cleavage at Asn\(^{464}\)
could not alone be sufficient to release PfAMA-1\(_{44}\) from the merozoite surface, since the
cleaved fragment would remain attached to the still membrane-bound C-terminal part of
domain III via this single disulfide bond – that is, the cleavage which produces the C-
terminus of PfAMA-1\(_{44}\) cannot be the same as that which releases it from the merozoite.
This new insight forced a re-evaluation of our previous model. We postulated the new
hypothesis that only the membrane-proximal cleavage is responsible for releasing PfAMA-1
from the merozoite membrane, and that the shed protein likely takes two forms; PfAMA-
1\(_{48}\), comprising a single polypeptide extending from Ile\(^{97}\) up to this membrane-proximal
cleavage site; and a second species which is identical to PfAMA-1\(_{48}\) except for the
presence of an internal “nick” within domain III, at Asn\(^{464}\). This “nick” would produce
PfAMA-1\(_{44}\), still bound to the remainder of domain III via the single disulfide bond (Fig. 1).
Subsequent experiments focused on testing this hypothesis.

*PfAMA-1\(_{44}\) is disulfide-bonded to a short C-terminal polypeptide fragment with a
C-terminus identical to that of PfAMA-1\(_{48}\).* SDS-PAGE of immunoprecipitated PfAMA-1\(_{44}\)
and PfAMA-1\(_{48}\) showed that the difference in molecular mass between the two species is
reduction dependent (Fig. 2A); in the absence of DTT the two proteins co-migrate. This is
consistent with PfAMA-144 being disulfide-bound to another species which, together with PfAMA-144, approximates the total mass of PfAMA-148. Under reducing conditions, this peptide would dissociate and migrate at the solvent front on SDS-PAGE and so would not be readily detectable.

To identify it, and to better analyze the structure of the shed species, larger amounts of protein were isolated on a mAb 4G2dc1 affinity column (Fig. 2B, left-hand lane), then further purified by gel filtration or RP-HPLC. The PfAMA-144 and PfAMA-148 species co-migrated precisely on both the gel filtration column (Fig. 2C) and on the RP-HPLC column (Fig. 2D), where the elution profile was characterized by a single major peak with a retention time of 43.5 min. A sample of the gel filtration peak (fraction 39 in Fig. 2C) was reduced and analyzed directly by electrospray ionization mass spectrometry (ESI-MS). Only two species were detectable (Fig. 3), one of molecular mass 48,336.85 ± 5.17 Da, probably representing PfAMA-148 (the major component of the mixture on SDS-PAGE), and the second of molecular mass 6,040.97 ± 1.19 Da. Identical results were obtained upon ESI-MS analysis of the RP-HPLC peak (not shown). Comparison of the high molecular mass signal with the predicted 3D7 PfAMA-1 sequence suggests that, if it corresponds to PfAMA-148, the species extends from its previously determined N-terminal residue of Ile97 through to Thr517 (the calculated mass of this sequence is 48,341.75 Da). The lower mass signal corresponds closely to the predicted mass of Asp465-Thr517 of the PfAMA-1 sequence (predicted m/z of the reduced, non-alkylated sequence is 6,038.87), suggesting that this is the sequence of the peptide hypothesized to be disulfide-bonded to PfAMA-144. PfAMA-144 was not detected, probably due to the fact that this is always present at substantially lower levels than PfAMA-148, as noted previously (17), and its charge envelope was likely
obscured by that of the more abundant species. This issue is returned to below.

A further sample of the RP-HPLC-purified PfAMA-144 and PfAMA-148 mixture was taken up in 8 M urea, reduced and alkylated to dissociate any disulfide-dependent interactions, then re-applied to the C4 RP-HPLC column. Three peaks were now evident, with retention times of 31.6, 43.6 and 45.5 min (Fig. 4A). SDS-PAGE of the major peak (not shown) demonstrated that it contained both PfAMA-144 and PfAMA-148, presumably in fully alkylated form. The minor 43.6 min peak was also found on SDS-PAGE (not shown) to contain both PfAMA-144 and PfAMA-148, probably representing residual unmodified material. The 31.6 min peak was examined directly by ESI-MS. A single, strong signal of molecular mass 6,324.91 ± 0.48 Da was detected, which corresponds closely to the predicted mass (6,325.2 Da) of the carbamidomethylated form of Asp465-Thr517 of the 3D7 PfAMA-1 sequence. A sample of this material was digested with Lys-C in 50% (v/v) 18O water and analyzed by MALDI-TOF (Fig. 4B). A strong but simple spectrum was obtained, comprising only seven major ions. All derived from sequence between Asp465-Thr517 of PfAMA-1 (see Fig. 1). Most significantly, only one ion of m/z 1119.496, corresponding to 509CVERRAEVT517 (the predicted m/z of the carbamidomethylated form of this peptide is 1119.558) lacked an 18O component to its spectrum, showing conclusively that this peptide was derived from the C-terminus of the fragment. Post source decay fragmentation of this peptide in the MALDI-TOF mass spectrometer (Fig. 4C) confirmed its identity beyond doubt.

The primary structure of PfAMA-144 and PfAMA-148 was finally confirmed by comparative MALDI-TOF analysis of Lys-C digests of SDS-PAGE purified, reduced and alkylated protein, performed in the presence of 18O water (Fig. 5). Each spectrum contained just a single species that was not labeled with 18O, and in each case this was absent from
the other spectrum. In the PfAMA-144 digest, the unlabelled ion at m/z 643.470 corresponds to 460RIKLN464 (calculated m/z 643.426), and is consistent with the Glu-C data discussed above in assigning the C-terminal residue of PfAMA-144 as Asn464. In the case of the PfAMA-148 digest, the unlabelled ion at m/z 1119.511 again corresponds to 509CVERRAEVT517, demonstrating unequivocally that the C-terminus of PfAMA-148 is identical to that of the small polypeptide associated with PfAMA-144.

PfAMA-148 and PfAMA-144 are shed in a monomeric form and at a constant molar ratio of about 2:1-The above data support the model proposed above, but do not give any information as to the higher order status of the shed fragments. The T. gondii microneme protein TgMIC2 is shed tightly bound to a distinct protein called TgM2AP (26), and several Plasmodium rhoptry proteins form multimeric complexes (e.g. 27). If the same were true of PfAMA-1, such noncovalent interactions could be sensitive to the conditions to which the protein was exposed during purification (e.g. low pH) and so might not be evident using these procedures. We explored this possibility by analytical gel filtration chromatography of material which had not been exposed to any potentially dissociating conditions. Freshly-harvested schizont rupture supernatants were concentrated and applied directly to a calibrated Superdex column and the position of elution of the PfAMA-1 fragments established by Western blot analysis. Fig. 6 shows that PfAMA-144 and PfAMA-148 co-eluted from the column at an elution volume consistent with a mass of ~46.5 kDa. This is close to the mobility of the proteins on non-reducing SDS-PAGE (Fig. 2A), and indicates that both forms of the shed protein – both PfAMA-148, and PfAMA-144 linked to its cognate C-terminal peptide – exist substantially or exclusively as monomers in solution and are not
released as part of a complex with any other proteins.

PfAMA-148 is always more abundant than PfAMA-144 in schizont rupture supernatants. To estimate the stoichiometry of the relationship, the radioactivity associated with immunoprecipitated, biosynthetically radiolabelled proteins separated on reducing SDS-PAGE was quantified by PhosphorImager analysis (not shown). In ten independent sets of counts, the mean ratio between the counts associated with the PfAMA-148 and PfAMA-144 species was found to be $2.72 \pm 0.29$. The sequence encompassed by PfAMA-148 (Ile$^{97}$-Thr$^{517}$) contains 10 Met and 16 Cys residues, whereas that encompassed by PfAMA-144 (Ile$^{97}$-Asn$^{464}$) contains 9 Met and 11 Cys residues. Assuming that the specific activities of the $[^{35}S]$methionine and $[^{35}S]$cysteine in Pro-mix are similar, and that both radioisotopes are incorporated with equal efficiency at all positions during biosynthetic labelling, our measurements suggest that the molar ratio between the two species is approximately 2.1:1. Our inability to detect PfAMA-144 in direct ESI-MS analysis of purified PfAMA-144 and PfAMA-148 (Fig. 3) probably reflects this stoichiometry.

Shedding of PfAMA-1 is mediated by a parasite protease which is indistinguishable from that responsible for secondary processing of MSP-1. In initial experiments focused on the provenance and nature of the protease responsible for cleaving PfAMA-1 from the merozoite surface, a suspension of metabolically radiolabelled mature schizonts (~85% parasitaemia) was dispensed into 24-well plates and supplemented with increasing amounts of fresh erythrocytes such that the final ratio of host cells to schizonts in individual wells varied from approximately
0.15:1 to 1000:1. Following a period of culture to allow merozoite release, supernatants were analyzed by immunoprecipitation. The total number of erythrocytes present had no detectable influence on the degree of PfAMA-1 shedding (not shown). In further experiments, the extent or specificity of shedding was found to be unaffected by the presence or absence of human serum or the serum substitute Albumax (not shown). Our data are consistent with shedding being mediated by a parasite protease.

To characterize this activity, we examined the sensitivity of PfAMA-1 processing in culture to a range of protease inhibitors or cytochalasin B and D, inhibitors of actin polymerisation. As shown in Fig. 7A, most of the compounds under test did not discernibly affect shedding of the PfAMA-144 and PfAMA-148 species. Shedding was slightly reduced in the presence of ALLN and ALLM, and more substantially inhibited in the presence of TLCK, TPCK and the serine protease inhibitor AEBSF, suggesting that these compounds directly affect PfAMA-1 processing. However, microscopic examination of these cultures following the incubation step revealed that the observed inhibition was likely due to a direct effect on schizont rupture, since in all these cases the cultures contained a significant number of morphologically abnormal, residual unruptured schizonts as compared to control cultures (not shown). PfAMA-1 shedding was unaffected by the presence of 2 µM cytochalasin B or D (note that preliminary experiments showed that invasion is completely blocked at 2 µM of either cytochalasin). In the presence of chymostatin, or the chelating agents EDTA and EGTA, schizont rupture occurred normally; here though, not only was shedding of PfAMA-144 and PfAMA-148 reduced, but much of the shed protein took the form of a higher molecular mass species of ~52 kDa, indicating aberrant processing. To further explore this, we examined the effect of EGTA in combination with additional calcium.
Fig. 7B lane 4 shows that merozoite release into culture medium supplemented with EGTA plus equimolar levels of CaCl₂ resulted in the normal pattern of PfAMA-1 processing and shedding. In contrast, supplementing harvested supernatants with CaCl₂ following the period of culture and merozoite release into medium containing EGTA, did not reverse the effect of EGTA (Fig. 7B lane 5). These results indicate that the observed effect of EGTA was directly due to its chelating activity, and show that, once shed, the ~52 kDa PfAMA-1 fragment produced in the presence of EGTA cannot subsequently be converted to PfAMA-1₄₄ and PfAMA-1₄₈ by the addition of excess calcium. This is consistent with the protease mediating formation of these latter species being active only at the merozoite surface. Inhibiting its activity still allowed PfAMA-1 to be shed, albeit less efficiently, as a result of an alternative cleavage at a different site. To identify this site, large amounts of the ~52 kDa form were purified from schizont rupture supernatants containing EGTA (Fig. 2B, right-hand lane), then analyzed by in-gel digestion and MALDI-TOF, or ESI-MS. Despite extensive efforts, it was not possible to establish the mass or C-terminus of this species (not shown).

To better characterize the protease which releases PfAMA-1 from the merozoite surface we turned to an assay originally developed for studying processing of MSP-1 (18,20). Purified merozoites in a calcium-containing buffer were incubated at 37°C in the presence of a range of protease inhibitors. PfAMA-1 shedding was then assessed by Western blot analysis of the merozoite supernatants. Fig. 7C (upper panel) shows that only a subset of inhibitors - the sulfhydryl-reactive compound pHMB, the serine protease inhibitors PMSF and DCI, and the chelating agent EDTA - had any effect on PfAMA-1 shedding. Interestingly, these reagents have been previously shown to inhibit secondary processing of MSP-1 (18,20). Reprobing the same blots with mAb X509, which recognizes the shed MSP-1₃₃ product of MSP-1 secondary processing, confirmed this (Fig. 7C lower
particularly striking was the observation that the relative sensitivity of shedding of both MSP-1 and AMA-1 to the irreversible serine protease inhibitors PMSF, DCI and AEBSF was similar; at the concentrations used, PMSF potently inhibited both processes, DCI had an intermediate effect, whilst AEBSF was completely ineffective. This led us to explore the effects on processing of a number of other more selective inhibitors (Fig. 7D). These included a peptidyl chloromethylketone, Z-GML-CH2Cl, which was designed and synthesized to be an inhibitor of MSP-1 secondary processing, and two compounds, 239f and 2312f, selected from a large compound library on the basis of their inhibition of MSP-1 processing. In every case, shedding of PfAMA-1 and shedding of MSP-1 was equally susceptible to the compounds under test. Collectively, our results strongly suggest that shedding of both proteins is mediated by the same protease.

Translocation of PfAMA-1 is not sensitive to cytochalasin D-Cytochalasin blocks invasion by malarial merozoites, arresting it at the point of tight junction formation (28,29) and supporting the widely held view of a role for an actinomyosin motor in propelling the parasite into the host cell. Cytochalasin also impairs anterior-to-posterior translocation of a number of microneme proteins (e.g. 30,31). The absence of any effect of cytochalasin on shedding of PfAMA-1 in the experiments described above led us to explore its effects on mobilization of the protein onto the merozoite surface. Schizonts were cultured in medium containing invasion-inhibitory concentrations of cytochalasin D for 1 h to allow merozoite release. Merozoites released over this period were then acetone-fixed and examined by IFA using mAb 4G2dc1 to determine the sub-cellular localization of PfAMA-1. As shown in Fig. 8, two distinct patterns of distribution of PfAMA-1 on individual merozoites were observed, consisting of either a solely apical staining pattern with the entire fluorescence signal concentrated in a discrete zone (sometimes appearing as two punctate dots) just forward of
the rhoptries, or alternatively an exclusively circumferential pattern consistent with a merozoite surface location. Merozoites exhibiting both patterns were present in the same thin films. However, the presence of cytochalasin D during merozoite release had no detectable effect on the proportion of free merozoites (~90% in all cases) that exhibited a circumferential pattern of mAb 4G2dc1 reactivity. Our results indicate that PfAMA-1 translocation onto the merozoite surface is not dependent upon an actinomyosin-based mechanism.
DISCUSSION

We have presented incontrovertible evidence that shedding of PfAMA-1 from the merozoite surface is mediated exclusively by proteolytic cleavage at a single site, Thr$^{517}$, just 29 residues N-terminal to the start of the predicted TM sequence (Fig. 9). A constant proportion (approximately one third) of the shed molecules also contain an internal proteolytic cleavage at Asn$^{464}$ within domain III, resulting in the formation of PfAMA-1$_{44}$, which remains bound to its cognate C-terminal fragment of domain III via a single disulfide bond. The reproducibility of this is striking, but its biological significance is unclear. It is also unclear whether both cleavage events are mediated by the same protease; certainly, there is no great sequence similarity surrounding the two cleavage sites. However as we have previously shown (17), once shed there is no further conversion of PfAMA-1$_{48}$ to PfAMA-1$_{44}$, indicating that – like the juxtamembrane cleavage - the cleavage within domain III which produces PfAMA-1$_{44}$ takes place exclusively at the parasite surface.

PfAMA-1 shedding is sensitive to precisely the same subset of serine protease inhibitors and chelating agents previously shown to inhibit secondary processing and shedding of the GPI-anchored surface molecule MSP-1 and its associated complex. Furthermore, the activity is equally sensitive to pHMB and a peptidyl chloromethylketone designed to target the MSP-1 processing protease, as well as to two compounds selected from a screen of a large, structurally diverse compound library. It seems most unlikely that shedding of the MSP-1 complex and PfAMA-1 are mediated by distinct merozoite surface proteases with indistinguishable physiochemical characteristics; much more likely is the presence at the merozoite surface of a single enzyme that is responsible for shedding of both. The most obvious argument against this is the lack of any obvious similarity between
the MSP-1 secondary processing site and that flanking Thr$^{517}$ in PfAMA-1, and the quite different anchoring structures of the two proteins. However, numerous studies on mammalian cell surface proteins that are subject to the action of 'sheddases' (usually but not always zinc metalloproteases) indicate that substrate recognition is not dependent primarily on the amino acid sequence flanking the juxtamembrane cleavage site; more important is thought to be the presence of an 8-10 residue-long stretch of conformationally unrestrained peptide surrounding the target bond, and its distance from the membrane (e.g. 32). Our data clearly implicate a serine protease and not a metalloprotease in shedding of PfAMA-1 and MSP-1, but the distance from the membrane at which PfAMA-1 is cleaved - 29 residues – is typical of sheddases. MSP-1 secondary processing occurs at a site approximately 96 residues upstream of the GPI anchoring sequence; however, as has been pointed out previously (2,33) the unusual U-shaped structure of the tandem EGF-like motif which comprises MSP-119 means that the secondary cleavage site probably lies in very close proximity to the GPI anchor, and is therefore ideally situated for interaction with a membrane-bound protease. Despite considerable sequence diversity within the MSP-119 sequence across Plasmodium species, its overall fold is conserved (34-36). It is also functionally conserved; gene targeting studies have shown that the P. falciparum MSP-119 sequence can be replaced with the divergent P. chabaudi sequence without any effects on invasion or secondary processing (37). It is conceivable that its compact structure serves mainly to orient the cleavage site at an appropriate distance from the membrane to allow protease binding. Interestingly, Brossier et al. (5) recently showed that proteolytic shedding of the T. gondii adhesin TgMIC2 is sensitive to mutations in a basic residue which lies 11 positions N-terminal to the TM sequence. This residue is conserved in a number of microneme proteins, leading the authors to suggest that the protease responsible, called
MPP1 (38) may mediate shedding of several microneme proteins. Our data from *P. falciparum* support this concept, and additionally raise the exciting possibility that resident surface proteins too may be shed by the same enzyme.

Inhibition of normal PfAMA-1 shedding invoked inefficient cleavage at an alternative site, presumably by a distinct protease. The resulting ~52 kDa fragment was larger than that shed under normal conditions, indicating that it resulted from cleavage at a site closer to, or even within, the TM sequence. We were unable to identify this alternative site. A recent report has suggested that some microneme proteins may be shed via cleavage at an intramembrane site (39). Our data indicate the presence of such an activity at the merozoite surface, but it clearly is not responsible for the bulk of PfAMA-1 shedding under physiological conditions.

There is now substantial evidence that movement of microneme components across the surface of apicomplexan zoites is controlled by interactions with a sub-plasmalemmal actinomyosin motor (e.g. 40). Exocytosis *per se* of microneme proteins is not necessarily coupled to their translocation; Bumstead and Tomley (31), for example, showed that cytochalasin D did not reduce secretion of the *E. tenella* tachyzoite protein EtMIC2 in a soluble form, but did prevent its capping over the parasite surface. We found that cytochalasin D at concentrations which completely block erythrocyte invasion affected neither shedding of PfAMA-1 nor its movement onto the merozoite surface. It is conceivable that, given the small dimensions of the *P. falciparum* merozoite, PfAMA-1 may redistribute spontaneously upon secretion by simple diffusion in the merozoite plasma membrane. Alternatively, its translocation may be *via* interactions with other motor proteins (41). Discharge of many microneme proteins is induced only upon contact with the appropriate host cell, and it has been suggested that this may be a mechanism of reducing exposure of these functionally critical molecules to host antibodies (42). Relocalisation of PfAMA-1 upon
merozoite release, in contrast, appears to be relatively unregulated. This may contribute to its accessibility to invasion-inhibitory antibodies.

Fraser et al. (43) have reported that domains I and II – but not the complete ectodomain - of the *P. yoelii* AMA-1 exhibit erythrocyte binding activity, leading these authors to suggest that a truncated form of AMA-1 may play an adhesive role during invasion. Our findings are difficult to reconcile with this model, showing as they do that, under physiological conditions either on the merozoite surface or in solution, authentic PfAMA-1 never exists in a form which lacks domain III. We have consistently failed to demonstrate that the shed forms of PfAMA-1 possess any erythrocyte binding activity (17). We have also been unable to demonstrate any capacity for a correctly-folded recombinant PfAMA-148 molecule (9) to bind to human erythrocytes, either in solution or when immobilised at high density on plastic or nitrocellulose, or when expressed at the surface of COS-7 cells with its cognate TM and cytoplasmic regions (C. Collins and M. Blackman, unpublished). Our gel filtration and immunoprecipitation data unambiguously demonstrate that PfAMA-1 is shed in a monomeric form, uncomplexed with any distinct proteins. This suggests that - whatever the function of PfAMA-1 - it does not involve high affinity interactions with other malarial proteins. However, we cannot rule out the possibility that the protein may exist in a multimeric form or be engaged in interactions with other merozoite proteins, only whilst occupying the merozoite surface, and that these interactions might be disrupted upon shedding.

In summary, we have demonstrated the presence at the merozoite surface of a serine protease activity capable of mediating the shedding of at least two distinct, functionally indispensable proteins by cleaving them at juxtamembrane sites which share no obvious sequence similarity. The molecular identification of this protease may be aided by the recently completed sequence of the *P. falciparum* genome (44), which has demonstrated
that the genome encodes no serine proteases of the chymotrypsin-like superfamily, and contains only three subtilisin-like serine protease genes (unpublished observations, M. Blackman). PfAMA-1 is of great interest as a potential malaria vaccine candidate. Nair et al. (13) have reported that antibodies against domain III can prevent erythrocyte invasion in vitro. It is conceivable that, as in the case of antibodies against MSP-119 (45), these antibodies may function by inhibiting the processing and shedding of PfAMA-1.

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**FOOTNOTES**

1The abbreviations used are: AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ALLN, N-acetyl-Leu-Leu-norleucinal; ALLM, N-acetyl-Leu-Leu-methioninal; CHAPS, 3-[[3-cholamidopropyl]-dimethylammonio]-1-propane-sulfonate; DAPI, 4,6-diamidino-2-phenylindole; DCI, 3,4-dichloroisocoumarin; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; GPI, glycosyl phosphatidylinositol; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MSP-1, merozoite surface protein-1; PAGE, polyacrylamide gel electrophoresis; PfAMA-1, *P. falciparum* apical merozoite antigen-1; pHMB, para-hydroxymercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; RP-HPLC, reversed-phase high pressure liquid chromatography; TLCK, N-tosyl-L-lysine chloromethylketone; TM, transmembrane; TPCK, N-tosyl-L-phenylalanine chloromethylketone; Z-GML-CH₂Cl, N-benzyloxycarbonyl-Gly-Met-Leu-chloromethylketone.
2 I. Wells and M. Blackman, manuscript in preparation.
FIGURE LEGENDS

FIG. 1. Primary structure and processing of PfAMA-1. A, PfAMA-1 contains sixteen conserved cysteine residues. The disulfide bond connectivities shown are based on the assignments of Hodder et al. (12) and Nair et al. (13), and suggest that the ectodomain comprises three independently folded domains. The downward arrow indicates the position of the C-terminus of PfAMA-1_{144} (this work), whereas the upward arrow indicates the C-terminus of PfAMA-1_{148}, i.e. the membrane-proximal site where PfAMA-1 is cleaved to completely release it from the merozoite surface (this work). Secretory signal and predicted TM domains are shown in dark grey, and the pro-sequence region is hatched. B, primary sequence of 3D7 PfAMA-1. Sequence encompassed by PfAMA-1_{166} (17) is shaded, the first Cys residue of domain III is double underlined, and the Glu-C peptide SKRIKLN forming the C-terminus of PfAMA-1_{144} (this work) is boxed. Positions indicated by arrows are as in A, and the sequence of the small polypeptide which is shed disulfide-bound to PfAMA-1_{144} lies between the arrows. Secretory signal and predicted TM sequences are underlined.
FIG. 2. Reduction-sensitivity and purification of shed forms of PfAMA-1. A, PfAMA-148 and PfAMA-144 shed following rupture of biosynthetically radiolabelled schizonts was immunoprecipitated with mAb 4G2dc1 and subjected to SDS-PAGE in the presence or absence of 0.1 M DTT. Reduction intermediates at the boundaries between lanes with and without reducing agent are visible. Positions of molecular mass marker proteins are indicated. B, Coomassie-stained SDS PAGE gel (run under reducing conditions) showing proteins eluted from the mAb 4G2dc1 affinity column following application of supernatants from schizonts allowed to undergo rupture in normal medium (left-hand lane) or medium containing 10 mM EGTA (right-hand lane). The ~52 kDa PfAMA-1 fragment predominantly shed in the presence of EGTA is indicated (asterisk). Note that this species is also evident at low abundance in the left-hand track. C, protein eluted from the affinity column was chromatographed under non-denaturing, non-reducing conditions on a Superdex HR 10/30 gel filtration column. Shown is a silver-stained gel of eluate fractions 34-42. PfAMA-144 and PfAMA-148 co-eluted from the column in fractions 38-40. D, alternatively, protein eluted from the affinity column was applied directly to a C4 RP-HPLC column under non-reducing conditions. Eluate fractions were collected at 1 min intervals The inset shows SDS-PAGE of fractions 43-45, stained with Coomassie blue. PfAMA-144 and PfAMA-148 co-eluted from the RP-HPLC column in fraction 44.

FIG. 3. ESI mass spectra of shed PfAMA-1 fragments. Proteins were purified by affinity chromatography and gel filtration (fraction 39 from Fig. 2C), then reduced with DTT. Two distinct charge state populations were evident (A6+-A8+ and B37+-B60+) corresponding to two polypeptide species with the indicated masses. For clarity, some consecutive B peaks
are not labelled.

**FIG. 4. Identification and C-terminal sequence of the small polypeptide disulfide-bonded to PfAMA-144.** A, PfAMA-144 and PfAMA-148 purified by RP-HPLC under non-reducing conditions (the major peak in Fig. 2D) was reduced and alkylated then re-applied to the RP-HPLC column. A novel 31.6 min peak (arrowed) was dried down and digested with Lys-C in 50% (v/v) 18O water. B, complete MALDI-TOF mass spectrum of the Lys-C digest. The most intense ions are labelled with their m/z values, and peaks unambiguously derived from PfAMA-1 are also labelled with their amino acid sequence. Insets show magnified views of the arrowed peaks. Only the peak corresponding to 509CVERRAEVT517 exhibits an unlabeled 16O isotope spectrum. C, post source decay sequencing of the peptide corresponding to 509CVERRAEVT517. The fragmentation spectrum is shown, with prominent b and y ions labelled. The inset shows positions at which fragmentation occurred to produce these ions.

**FIG. 5. The C-terminus of PfAMA-148 is identical to that of the small polypeptide associated with PfAMA-144.** MALDI-TOF mass spectra of Lys-C digests of PfAMA-144 (upper spectrum) and PfAMA-148 (lower spectrum) performed in 50% (v/v) 18O water are shown overlaid. Insets show magnified views of peaks corresponding to peptides 460RIKLN464 (m/z 643.470, predicted m/z 643.426) and 509CVERRAEVT517 (m/z 1119.511, predicted m/z in carbamidomethylated form 1119.558); note that these peaks are unique to the PfAMA-144 and the PfAMA-148 spectra respectively. The peak at m/z 1671.814, corresponding to the domain II peptide 377SAFLPTGAFKADRYK391, is highlighted as a
conspicuous example of a peak which is present in both spectra and displays the $^{18}$O-labelled spectrum typical of an internal peptide.

**FIG. 6.** PfAMA-144 and PfAMA-148 co-migrate as monomers on non-denaturing gel filtration. A, standard curve showing masses and positions of elution of molecular mass marker proteins on a Superdex 200 HR 10/30 column. The column void volume ($V_0$) is indicated, as is the position of elution of fraction 38 (asterisk). B, Western blot of eluate fractions 30-43 (0.4 ml each) collected following gel filtration of concentrated schizont rupture supernatants. The peak of elution of PfAMA-144 and PfAMA-148 was in fraction 38, corresponding to a mass of ~46.5 kDa.

**FIG. 7.** The protease responsible for shedding of PfAMA-1 is indistinguishable from that which mediates secondary processing of MSP-1. A, immunoprecipitation of shed PfAMA-1 fragments from schizont rupture supernatants following culture in the presence of the indicated compounds; supernatants were harvested at once (start) or after 5 h of culture. DMSO (0.1% v/v) and methanol (1% v/v) acted as solvent-only controls. B, shedding of PfAMA-1 upon rupture of biosynthetically radiolabelled schizonts was determined as in A. Culture medium used was supplemented with 10 mM EGTA alone, or additionally with 10 mM CaCl$_2$, which was added either together with the EGTA prior to the 5 h culture period (lane 4), or following the period of culture in EGTA-containing medium (lane 5). The ~52 kDa species shed in the presence of excess chelating agent is arrowed. C, processing of
PfAMA-1 and MSP-1 in isolated merozoites. Shed fragments of PfAMA-1, or the MSP-1 product of secondary processing, were detected in merozoite supernatants by Western blot using the anti-PfAMA-1 antiserum or mAb X509 respectively; supernatants were either harvested at once (start) or after 1 h incubation at 37°C in the presence of the indicated compounds. D, further processing assays, performed as in C. Z-GML-CH₂Cl but not another chloromethylketone, TLCK, inhibits processing of both MSP-1 and PfAMA-1. Similarly, compounds 239f and 2312f but not two structurally related compounds, 247h and 2411e, inhibit processing of both MSP-1 and PfAMA-1. Solvent alone had no effect on either processing activity (not shown). Structures of compounds 239f and 2312f are shown.

**FIG. 8. Cytochalasin D does not prevent translocation of PfAMA-1 onto the merozoite surface.** PfAMA-1 was detected both in an apical location (left-hand panels) or around the circumference of free merozoites (right-hand panels) whether released in the absence (control) or presence of invasion-inhibitory concentrations of cytochalasin D. The relative ratios of the two forms was unaffected by the presence of cytochalasin D. Acetone-fixed merozoites were probed sequentially with mAb 4G2dc1, FITC-conjugated sheep anti-mouse IgG, Texas Red-conjugated mAb 209.3 (which recognises the rhoptry protein RAP-1), and DAPI, which stains merozoite nuclei blue.

**FIG. 9. New model for translocation and proteolytic processing of PfAMA-1.** The schematic is based on the present work and Howell *et al.* (17). Following translocation onto the merozoite surface by an actin-independent mechanism, PfAMA-1₆₆ is shed in two alternative forms; in both cases, release is mediated by a common juxtamembrane cleavage at Thr⁵¹⁷. The intact form of the shed molecule exists as PfAMA-1₄₈. A proportion (~33%) of the shed molecules also contain an additional cleavage at Asn⁴₆⁴ within domain III,
producing PfAMA-144 which remains bound via a single disulfide bond to the small polypeptide comprising the remainder of domain III, and shown in this work to correspond to Asp^{465}-Thr^{517}. Disulfide bonds are represented by thin lines.
A single malaria merozoite serine protease mediates shedding of multiple surface proteins by juxtamembrane cleavage

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