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Sequential Processing of Merozoite Surface Proteins during and after Erythrocyte Invasion by *Plasmodium falciparum*

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***Plasmodium falciparum* causes malaria disease during the asexual blood stages of infection when merozoites invade erythrocytes and replicate. Merozoite surface proteins (MSPs) are proposed to play a role in the initial binding of merozoites to erythrocytes, but precise roles remain undefined. Based on electron microscopy studies of invading *Plasmodium* merozoites, it is proposed that the majority of MSPs are cleaved and shed from the surface during invasion, perhaps to release receptor-ligand interactions. In this study, we demonstrate that there is not universal cleavage of MSPs during invasion. Instead, there is sequential and coordinated cleavage and shedding of proteins, indicating a diversity of roles for surface proteins during and after invasion. While MSP1 and peripheral surface proteins such as MSP3, MSP7, serine repeat antigen 4 (SERA4), and SERA5 are cleaved and shed at the tight junction between the invading merozoite and erythrocyte, the glycosylphosphatidylinositol (GPI)-anchored proteins MSP2 and MSP4 are carried into the erythrocyte without detectable processing. Following invasion, MSP2 rapidly degrades within 10 min, whereas MSP4 is maintained for hours. This suggests that while some proteins that are shed upon invasion may have roles in initial contact steps, others function during invasion and are then rapidly degraded, whereas others are internalized for roles during intraerythrocytic development. Interestingly, anti-MSP2 antibodies did not inhibit invasion and instead were carried into erythrocytes and maintained for approximately 20 h without inhibiting parasite development. These findings provide new insights into the mechanisms of invasion and knowledge to advance the development of new drugs and vaccines against malaria.**

Plasmodium falciparum is the causative agent of the majority of malaria morbidity and mortality worldwide. Pathogenesis results from blood-stage infection, where the merozoite stage of the parasite invades and then replicates in red blood cells (RBCs). Invasion occurs through a series of stepwise mechanisms: initial contact, reorientation, commitment to invasion, tight-junction formation, and invasion driven by an actin-myosin motor (1–5). Initial contact is thought to be mediated by merozoite surface proteins (MSPs) via multiple weak interactions with receptors on the surface of RBCs, but the roles of individual merozoite surface proteins remain largely undefined (6). Following initial contact, commitment to invasion occurs with the reorientation of the parasite to the apical pole, allowing the formation of a tight junction between the merozoite and the RBC surface via interactions between AMA1 and RON2 (3, 7). Invasion into RBCs is then driven by the parasite actin-myosin motor (8), with the tight junction progressing rearwards as the merozoite enters the RBC. Studies of invasion by *Plasmodium knowlesi*, *P. berghei*, *P. gallinaceum*, and *P. falciparum* using electron microscopy (EM) have suggested that the entire surface coat of the merozoite is shed during invasion around the point of the tight junction (4, 9–11). The shedding of surface proteins is thought to be required to disrupt receptor-ligand interactions, allowing invasion to proceed. This phenomenon also occurs in other Apicomplexa parasites, including *Toxoplasma*, *Neospora*, *Eimeria*, and *Cryptosporidium* (12). Based on these studies, it is widely assumed that the majority of, if not all, merozoite surface proteins are cleaved and shed during invasion of the *P. falciparum* merozoite (1, 13, 14). Indeed, to the best of our knowledge, it has not been proposed in the literature that some surface proteins may not be shed and instead may be internalized into RBCs without processing.

This concept of surface coat shedding is supported by studies showing the cleavage and shedding of MSP1 and the associated proteins MSP6 and MSP7 during invasion (15–17) and reports of several other merozoite surface proteins being detected in culture supernatants after invasion, suggesting that they are shed from the surface (17–20). However, due to a lack of methods to study merozoites during invasion, shedding of merozoite surface proteins at the tight junction between the merozoite and RBC has been directly visualized only with MSP1 (3, 21, 22). MSP1 is found on the surface of the merozoite via the C-terminal glycosylphosphatidylinositol (GPI)-anchored MSP1-42 fragment (15–17). During invasion, MSP1-42 is cleaved by a subtilisin protease, PfSUB2, which also cleaves AMA1 (23), releasing the majority of the complex at the point of the tight junction with the RBC (1–5, 22). The remaining MSP1-19 fragment, representing <10% of the protein, is carried into the RBC (6, 15), where it is thought to be involved with formation of the food vacuole in ring-stage para-

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sites (24). Cleavage and shedding of MSP1 are essential, since inhibitors, including antibodies, that block these processes disrupt invasion (12, 15, 25–28). MSP1 is believed to mediate initial contact with RBCs via binding to surface receptor band 3 and possibly heparin-like molecules (29–31). It is possible that the cleavage and shedding of MSP1 during invasion are required to release the receptor-ligand interactions between this protein and the RBC surface. To date, there are no published reports of more global analyses of merozoite surface proteins to assess the loss of the surface coat during invasion.

Merozoite surface proteins have long been considered attractive targets for malaria vaccine development, and several proteins have progressed to phase 1 and 2 clinical trials (32, 33). Merozoite surface proteins also appear to be important targets of acquired human immunity, particularly antibodies (34–38). Therefore, an understanding of their role, localization, and fate during invasion is important in the context of identifying key immune targets and prioritizing candidates for vaccine development and understanding how specific candidates can be targeted by vaccine-induced responses. Furthermore, while it is thought that surface proteins are likely to be involved in initial contact with RBCs, specific receptor-ligand interactions for merozoite surface proteins other than MSP1 have yet to be identified. Characterization of processing and shedding patterns of other merozoite surface proteins during invasion may also inform the roles of these proteins during the invasion process.

Here, we aimed to conduct a broader characterization of surface shedding of merozoite proteins by studying two GPI-anchored proteins, MSP2 and MSP4, and four peripherally associated proteins, MSP3, MSP7, serine repeat antigen 4 (SERA4), and SERA5, during and immediately after invasion. All proteins except SERA4 have been considered malaria vaccine candidates; MSP2 and MSP3 have progressed to phase 2 clinical trials, and SERA5 has progressed to phase 1 clinical trials (39–41). MSP2 is the second most abundant merozoite surface protein in terms of copy number (42), and while a function for MSP2 has not been identified, this protein is refractory to genetic deletion and is thought to be essential (43). MSP2 is highly polymorphic; however, all alleles can be grouped as either 3D7-like or FC27-like based on tandem sequence repeats and flanking nonrepetitive dimorphic sequences, with both alleles sharing N- and C-terminal regions (44–46). Currently, the fate of MSP2 during invasion is unclear, and there is no evidence of MSP2 in culture supernatants (20, 47). MSP4, like MSP1-19, contains an epidermal growth factor (EGF) domain adjacent to the C-terminal GPI anchor (48) that is essential for the conformation and immunogenicity of the protein (49). MSP4 has been tested in multiple vaccine trials in mouse models, with significant efficacy (50–55). MSP4 is refractory to genetic deletion *in vitro* and is assumed to be essential for asexual replication (43), but its role and localization during invasion have not been defined. MSP7 is bound to MSP1 on the merozoite surface and is thought to be shed during invasion along with the majority of MSP1 (16). The binding partners of the peripheral surface proteins MSP3, SERA5, and SERA4 are not established.

New methods to isolate viable *P. falciparum* merozoites provide valuable approaches to imaging of merozoites and important proteins during the process of invasion by immunofluorescence (IF) microscopy (3, 6, 22). Here, we used these approaches to localize a number of merozoite surface proteins during and immediately after invasion and to characterize the cleavage and shedding of these proteins. We aimed to investigate whether the shedding of the merozoite surface coat during invasion involves the specific processing of individual proteins, reflecting the different roles and functions of merozoite surface proteins during invasion rather than shedding being a nonspecific global process resulting in the complete loss of the merozoite surface protein coat. A detailed understanding of invasion is important because merozoite proteins are leading vaccine candidates (32–34), and targeting of invasion may be an effective strategy in antimalarial drug development (6, 56). We show that loss of the merozoite surface coat is not a global event and instead involves specific processes resulting in the sequential cleavage of individual proteins both during and after invasion, indicating the diversity of roles of different proteins.

MATERIALS AND METHODS

Parasite culture and merozoite isolation. *P. falciparum* isolates were cultured as described previously (57, 58), in culture medium of RPMI-HEPES (pH 7.4) supplemented with 50 μg/ml hypoxanthine, 20 μg/ml gentamicin, 25 mM sodium bicarbonate ($NaHCO_3$), and 0.5% Albumax II (Gibco). RBCs from group O^+ blood donors were used to culture parasites. Cultures were gassed with 1% O_2, 4% CO_2, and 95% N_2 and incubated at 37°C. Parasites were initially synchronized by using 5% D-sorbitol treatment for 5 min, as described previously (59). Following sorbitol treatment, cultures were further synchronized by using the invasion-inhibitory properties of heparin (29). Parasite strains 3D7 and D10 were used as indicated. Viable merozoites were isolated as described previously (6, 22). Highly synchronized late-stage schizonts were magnet purified via a MACS magnet separation column (Miltenyi Biotec) and treated with E64 until mature merozoites were formed. Merozoites were isolated by membrane filtration, incubated with uninfected RBCs, and prepared for imaging for IF microscopy or EM as described previously (3, 6, 22).

Immunofluorescence microscopy and electron microscopy. IF microscopy and EM were performed as described previously (3, 6, 22). Cells were fixed with 4% formaldehyde–0.0075% glutaraldehyde coated onto glass slides and labeled with antibodies as indicated. Labeling was detected by an Alexa 594/488-conjugated secondary antibody (Molecular Probes). Slides were mounted in VectaShield (Vector Laboratories) with 0.1 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen) to label the parasite nucleus. Images were obtained by using a Plan-Apochromat (100×/1.40) oil immersion phase-contrast lens (Carl Zeiss) on an AxioVert 200M microscope (Carl Zeiss) equipped with an AxioCam Mrm camera (Carl Zeiss). Images were processed by using Photoshop CS4 (Adobe). In cases where brightness and contrast were changed, processing was applied to whole images and controls equally. For EM, invading merozoites were fixed in 1% glutaraldehyde in RPMI-HEPES on ice for 30 min. Samples were pelleted in low-melt agarose before being transferred into water, dehydrated in ethanol, and embedded in LR Gold resin (ProSciTech). Following polymerization by benzoyl peroxide (SPI-Chem), 100-nm sections were prepared by using an Ultracut R ultramicrotome (Leica). Sections were poststained with saturated aqueous uranyl-acetate and then 5% triple lead and observed at 120 kV on a CM120 BioTWIN transmission electron microscope (Philips).

To test for the internalization of MSP2 antibodies, MSP2 monoclonal antibodies (MAbs) were incubated with merozoites and uninfected RBCs for 10 min. Cultures were washed twice in cold phosphate-buffered saline (PBS) containing complete protease inhibitors and then fixed and processed for IF microscopy. Control labeling was carried on in the absence of primary or secondary antibodies for all antigens. In all cases, laser intensity used to image positive samples showed no fluorescence in controls.

Antibodies. MSP2-3D7 rabbit polyclonal antibodies for N-terminal purification were raised against full-length 3D7 recombinant MSP2. Monoclonal antibodies to MSP2 were generated by vaccination with full-length 3D7 or FC27 MSP2 (60). Rabbit and mouse anti-PfRON4 antibod-

ies (61) and rabbit anti-MSP3 antibodies (62) were kindly provided by Alan Cowman, Walter and Eliza Hall Institute. MSP4 antibodies to different regions and full-length MSP4 were generated as described previously (49). MSP7 polyclonal rabbit antibodies were raised in rabbits (63). Rabbit antibodies to MSP1-19 (64) were kindly provided by Brendan Crabb, Burnet Institute. The SERA4 central domain was prepared in a manner similar to that for the central domain of SERA5 (65, 66), and the *in vitro* refolded protein was used to produce antibodies in rabbits. The cysteine-rich region of the C-terminal fragment of SERA5 (R914 and V997) was oxidatively refolded in a manner similar to that for the SERA5 central domain. The monoclonal antibody used in this study was found to target a disulfide bond-stabilized conformational epitope in this domain fragment (data not shown). Antibodies were generated by immunization of experimental animals with purified recombinant proteins at the Walter and Eliza Hall Institute Antibody Service. Procedures were consistent with the policies of the National Health and Medical Research Council, Australia (83), and studies were approved by the Animal Ethics Committee of the Walter and Eliza Hall Institute, Australia (protocol AEC 2011.009r0).

Purification of MSP2 N-terminal specific antibodies. MSP2 N-terminal specific antibodies were purified from polyclonal 3D7 MSP2 rabbit serum raised by vaccination with full-length 3D7 MSP2 recombinant protein. A peptide corresponding to the first 25 amino acids of the N terminus of MSP2 (conserved between 3D7 and FC27 sequences) and the next 4 additional amino acids of the FC27-specific region (H-IKNESKYSNTFINNAYN MSIRRSMANEGSN-NH_2) was purchased from Mimotopes (90% minimum purity by high-performance liquid chromatography [HPLC]). Peptide was solubilized in 50% dimethyl sulfoxide (DMSO) at 2.75 μg/ml and then diluted to 687.5 μg/ml and coupled to AminoLink Plus column resin overnight at 4°C. Unbound peptide was collected, the column was washed 2 times in PBS, and the coupling reaction was completed with 50 mM $NaCNBH_3$. The column was blocked according to the manufacturer's instructions with quenching buffer and 50 mM $NaCNBH_3$ and then washed extensively with PBS. Polyclonal MSP2 3D7 rabbit serum was incubated on the column overnight. The column was washed 12 times with PBS, and the antibodies were then eluted with 0.1 M glycine (pH 2.6) into tubes containing 100 μl of 1 M Tris (pH 8.8). Eluted antibodies were dialyzed against PBS overnight by using SnakeSkin dialysis tubing (Thermo Scientific) and then concentrated 8-fold through Amicon microspin columns with a molecular mass cutoff of 10,000 kDa. Reactivity of rabbit polyclonal serum before and after purification was tested by an enzyme-linked immunosorbent assay (ELISA) using an MSP2 peptide array.

MSP2 peptide ELISA array. An MSP2 peptide array of 84 biotinylated 13-mer peptides overlapping by 8 amino acids, covering the entire 3D7 and FC27 MSP2 sequence, was purchased from Mimotopes (60). Peptides were solubilized in 80% DMSO to a concentration of 2 to 6 mg/ml. Ninety-six-well Nunc flat-bottom plates were coated overnight with 1 μg/ml streptavidin in PBS. Blocking was performed for 2 h with 1% casein at 37°C, followed by incubation with peptides diluted in PBS to 4 to 12 μg/ml. MSP2 MAbs at a 1:1,000 serum dilution were incubated for 1 h at room temperature, and binding was detected with anti-mouse horseradish peroxidase (HRP)-conjugated antibody (Invitrogen) at a 1:2,500 dilution. Plates were washed 3 times with PBS–0.05% Tween between incubations, the ELISA plate was exposed with 2,2-azinobis(3-ethylbenzthiazolinesulfonic acid) (ABTS) (Sigma-Australia) for 30 min and then stopped with 1% SDS, and the absorbance was read at 405 nm.

Growth inhibition assays and invasion inhibition assays. High-throughput growth inhibition assays (GIAs) were performed as described previously (58, 67–69). Duplicate suspensions of synchronized parasites at 0.2 or 2% parasitemia (one- or two-cycle assays; 32-h trophozoite stage) and 1% hematocrit were incubated with antibodies in 96-well sterile U-bottom plates (Falcon). Plates were incubated as described above for parasite culture for 44 h for one-cycle assays or 72 h for two-cycle assays and analyzed by flow cytometry with staining of parasites with 10 μg/ml ethidium bromide (EtBr) (Bio-Rad) for 1 h in darkness. Parasitemia was measured by using a BD FACSCalibur or BD FACSCantoII flow cytometer. Samples were analyzed by using FlowJo (Tree Star) gating on intact erythrocytes and then determining parasitemia by EtBr-positive erythrocytes. Inhibitory effects of compounds were normalized as percent growth of controls for each assay.

Invasion inhibition assays (IIAs) with isolated merozoites were conducted as described previously (6, 22). Merozoites were isolated from D10 PfPHG (68) and incubated with MSP2 MAbs and uninfected RBCs. Following invasion, cultures were washed twice and returned to culture medium. Parasites were analyzed by flow cytometry at 40 h postinvasion as described above for GIAs, with parasites being gated as EtBr- and green fluorescent protein (GFP)-positive cells. To investigate the ability of MAbs to induce a growth delay, parasites were analyzed for mean fluorescence intensity (MFI) of GFP and EtBr at 30 and 40 h postinvasion (70).

Protein extraction and Western blotting. Synchronized schizont-stage (40 to 48 h), or early-ring-stage (0 to 6 h postinvasion) parasite cultures were used for protein extraction. Uninfected erythrocytes were lysed with 0.15% saponin (Kodak) on ice for 10 min. The remaining pellets were washed three times with cold PBS. Culture supernatant proteins were collected from spent media from synchronized schizont parasite cultures after reinvasion of RBCs following schizont rupture. At all stages of protein extraction, Complete protease inhibitor (Roche) was added at an excess concentration to ensure minimization of nonspecific proteolysis. Proteins were solubilized in nonreducing sample buffer and separated by SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and analyzed by Western blotting on a chemiluminescence system.

RESULTS

Peripherally associated merozoite surface proteins MSP3, MSP7, SERA4, and SERA5 are shed at the tight junction during invasion. To assess the shedding of the merozoite surface coat, we localized both peripherally associated and GPI-anchored proteins during invasion. To assess the shedding of individual merozoite surface proteins, invading *P. falciparum* merozoites were fixed for microscopy and visualized at attachment to the RBC membrane, during the process of invasion, and immediately postinvasion by orientation with the *P. falciparum* rhoptry neck protein PfRON4, which is a marker of the tight junction (3). Using these approaches, we have previously visualized the shedding of the N-terminal regions of MSP1 (3, 22). As a first step, we localized the peripherally associated proteins MSP3, MSP7, SERA4, and SERA5 during invasion (Fig. 1). As expected, these proteins were shed from the surface of merozoites during invasion at the point of tight junction with RBCs. This was seen with positive labeling of merozoites at the initial stages of invasion with polyclonal antibodies specific to these proteins. As invasion progressed, labeling was lost from the region of the merozoite that had already passed through the tight junction into the RBC, indicating the shedding of individual proteins (Fig. 1). The shedding of MSP3, MSP7, SERA4, and SERA5 at the point of tight junction with the RBC is consistent with the presence of these proteins in culture supernatants following invasion.

The GPI-anchored protein MSP2 is carried into invaded RBCs and then rapidly degraded. To localize MSP2 during and after invasion, we used merozoites from 3D7 or FC27 (D10 clone) parasites in order to include the two major allelic forms of MSP2 and labeled invading merozoites with MAbs to distinct regions of MSP2; MAbs were described in detail previously (60). The epitopes recognized by these MAbs were distributed among different regions of MSP2, including the 3D7 allele-specific region (MAbs 111, 9D11, and 2F2), the FC27 allele-specific region (MAb

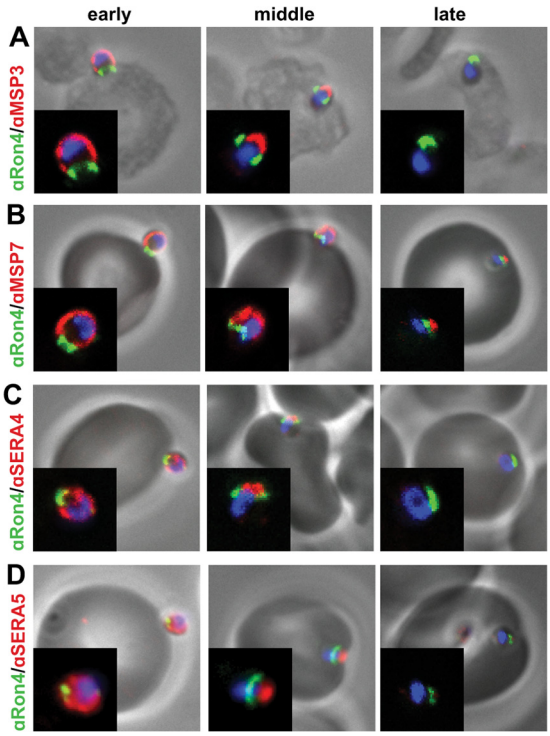

FIG 1 Peripherally associated proteins MSP3, MSP7, SERA4, and SERA5 are shed during invasion. Invading merozoites were labeled with PfRON4 as a marker of the tight junction (green) and colabeled with antibodies directed against MSP3 (A), MSP7 (B), SERA4 (C), or SERA5 (D). All proteins were shed at the point of tight junction, with antibody reactivity to the merozoite surface being observed only on the proportion of the merozoite external to the RBC. All primary antibodies were used at a 1:100 serum dilution, and secondary antibodies were used at a 1:500 dilution.

8G10), and two regions of the conserved C-terminal region (MAbs 6C9, 9H4, 1F7, 9G8, and 4D11) (Fig. 2; see also Table S1 in the supplemental material). The specificity of the MAbs to MSP2 was previously confirmed via Western blotting; all MAbs were specific to MSP2, with no cross-reactivity to other parasite proteins, except for MAb 9G8, which has some cross-reactivity to spectrin (60). 8G10 was reported previously in other studies to be cross-reactive to some extent with other parasite proteins, which have not been identified (71). As we lacked a MAb against the N-terminal region of MSP2 that reacted with the native surface-located antigen by IF microscopy, N-terminally specific antibodies were purified from rabbit polyclonal serum that had been generated by vaccination with full-length 3D7 MSP2. When tested by ELISA using an overlapping peptide array covering the full-length MSP2 protein, the affinity-purified N-terminal polyclonal antibodies reacted only with the N-terminal peptides of MSP2 (see Fig. S1A and S1B in the supplemental material). These antibodies were not cross-reactive with other parasite proteins; on Western blots of parasite lysates, they labeled a single 40-kDa band indicative of MSP2 (see Fig. S1C in the supplemental material).

Labeling of MSP2 during invasion indicated that MSP2 was not cleaved and shed during invasion, in contrast to MSP1 and the peripheral surface proteins described above. Antibodies to N-terminal, central variable, and C-terminal regions of MSP2 labeled the parasite evenly on both sides of the tight junction at early, middle, and late stages of invasion. These striking findings indicate that no part of MSP2 was lost during invasion, suggesting that this protein does not undergo processing during invasion and is instead internalized during invasion (Fig. 3A; see also Fig. S2 in the supplemental material), which was unexpected based on the widely described paradigm of surface protein shedding. The fate of MSP2 in the immediate period following invasion was investigated by fixing newly invaded parasites 10 min after mixing isolated merozoites with RBCs. Parasites were colabeled with antibodies to MSP1-19, which is known to remain on the parasite postinvasion, and labeling with these antibodies was clearly seen in newly invaded parasites, as expected. However, little or no MSP2 labeling of newly invaded parasites was seen by using any of the MSP2 antibodies, suggesting that MSP2 was rapidly lost postinvasion (Fig. 3B). In contrast, the MSP2 antibodies labeled extracellular merozoites in the same IF microscopy preparations, confirming antibody reactivity (for example, see merozoite labeling with N-terminal MSP2 rabbit antibodies in Fig. 3B, top). The loss of MSP2 labeling in newly invaded parasites, despite the labeling of merozoites during invasion and immediately postinvasion, suggests that MSP2 is carried into RBCs but is then rapidly degraded. In a minor proportion of invaded cells, MSP2 antibody fluorescence was seen, but labeling was limited to discrete dots that had no clear indication of colocalization with MSP1-19. This minimal MSP2 labeling observed was above background fluorescence levels, as assessed by labeling with secondary antibodies alone or control antibodies (data not shown). 8G10, which is reactive with the FC27 central variable region, labeled newly invaded rings to a higher degree than other antibodies, but this labeling was still minimal compared to the labeling of merozoites prior to, during, or immediately after invasion. This may indicate that the epitopes recognized by this antibody are more accessible or degraded more slowly than other epitopes, or the labeling seen may reflect the cross-reactivity of this MAb, which was reported previously (71).

MSP4 is carried into invaded RBCs and maintained in early ring forms. The same experimental approach was used to assess the fate of MSP4, another essential GPI-anchored merozoite surface protein, during invasion. To study MSP4, we used rabbit polyclonal antibodies that were raised against different regions of MSP4 with recombinant protein, as described previously (49) (Fig. 2B). The specificity of the antibodies was investigated by using Western blots of 3D7 and D10 schizont protein extracts. MSP4 full-length antibodies were specific for MSP4 in 3D7 parasites reacting only to a single band of approximately 40kDa. This antibody showed some cross-reactivity with a non-MSP4 protein in D10 parasites, as seen with the labeling of a protein at approximately 140 kDa. MSP4A antibodies were specific to MSP4 in D10 parasites but showed some cross-reactivity in 3D7 parasites with an approximately 55-kDa protein. MSP4B and MSP4D antibodies were specific to MSP4 in both 3D7 and D10 parasite protein extracts, while MSP4C antibodies were cross-reactive to non-MSP4 proteins in both parasite strains, as determined by the multiple bands seen in the Western blot (Fig. 2C).

As seen for MSP2, labeling of merozoites with antibodies to full-length MSP4 was clearly seen at all stages of invasion (early, middle, and late), indicating that at least part of MSP4 is carried into RBCs (Fig. 4A). Subsequently, invading D10 merozoites were labeled with antibodies raised against the four regions of MSP4. All antibodies labeled invading merozoites on both the intra- and extracellular surfaces in the mid-invasion stage, indicating that

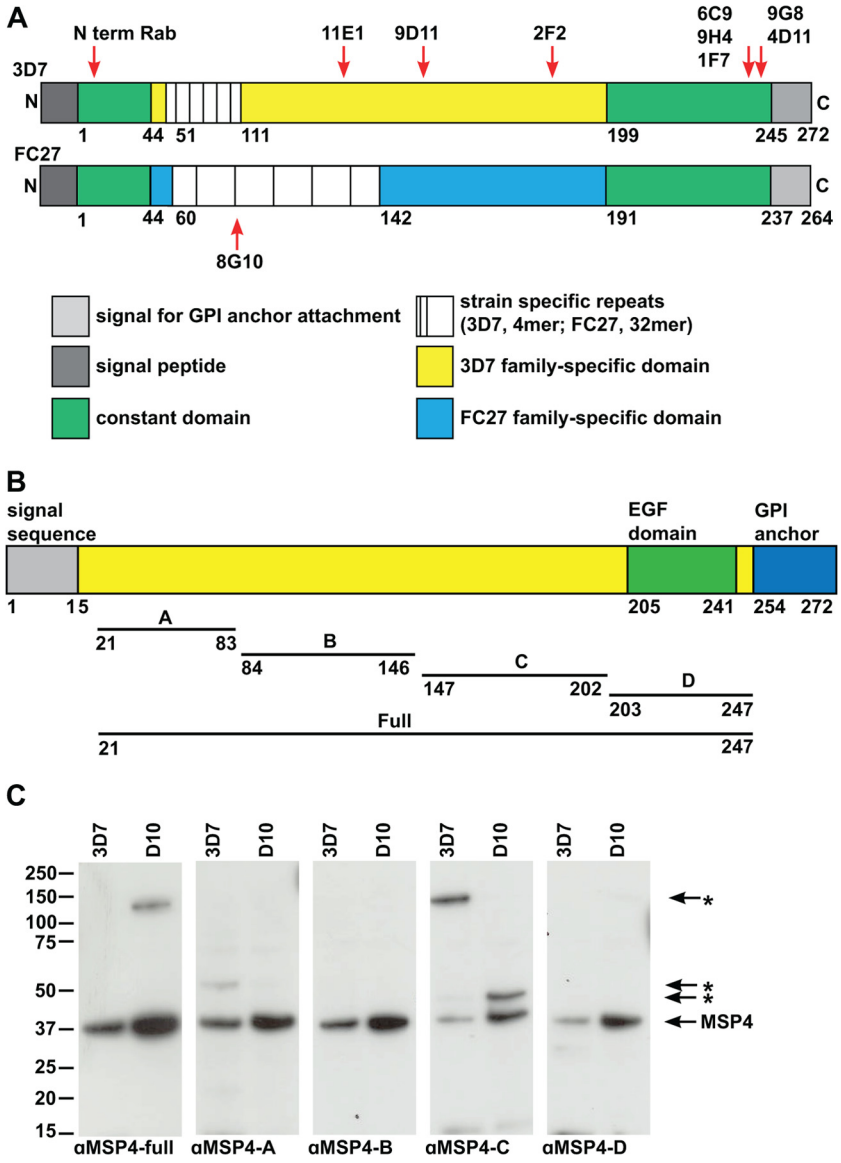

FIG 2 Schematic diagram of MSP2 and MSP4 and analysis of MSP4 antibodies. (A) MSP2 alleles are grouped as 3D7-like or FC27-like based on the allele-specific variable central regions. 3D7 family MSP2 alleles share 4-mer repeat sequences, while FC27 family MSP2 alleles share 12-mer and 32-mer repeats. Both alleles share N-terminally and C-terminally conserved regions. MSP2 is GPI anchored at the C terminus of the protein. Reactivities of MSP2 MAbs used in this study are indicated with red arrows. (B) MSP4 is a C-terminally GPI-anchored protein with a molecular mass of approximately 40 kDa. Adjacent to the C-terminal GPI anchor is an EGF domain (45). Antibodies to MSP4 were raised to the regions indicated. (C) Specificity of antisera was tested in Western blots against 3D7 and D10 schizont extracts. MSP4 is indicated, and cross-reactive bands are shown with asterisks.

N-terminal (anti-MSP4A) and C-terminal (anti-MSP4D) regions of MSP4 were carried into RBCs during invasion, without any detectable cleavage and shedding (Fig. 4B). To investigate the fate of MSP4 postinvasion, 3D7 merozoites were incubated with erythrocytes and fixed after 10 min, 2 h, and 5 h of incubation, and cells were labeled with full-length MSP4 and MSP1-19 antibodies. MSP4 labeling of intraerythrocytic parasites was detected up to 5 h postinvasion, with no clear difference in staining seen between the 10-min and 2- and 5-h time points (Fig. 4C). Indeed, MSP4 appeared to be maintained throughout the life cycle of the parasite, with a 40-kDa band being detected by MSP4 antibodies for ring- and trophozoite-stage parasites via Western blotting (Fig. 5A; see also Fig. S3A in the supplemental material).

Differential cleavage of surface proteins was confirmed by Western blotting and electron microscopy. To confirm the localizations of MSP2 and MSP4 during and after invasion that were observed by microscopy, proteins from late-stage schizonts (44 to 48 h) and ring-stage parasites (0 to 6 h postinvasion) and culture supernatants from 3D7 strain parasites were analyzed by Western blotting. MSP4 antibodies labeled a single band of the same size in both schizont- and ring-stage preparations, which was not present in supernatant fractions, supporting the conclusion that MSP4 does not undergo cleavage or shedding during invasion (Fig. 5A). Furthermore, there was no evidence of smaller processed fragments of MSP4 in the ring or supernatant fractions (see Fig. S3B in the supplemental material). MSP2 antibodies labeled a protein

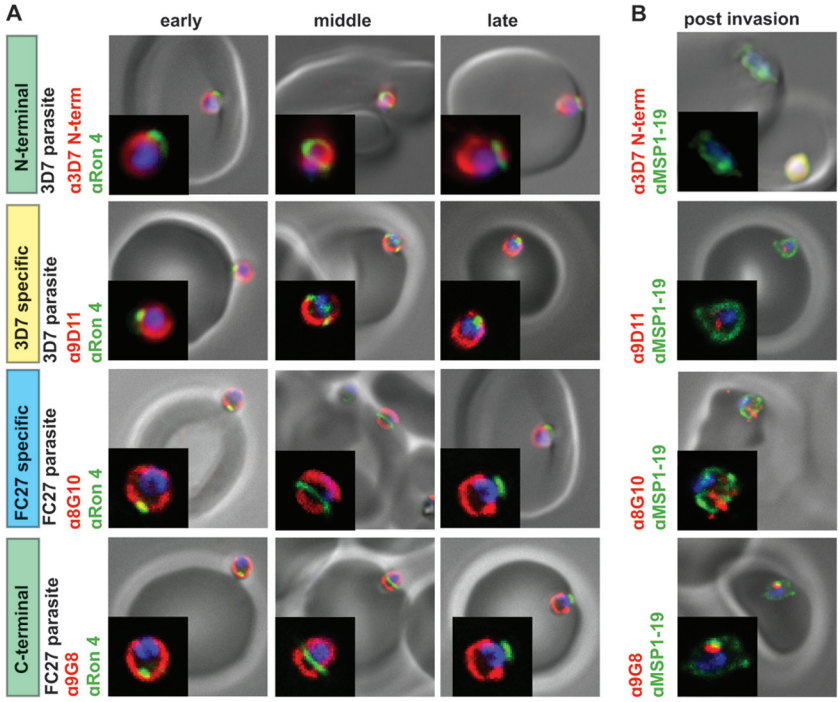

FIG 3 GPI-anchored MSP2 is carried into invaded RBCs and rapidly degraded. (A) Invading merozoites were labeled with the tight junction marker PfRON4 (green) and colabeled with antibodies to different regions of MSP2 (red). All regions of MSP2 were carried through the tight junction into the RBC, with labeling being visible on both sides of PfRON4 at the tight junction. (B) Newly invaded rings (10 min postinvasion) were labeled with antibodies to MSP1-19 (green) and antibodies to different regions of MSP2 (red). MSP2 was rapidly degraded postinvasion, with little or no labeling being visible in rings. Images are single slices from deconvoluted stacks. Representative images of antibodies to the different regions of MSP2; other antibodies tested showed the same labeling patterns (see Fig. S2 in the supplemental material). The MSP2 allele of the parasite strain used in each row of images is indicated as 3D7 parasite or FC27 parasite (D10 parasite strain). Antibody dilutions used were 1:500 for secondary antibodies, 1:250 for 8G10, 1:100 for PfRON4, and 1:50 for N-terminal purified antibodies, 9D11, and 9G8.

only in schizont protein extracts, confirming the lack of MSP2 cleavage and shedding into the supernatant and the degradation of MSP2 following invasion (noted by the absence of MSP2 labeling in ring-stage protein extracts) (Fig. 5A). In control experiments, EBA175 was detected in both schizont extracts and supernatant fractions, which indicated that the supernatants did contain parasite antigens and that the ring-stage preparations were relatively free of schizont contamination (see Fig. S3B in the supplemental material).

Differential cleavage of surface proteins was also explored with electron microscopy. Preparations of 3D7 merozoites and erythrocytes were fixed to capture invading merozoites, similar to IF microscopy described above. Merozoites before, during, and immediately after invasion were positively labeled with MSP2 N-terminal rabbit antibodies (Fig. 5B), supporting the IF microscopy results showing that MSP2 protein is not cleaved and shed during invasion. Attempts to label electron micrographs of invading merozoites with MSP4 antibodies were hampered by poor staining and high background labeling.

MSP2 MAbs but not rabbit antibodies to MSP4 are internalized during merozoite invasion and maintained for approximately 24 h. It was previously reported that antibodies to MSP1-19 are able to be internalized during invasion along with the MSP1-19 protein (24, 25, 70). To assess whether this was also the case for the internalized proteins MSP2 and MSP4, MSP2 MAbs and MSP4 rabbit antibodies were incubated with merozoites and RBCs in invasion assays. The MSP2 MAbs had little or no inhibitory activity in standard growth inhibition assays (GIAs) (58) performed over one or two cycles of replication using isolates that express either the FC27 or 3D7 form of MSP2 and in direct invasion inhibition assays (IIA) with purified merozoites (22) (11E1 and 6D8, 700 μg/ml; 9G8, 420 μg/ml; 1F7, 2F2, 4D11, 6C9, 9D11, and 8H4, 250 μg/ml for GIA and 500 μg/ml for IIA) (Fig. 6A to C). Furthermore, we found no evidence that the MAbs delayed intraerythrocytic parasite development; this was assessed by the mean fluorescence intensity of parasites invading in the presence of MAb compared to the control (Fig. 6D). The noninhibitory activity of MSP2 MAbs is consistent with previous studies of antibodies targeting this protein (40, 72). MSP4 rabbit antibodies have been tested for activity in GIAs previously, with full-length MSP4 and MSP4A fragment antibodies showing modest, but significant, growth-inhibitory activity (73). The activity of MSP4 rabbit antibodies was confirmed here by a direct IIA, with a modest inhibition of D10 parasite strains being observed (Fig. 6E).

To examine the internalization of MSP2 MAbs and MSP4 rabbit antibodies during invasion, invading merozoites were incubated with antibodies and RBCs for 10 min before cells were processed for IF microscopy. For MSP2 MAbs, parasites were colabeled with polyclonal rabbit MSP1-19 antibodies to visualize the developing parasites postinvasion, and MSP2 MAbs were detected with anti-mouse secondary antibodies. Both extracellular merozoites and newly invaded ring-stage parasites were positively labeled by anti-mouse antibodies, showing that MSP2 MAbs bound extracellular merozoites and were then internalized during

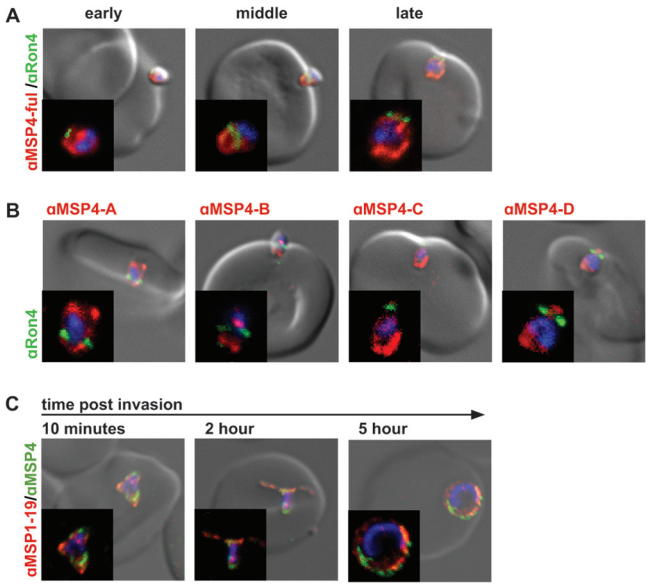

FIG 4 MSP4 is carried into RBCs during invasion without cleavage. (A) Invading 3D7 merozoites were labeled with PfRON4 (green) and antibodies raised against full-length MSP4 (red). MSP4 labeling was visible on both sides of the tight junction for early-, mid-, and late-stage invading parasites. (B) D10 strain merozoites were labeled with PfRON4 (green) and antibodies raised against different regions of MSP4 (red). All MSP4 antibodies labeled merozoites on both sides of the tight junction. Images are representative of mid- and late-stage invading parasites. (C) MSP4 is present at 5 h postinvasion. Isolated 3D7 strain merozoites were allowed to invade and fixed at 10 min and 2 and 5 h postinvasion. Ring-stage parasites were labeled with MSP1-19 antibodies (red) and full-length MSP4 (green). Positive MSP4 labeling was seen at 5 h postinvasion. Antibody concentrations used were 1:500 for secondary antibodies and 1:50 for all MSP4 antibodies.

invasion. The labeling of internalized MSP2 MAb was colocalized with labeling of MSP1-19 by polyclonal rabbit antibodies, further suggesting that MAbs to MSP2 were bound to the parasite surface postinvasion (Fig. 7A, top; see also Fig. S4 in the supplemental material). Internalization of MAbs was a result of specific binding to MSP2 rather than via a "bystander" effect; antibodies directed against the conserved C-terminal region of MSP2 (6C9, 9H4, 1F7, 4D11, and 9G8) were internalized by parasites expressing either 3D7 or FC27 allelic forms of MSP2, while antibodies directed against the 3D7-specific region (MAbs 11E1, 9D11, and 2F2) were internalized only with merozoites expressing the 3D7 allele (Fig. 7B; see also Fig. S4 in the supplemental material). MAb 8G10, specific to the FC27 allele of MSP2, showed no evidence of internalization during invasion with either FC27 or 3D7 MSP2-expressing parasites, which may indicate that the FC27-specific region is unable to be bound by the MAb during invasion or that the MAb does not have sufficient affinity for the MSP2 protein during invasion to be internalized. In no cases was labeling observed on merozoites that had invaded in the absence of MSP2 MAb (PBS control), indicating that fluorescence was not due to cross-reactivity of secondary antibodies.

For MSP4 rabbit antibodies, parasites were colabeled with polyclonal mouse MSP1-19 antibodies to visualize the developing parasites postinvasion, and MSP4 antibodies were detected with anti-rabbit secondary antibodies. In contrast to MSP2 MAbs, positive labeling by anti-rabbit antibodies was seen only on extracellular merozoites and not newly invaded ring-stage parasites, indicating that MSP4 antibodies were not internalized during invasion (see Fig. S5 in the supplemental material). The lack of internalization of the MSP4 antibodies maybe due to the fine specificity of antibodies or due to the inhibitory activity of these antibodies that is not seen in the MSP2 MAbs tested.

The fate of internalized MSP2 MAbs during intraerythrocytic development was investigated by allowing merozoites to invade RBCs in the presence of the 9G8 MAb (C terminal), with invaded parasites being fixed at 5, 14, 19, and 24 h postinvasion for microscopy. The internalized 9G8 MAb was detected for an extended period postinvasion, at 5, 14, and 19 h but not at 24 h, indicating that the internalized MAb had been degraded between 19 and 24 h postinvasion (Fig. 7A). It was striking that the internalized MAbs were maintained for an extended period postinvasion despite the rapid degradation of MSP2 protein postinvasion (Fig. 3 and 5). By IF microscopy, internalized MSP2 MAbs colocalized with MSP1-19 (as detected with polyclonal rabbit antibodies) at all time points. MSP1-19 has been reported to be found in the forming food vacuole in ring-stage parasites (24). It was notable that internalized MSP2 MAbs could be detected many hours after MSP2 protein was normally degraded. It is possible that the internalized MAbs remain trapped between the parasite membrane and the invaginated RBC membrane, despite the absence of MSP2, until the antibodies are eventually degraded, possibly following the formation of a central food vacuole. Despite the inter-

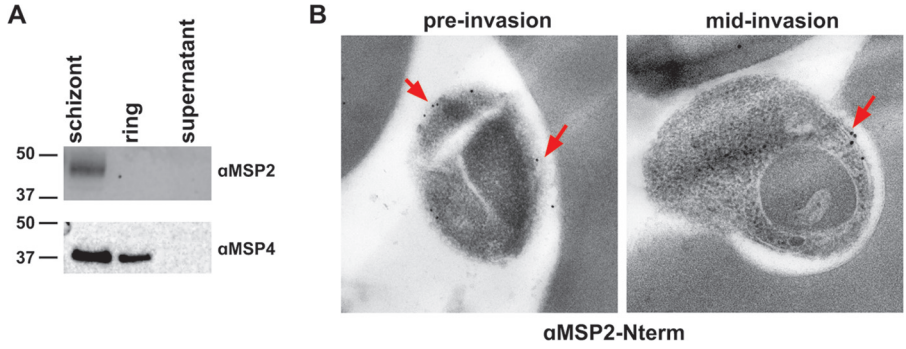

FIG 5 Differential cleavage and shedding of MSP2 and MSP4 confirmed by Western blotting and electron microscopy. (A) Protein extracts from late-stage schizonts (44 to 48 h) and newly invaded ring-stage parasites (0 to 6 h) and culture supernatants from 3D7 parasites were analyzed by Western blotting with antibodies to MSP4 and MSP2. (B) 3D7 strain merozoites were fixed during invasion for EM and labeled with MSP2 N-terminal antibodies. A representative image of a merozoite prior to and during invasion is shown. Labeling is indicated with red arrows.

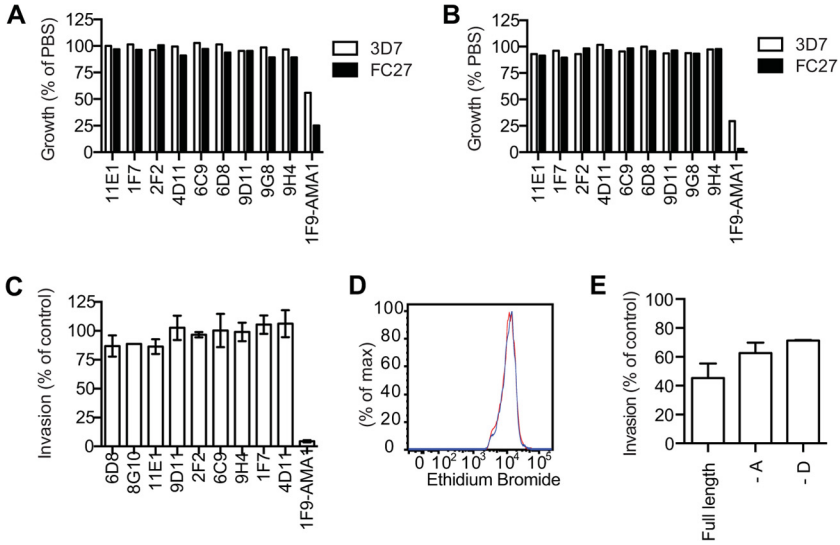

FIG 6 MSP2 MAbs have no invasion inhibition or growth delay activity. (A and B) MSP2 MAbs were tested for growth inhibition activity by a one-cycle (A) or a two-cycle (B) GIA against parasites expressing 3D7 MSP2 or FC27 MSP2 at the following concentrations: 700 μg/ml for 11E1 and 6D8; 420 μg/ml for 9G8; and 250 μg/ml for 1F7, 2F2, 4D11, 6C9, 9D11, and 8H4. MAb 1F9 directed against AMA1 was used as a positive control for inhibition. Data are from one assay in duplicate and are shown as mean growth and range. (C) MSP2 MAbs were tested in invasion inhibition assays against FC27 MSP2-expressing parasites at 500 μg/ml. No invasion inhibition activity was observed. Data are means ± standard errors of the means for 2 to 4 assays in duplicate. (D) Mean fluorescence of GFP and EtBr is known to increase with the age of parasite cultures, so comparison of mean fluorescence intensities between cultures invaded in the presence of MSP2 MAbs and control cultures can detect a growth delay. No growth delay was detected at 30 or 40 h, as shown by the identical mean fluorescence intensity (MFI) of parasites labeled with ethidium bromide or GFP following invasion inhibition assays. No growth delay phenotype was observed for all MSP2 MAbs. The graph is a representative plot of MFIs of parasites incubated with MAb 4D11 (red) compared to the PBS control (blue), stained with EtBr. (E) MSP4 rabbit antibodies (MSP4-full, MSP4A, and MSP4D) were tested in invasion inhibition assays at a 1:10 serum dilution. Data are mean invasions and ranges of two assays in duplicate.

nalization of MAbs, no growth inhibition or growth delay was detected (Fig. 6). This is in contrast to previous reports showing that antibodies to MSP1-19 can be internalized and result in delayed or inhibited intraerythrocytic parasite development in some cases (70).

DISCUSSION

Here, we provide evidence that there is a coordinated process of sequential cleavage of merozoite surface proteins during invasion rather than the complete shedding of all surface proteins at the tight junction, which was previously proposed based on EM studies (4, 9–11). Shedding is instead a specific process, with differential cleavage and loss of merozoite surface proteins during and after invasion. Our novel findings suggest that individual merozoite surface proteins have different roles during invasion and during intraerythrocytic development (Fig. 8). Some merozoite surface proteins are shed during invasion, suggesting that these proteins may play a role in initial invasion events (e.g., N-terminal MSP1, MSP7, MSP3, SERA4, and SERA5). Other proteins, such as MSP2, may function during invasion and are then rapidly degraded postinvasion, indicating that they do not function in further intraerythrocytic development. Finally, other proteins are internalized without processing and persist postinvasion (e.g., MSP4) or persist postinvasion as a processed form (e.g., MSP1-19) and may have important roles in intraerythrocytic development of parasites. These findings suggest that the merozoite surface is gradually remodeled by processing events commencing with initiation of invasion, through immediate postinvasion processes, and subsequently through intraerythrocytic development, which significantly advances our understanding of erythrocyte invasion and merozoite surface protein processing. We believe that these studies are the first to identify proteins (MSP2 and MSP4) that remain on the surface of the merozoite during invasion without processing, and we demonstrate the striking postinvasion processing of a merozoite antigen (MSP2).

Our study has shown that at least two GPI-anchored proteins, MSP2 and MSP4, appear to be carried into RBCs intact, in contrast to the peripheral surface proteins (MSP3, MSP7, SERA4, and SERA5) and the MSP1 complex (3, 22, 25), which are shed during invasion, at the point of the tight junction. The shedding of peripheral surface proteins is likely due to the cleavage and subsequent shedding of the membrane-bound binding partners of these proteins; for example, MSP7 is part of the MSP1 complex, which is shed due to cleavage of MSP1-42 to the MSP1-19 form by PfSUB2 (23). In contrast, for MSP2 and MSP4, antibodies to these proteins used for IF microscopy of invading merozoites labeled the merozoite surface equally on both sides of the tight junction, showing that these proteins were carried into the RBC during invasion. Importantly, this was confirmed with a number of antibodies for different regions of both MSP2 and MSP4, indicating that these proteins are not cleaved. Along with these proteins, the GPI-anchored fragment of MSP1, MSP1-19, is well characterized as being carried into RBCs and can be detected on ring-stage parasites. It is possible that the GPI anchor of these proteins is important for the passage of these proteins through the tight junction during invasion, and any shedding of GPI-anchored proteins requires specific cleavage and processing.

Interestingly, MSP2 was immediately degraded postinvasion, with little or no protein being detectable by IF microscopy in parasites fixed for imaging at 10 min postinvasion. This result is

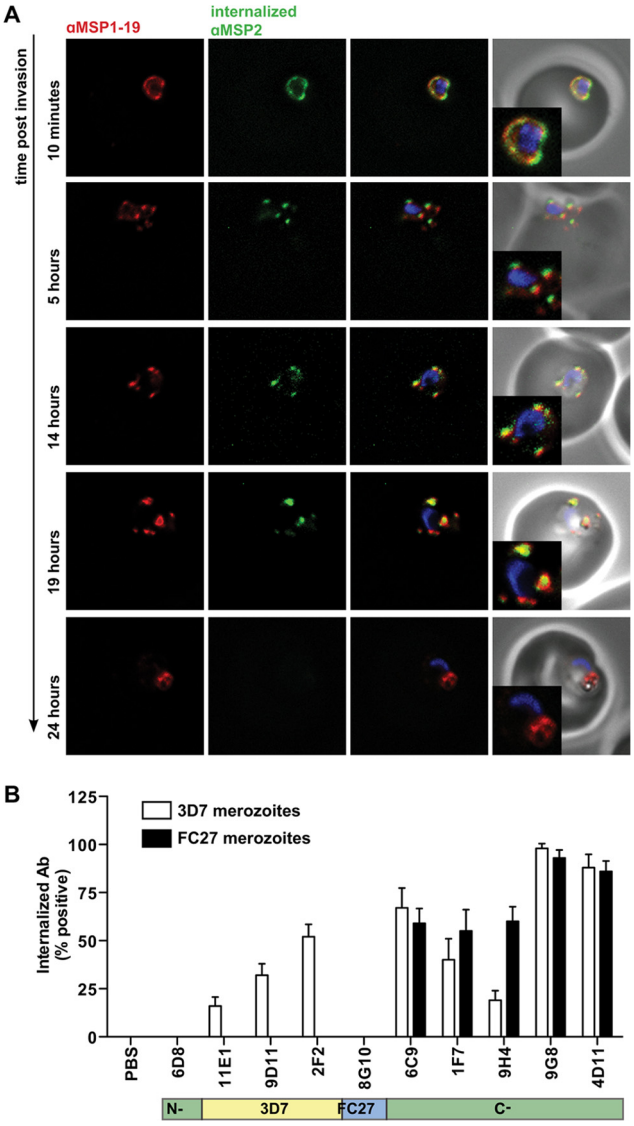

FIG 7 Antibodies to MSP2 are internalized during invasion and maintained for approximately 24 h. Isolated merozoites expressing the 3D7 or FC27 form of MSP2 were incubated with each of the different MSP2 MAbs and RBCs and allowed to invade. Following 10 min of incubation, parasites were washed to remove excess antibody and fixed for immunofluorescence assays postinvasion. (A) Parasites were labeled with polyclonal rabbit antibodies to MSP1-19 and secondary antibodies (anti-rabbit Alexa594 to detect MSP1-19 labeling and anti-mouse Alexa488 to detect internalized MSP2 MAbs, with anti-MSP1-19 at a 1:200 dilution and secondary antibodies at a 1:500 dilution). Internalized MSP2 MAbs were detected up to 19 h postinvasion. Images are from representative antibodies to the C-terminally conserved region. (B) Positive labeling for internalized MAbs was quantified in parasites expressing the 3D7 and FC27 forms of MSP2 with the panel of MSP2 MAbs. Percent positive merozoites and standard errors of the proportion are graphed for each parasite strain. The region of reactivity of the MAb is represented below the graph. No labeling was detected in merozoites that had invaded RBCs in the absence of a specific MSP2 MAb (PBS control), and labeling was detected only for 3D7-specific MSP2 MAbs in parasites expressing the 3D7 and not the FC27 form of MSP2, indicating that internalization was specific.

consistent with previous reports that MSP2 is not detectable in ring-stage parasites (47, 74, 75) and is not found in culture supernatant fractions (20, 47). Importantly, both the 3D7 and FC27 allelic forms of MSP2 showed the same phenotype of being carried into RBCs followed by rapid degradation. The 3D7 and FC27 alleles of MSP2 share N- and C-terminal regions but vary significantly in the central strain-specific region of the protein (46, 76–78). The observation that both MSP2 variants show the same localization and very precise timing of degradation postinvasion suggests that the functions of the 3D7 and FC27 forms of MSP2 are the same, despite any structural differences. The internalization of MSP2 protein during invasion observed by IF microscopy was confirmed by EM of invading merozoites and by demonstrating the specific internalization of MSP2 MAbs during invasion. In contrast to the rapid degradation of MSP2, MSP4 was maintained for at least 5 h in the developing ring parasite, and the presence of uncleaved protein postinvasion was confirmed by Western blotting. Furthermore, the MSP4 protein was detected in trophozoite-stage parasites via Western blotting. A previous report failed to detect MSP4 by IF microscopy on early-ring-stage parasites (48). However, the precise age of the rings examined was not given in that study, and it may be that as the parasite matures, MSP4 is degraded and may not be detected via IF microscopy.

Merozoite surface proteins have been implicated in initial contact events between the merozoite and erythrocyte surface (1, 6). Indeed, shed proteins such as MSP1 (aside from the internalized GPI-anchored MSP1-19 component), MSP3, MSP7, SERA4, and SERA5 may have roles at this stage, and the shedding of these proteins may indicate that the role of these proteins in invasion is complete. In contrast, nonshed proteins may have roles distinct from initial contact; the localization of MSP2 during and after invasion suggests that MSP2 may function during the invasion process, while the localization of MSP4 suggest that this protein, like MSP1-19, may function postinvasion during early intraerythrocytic development of the parasite. While both MSP2 and MSP4 are thought to be essential blood-stage antigens (43), only MSP2 is under extensive immune selection pressure (39, 79) and appears to be an important target of human immunity (37, 80). The presence of polymorphisms for immune evasion provides additional support for an important role of MSP2 during or around the time of invasion and its significance as a target of human immunity. Our findings are also significant for understanding immunity and vaccine development because they indicate that MSP2 is seen by the immune system only as it is presented on the merozoite surface, and it is not found as a processed or secreted form. This emphasizes the importance of understanding the native structure of MSP2 and the interaction with antibodies (60). Several other GPI-anchored merozoite surface proteins have been identified; the localization of these proteins during invasion to ascertain cleavage and shedding profiles may inform our understanding of the roles of these specific proteins during invasion.

This work has also demonstrated that antibodies to MSP2 can bind the merozoite surface and then be internalized during merozoite invasion. The ability of antibodies to be carried into invaded RBCs was observed previously for MSP1-19, with internalization of noninhibitory monoclonal antibodies (24, 25) as well as inhibitory polyclonal rabbit antibodies, which can result in a specific growth delay (70). Other studies have reported that antibodies to MSP1 are able to disrupt intraerythrocytic development as well as inhibit invasion (81), a process that may be due to the internalization of antibodies during invasion. Although internalized MSP2 antibodies were maintained within the parasite for between 19 and 24 h, no invasion-inhibitory or growth-inhibitory activity was observed in either one-cycle direct invasion inhibition assays or

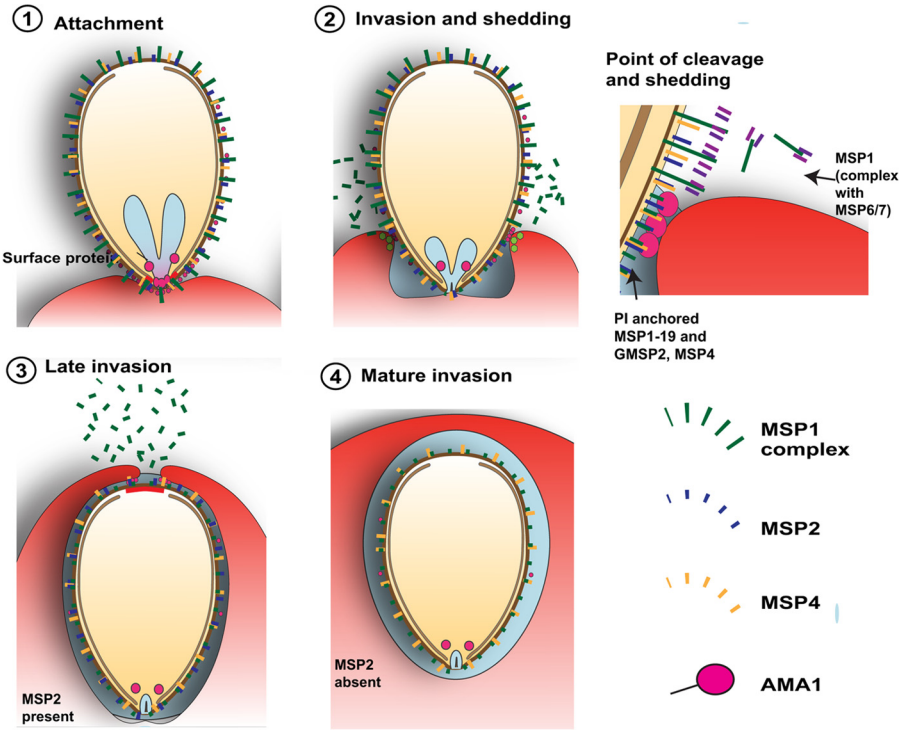

FIG 8 Coordinated cleavage and shedding of major merozoite surface proteins during invasion. At initial attachment and reorientation of the merozoite, the merozoite surface coat includes numerous proteins (1). The most abundant of these is MSP1, which is found in a complex with other antigens (indicated in green and purple). MSP2 is indicated in blue, and MSP4 is indicated in yellow. AMA1 and the tight junction are represented by pink circles. During invasion, some but not all merozoite surface proteins are cleaved and shed (2). Shed proteins include the majority of the MSP1 complex, with MSP1-19 being carried into the RBC along with MSP3 and SERA4/5 (not indicated). MSP2 and MSP4 are not cleaved and shed at the point of the tight junction and are instead carried into the RBC. Shedding continues to occur as the merozoite invades the RBC (3). Between late invasion and mature invasion, MSP2 protein is lost; however, MSP4 and MSP1-19 are maintained into early intraerythrocytic development (4).

one- and two-cycle standard growth inhibition assays. It is possible that *in vivo*, internalized antibodies would have some negative impact on parasite growth that is not measurable in the *in vitro* assays used here. However, it is also possible that internalized MSP2 antibodies have no role in inhibiting intraerythrocytic development, as MSP2 is rapidly degraded and presumably has no role in the subsequent development of the parasite postinvasion. This is in contrast to MSP1-19, which has been hypothesized to have a role in food vacuole formation, with internalized antibodies to MSP1-19 having the potential to disrupt function postinvasion (24, 70). It is interesting to speculate that the ability of the merozoite to carry antibodies bound to surface proteins, such as MSP2, into the invaded RBC without apparent disruption of invasion or growth may represent an adaption of the parasite to render antibodies to the merozoite surface nonfunctional, thereby contributing to immune evasion. Although antibodies to MSP2 do not inhibit invasion directly, they do function to opsonize merozoites and promote antibody-dependent cellular inhibition mediated by monocytes (40, 72), which is thought to be an important mechanism contributing to acquired immunity (82). In contrast to MSP2, the internalization of rabbit antibodies to MSP4 was not detected. It is possible that MSP4 antibodies are unable to be internalized due to the mild inhibitory activity of these antibodies, as seen in growth inhibition and direct invasion inhibition assays (73). However, it is also possible that the position of MSP4 during invasion prevents the internalization of antibodies targeting this protein or that this observation is due simply to the specific antibodies tested. Further studies on the internalization of multiple antibodies targeting specific proteins are needed to further understand the mechanisms mediating antibody internalization. Likewise, while the observations presented here indicate that internalized MSP2 antibodies had no impact on parasite development, more in-depth analysis is needed to fully understand the implications of internalized antibodies for protein processing, function, and localization.

Differential cleavage and shedding of merozoite surface proteins and the ability of antibodies to be internalized during invasion raise questions about the mechanisms of shedding and the nature of the tight junction. While PfSUB2 processing is required for the cleavage and subsequent shedding of some surface proteins (23), the timing of these events remains unclear. It was hypothesized previously that the cleavage of MSP1-42 would occur at the tight junction and occur concurrently with shedding of MSP1 (23). However, the localization of PfSUB2 during this period appears to be different from the point of shedding; PfSUB2 appears to track to the posterior of the invading merozoite before MSP1 shedding occurs (3). It is clear that the tight junction alone does not force the shedding of proteins, as some merozoite surface proteins and antibodies can be internalized during the invasion process. Antibodies are approximately 150 kDa; for internalization to occur, the tight junction between the merozoite and RBC must not be a tight seal of proteins but instead must consist of either transient interactions which can allow passage of antibodies and surface proteins or gaps between protein-protein interactions

that allow molecules to pass through. The tight junction appears as a complete ring structure by IF microscopy of PfRON4 and AMA1 (3) and as a region of close proximity between the merozoite and RBC membranes by EM (4). Our results for internalization of antibodies, and MSP2 and MSP4 without obvious processing, suggest that the tight junction is able to specifically exclude some proteins but not others. It remains possible that cleavage of surface proteins and the mechanisms of shedding require a further unknown factor to mediate the specific release of proteins from the merozoite surface. Clearly, the processes involved in the cleavage and shedding of the surface coat are specific and not universal or nonselective; instead, they act against a subset of proteins. A further understanding of the molecular basis of merozoite protein processing might reveal attractive targets for the development of antimalarial inhibitors, and several proteases that inhibit invasion have been reported in the literature (6, 13). Furthermore, with merozoite surface proteins being the targets of vaccine development, clear characterization of individual proteins during and after invasion informs vaccine development by identifying periods when parasite proteins are most likely to be targeted by the immune system (32, 33). In conclusion, these studies advance our understanding of the mechanisms of invasion, demonstrating that the loss of the merozoite surface coat is not a global event and instead involves specific processes resulting in the sequential cleavage of individual proteins both during and after invasion, indicating the diversity of roles of different proteins (Fig. 8). Invasion of RBCs involves a complex series of events, and an understanding of key processes and interactions may reveal targets for drug development and will facilitate the prioritization of antigens for vaccine development.

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We declare that we have no conflicts of interest.

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