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REVIEW

Host Cytoskeleton Remodeling throughout the Blood Stages of *Plasmodium falciparum*

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SUMMARY The asexual intraerythrocytic development of *Plasmodium falciparum*, causing the most severe form of human malaria, is marked by extensive host cell remodeling. Throughout the processes of invasion, intracellular development, and egress, the erythrocyte membrane skeleton is remodeled by the parasite as required for each specific developmental stage. The remodeling is facilitated by a plethora of exported parasite proteins, and the erythrocyte membrane skeleton is the interface of most of the observed interactions between the parasite and host cell proteins. Host cell remodeling has been extensively described and there is a vast body of information on protein export or the description of parasite-induced structures such as Maurer's clefts or knobs on the host cell surface. Here we specifically review the molecular level of each host cell-remodeling step at each stage of the intraerythrocytic development of *P. falciparum*. We describe key events, such as invasion, knob formation, and egress, and identify the interactions between exported parasite proteins

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and the host cell cytoskeleton. We discuss each remodeling step with respect to time and specific requirement of the developing parasite to explain host cell remodeling in a stage-specific manner. Thus, we highlight the interaction with the host membrane skeleton as a key event in parasite survival.

KEYWORDS malaria, cytoskeleton, erythrocytes, gametocytes, remodeling

INTRODUCTION

Plasmodium

Malaria is caused by intracellular apicomplexan parasites of the genus *Plasmodium*. This infectious disease is transmitted by the bite of an infected female *Anopheles* mosquito. Despite many efforts toward its elimination, malaria remains a major global health burden, causing roughly 430,000 deaths and 210 million infections per year (1, 2).

Six *Plasmodium* species cause human malaria: *P. falciparum*, *P. vivax*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. malariae*, and *P. knowlesi* (1, 3). Of these, *Plasmodium vivax* is the most widespread species, while the most severe form of malaria is caused by *P. falciparum* (4).

The life cycle of *P. falciparum*. Throughout its life cycle, *P. falciparum* alternates between two hosts, the arthropod vector and the human host. During a blood meal of an infected female *Anopheles* mosquito, extracellular sporozoites are transmitted into dermal tissue, subsequently reaching blood vessels from where they are transported to the liver. Sporozoites transverse and invade hepatocytes, where they replicate and develop into merozoites that are released into the peripheral blood. Once in the blood, merozoites invade erythrocytes and the asexual replication cycle starts, which is responsible for all clinical symptoms of malaria. During each intraerythrocytic developmental cycle, a few parasites commit to sexual development and become gametocytes. Mature gametocytes are transmitted to mosquitoes during a blood meal. In the mosquito midgut, male and female gametocytes become gametes, which then fuse into a zygote. After development in the mosquito, sporozoites are formed, migrate to the salivary glands, and can be transmitted to the next human host (1, 5).

Why remodel the host cell? *P. falciparum* invades its host cell to replicate and to be transmitted, and all changes during the 48-h intraerythrocytic cycle are consequences of this fact. Host cell invasion requires the parasite to actively penetrate the erythrocyte membrane and cytoskeleton. Replication and formation of daughter cells lead to substantial deformation of the once-discoid red blood cell (RBC), which becomes more spherical, with the consequence that the infected red blood cell (iRBC) can no longer pass through the spleen. To avoid splenic clearance, the parasite sequesters at the endothelial lining of the capillaries in deep tissue. This cytoadhesion requires the parasite to insert antigens into the host cell membrane and to anchor them in knob complexes to the iRBC cytoskeleton. At the end of the intraerythrocytic cycle, the merozoites are released from their host cell, and the iRBC cytoskeleton and membrane are destroyed. Each stage therefore has its own requirements; through remodeling of the host cell the parasite fulfills those requirements, and the exported parasite proteins play a key role in these processes.

A number of reviews mention how extensively *P. falciparum* remodels the host cell but focus on exported parasite proteins, the machinery required for export, and the establishment of exomembrane structures which aid in protein trafficking (5–8). In this review, we focus on the iRBC cytoskeleton because it is the target or interface of many known host-parasite interactions and is continually remodeled throughout the intraerythrocytic asexual and sexual development (Fig. 1). The erythrocyte cytoskeleton is remodeled during invasion, knob formation, and egress or during gametocyte maturation. Each step has been shown to require specific modifications of the cytoskeleton, and here we review the different modifications and the key players involved in each stage or specific event. We highlight and discuss the specific steps involving host-parasite interactions at the iRBC cytoskeleton.

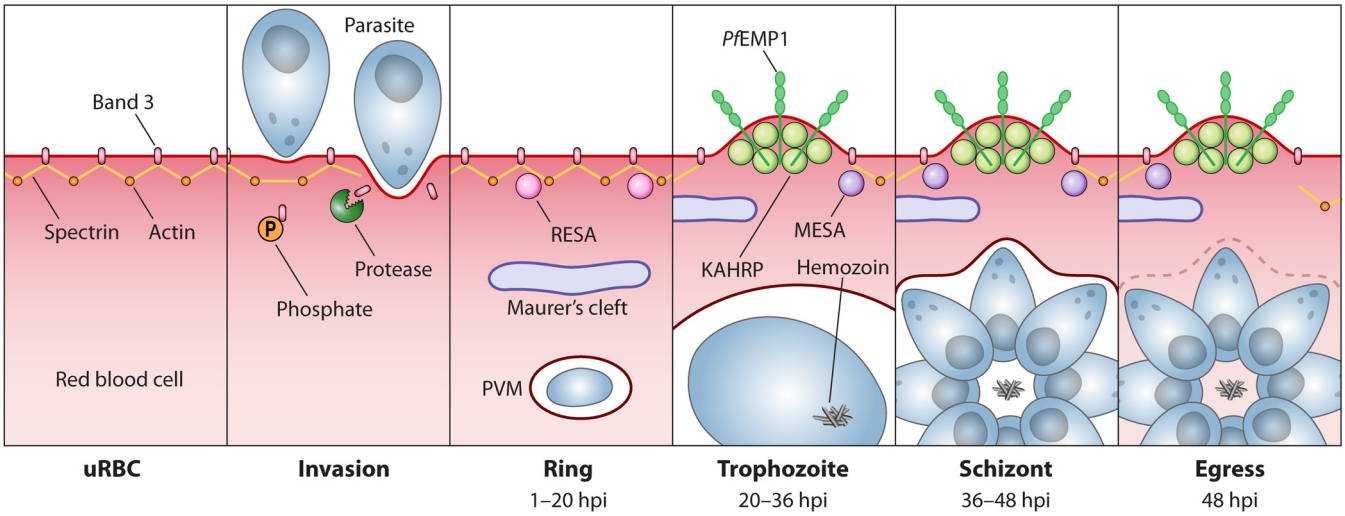

FIG 1 Overview of the intraerythrocytic cycle of *P. falciparum*. A summary of some of the parasite-mediated changes occurring at the erythrocyte cytoskeleton throughout the asexual blood stages is shown. The first box shows the spectrin-actin network connected to the RBC membrane vertically linked to proteins such as band 3 in the uninfected RCB (uRBC). During invasion, the RBC cytoskeleton is locally rearranged by kinase and protease activity (box 2). During the ring stage, first exported parasite proteins target the iRBC cytoskeleton, and parasite-induced structures such as Maurer's clefts are found (box 3). The maturing parasite forms knobs, surface protrusions which anchor PfEMP1 (box 4), and merozoites are formed during the last hours of the intraerythrocytic life cycle (box 5). During egress, the PVM is permeabilized, and the iRBC cytoskeleton and membrane are degraded to release the newly formed merozoites into the bloodstream (box 6).

Protein export. To initiate host cell changes, the parasite must export a large number of proteins to refurbish the iRBC. In this process, all exported proteins need to pass through the parasite and parasitophorous vacuole (PV) membranes. The identification of the *Plasmodium* export element (PEXEL), a pentameric amino acid motif, was a major breakthrough and allowed the prediction of a large number of exported proteins (9, 10). Currently, proteins carrying a PEXEL, a more relaxed PEXEL (11), or a noncanonical PEXEL (12) have allowed the prediction of over 460 exported proteins. In addition, there is another group of exported proteins lacking a known export motif, and these are referred to as PEXEL-negative exported proteins (PNEPs) (13). Exported proteins have been implicated in the genesis of new organelles or functional complexes or structures, such as the *Plasmodium* translocon of exported proteins (PTEX) (14), Maurer's clefts (MCs) (15), J dots (16, 17), knobs (18), and the new permeability pathway (19, 20), as previously reviewed (8, 21, 22). Many of these proteins are also involved in remodeling of the cytoskeleton, and export of these proteins leads to the pathology of *P. falciparum* malaria.

The Human Red Blood Cell Cytoskeleton

The erythrocyte membrane skeleton is a two-dimensional hexagonal lattice formed by $(\alpha 1\beta 1)_2$-spectrin tetramers (about 180 nm in extended length) which are connected at their ends by short actin filaments (35 nm). These junctions are stabilized by band 4.1, forming a ternary complex (23–26). Multiple other proteins either stabilize this meshwork or support its attachment to the red cell membrane. Proteins involved in actin binding and turnover are adducin, tropomodulin, tropomyosin, and others, as reviewed in reference 25. The spectrin tetramer is bridged in its middle to band 3, an integral membrane protein, via ankyrin, keeping the skeleton close to the cell membrane (23, 24, 27, 28). Another link between the skeleton and the membrane is formed by the interaction between the cytoplasmic face of glycophorin A and band 4.1, which in turn associates with actin (23). An alternative vertical connection between membrane and skeleton is facilitated by p55 linking glycophorin C and band 4.1 (29). The highly elastic properties and unique biconcave shape of the erythrocyte membrane skeleton allow for passive deformation, which occurs during the passage through the spleen (23, 30).

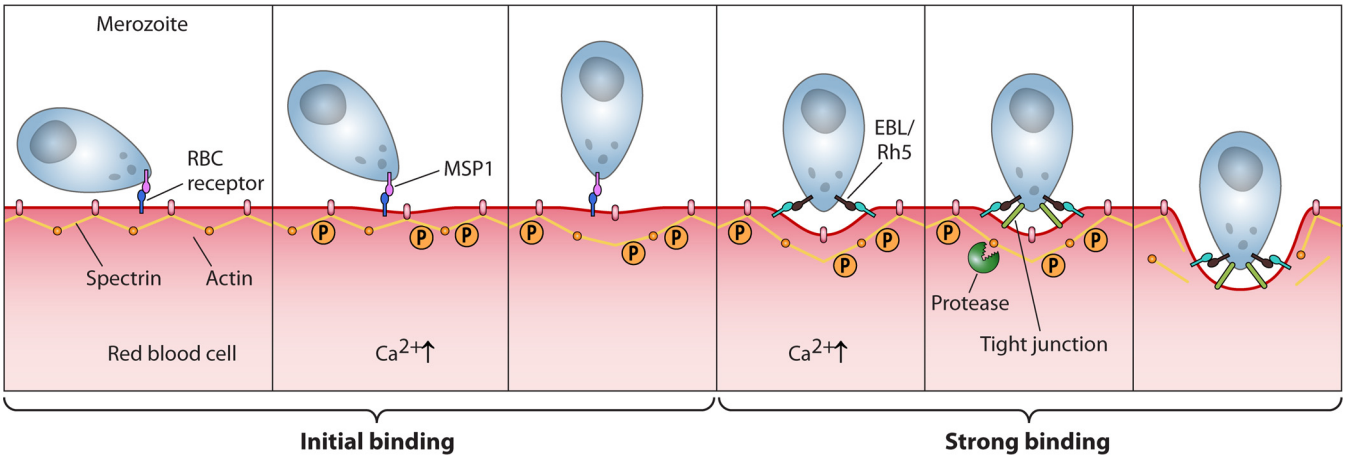

FIG 2 Invasion. Individual steps of invasion are shown, focusing on changes to the erythrocyte cytoskeleton. The initial contact between merozoite and erythrocyte is mediated by binding of MSP1 to its RBC receptor (box 1), which then causes a reorientation of the merozoite. An increase in intracellular calcium leads to local reorganization of the erythrocyte cytoskeleton (boxes 2 and 3). Once the parasite has reoriented its apical end facing the RBC membrane, stronger binding by EBL and Rh5 complex proteins occurs (box 4). A tight junction is formed, and proteases locally degrade the cytoskeleton to create an opening for the inward-pushing parasite (boxes 5 and 6).

LIFE CYCLE STAGE- AND EVENT-SPECIFIC REMODELING OF THE HOST CELL CYTOSKELETON

In a simplified model, the mature human erythrocyte can be divided into three major components, i.e., the membrane, membrane skeleton, and cytosol, of which all are important sites for the intracellular development of the parasite. During invasion and egress, the cytoskeleton and erythrocyte membrane have to be crossed, and during other stages, the cytoskeleton and membrane need to be modified to accommodate the needs of a growing parasite that tries to evade the host immune system.

Invasion

Erythrocyte invasion is a fast and well-orchestrated process that is completed within less than 2 min (Fig. 2). The initial contact is not yet well understood, but merozoite surface protein 1 (MSP1) seems to mediate the initial binding between merozoite and erythrocyte (31). This causes a weak deformation of the erythrocyte at the site of merozoite attachment (31–34) and could be the result of cytoskeleton phosphorylation induced by increased Ca^{2+} (32, 35). Such changes in Ca^{2+} concentration could lead to altered cell morphology through modulating protein phosphorylation, which in turn regulates protein-protein interactions at the erythrocyte cytoskeleton. However, recent data challenged this hypothesis by providing evidence of an absence of Ca^{2+} signals during invasion (36).

The initial contact is followed by reorientation and stronger binding mediated by members of two *Plasmodium* protein families, the erythrocyte-binding-like (EBL) proteins or the *P. falciparum* reticulocyte-binding protein homologs (PfRh), to glycophorin A, B, or C or to the complement receptor 1 on the erythrocyte surface (31). This interaction between the Rh5 complex and Basigin leads to an influx of Ca^{2+} into the erythrocytes, which triggers phosphorylation of membrane skeleton proteins (34) such as spectrin (37) or band 3 (38, 39). Once phosphorylated, band 3 dissociates from ankyrin and spectrin, thereby weakening the cytoskeleton and detaching it from the membrane at the site of entry (38, 40). It has been shown that binding of recombinant Rh5 to RBCs increases phosphorylation of ankyrin and adducin, and it has been speculated that this leads to dissociation from the skeleton and its weakening but also increases the overall size of the spectrin meshwork (34). The importance of band 3 as one factor in invasion is evident in the higher resistance to *P. falciparum* invasion of ovalocytic erythrocytes which carry a 27-bp deletion in the band 3 gene (41, 42). This might be linked to increased ATP depletion by the mutated anion transporter band 3 (43).

Another mechanism emerged for protein depletion and detachment of the cytoskeleton at the site of entry. Band 3, ankyrin, adducin, and band 4.1 are proteolytically cleaved (44, 45), and proteases potentially involved are chymotrypsin-like protease (44), the parasite serine protease gp76 (46, 47), falcipain 1 (48), or plasmepsin II (49, 50). Some of these proteases are also found in schizonts, and it is not clear whether they are involved in egress or whether they are stored in apical organelles of newly formed merozoites to be used later. Some of these proteases have been tested only on substrates *in vitro*, and their true function has not been clearly elucidated.

It is intriguing that band 4.1 can be proteolytically cleaved during the invasion process (45). However, when band 4.1 is already absent due to its gene deletion in hereditary elliptocytosis, invasion of merozoites is less efficient. Band 4.1 links the spectrin-actin cytoskeleton to glycophorin C and thus to the membrane and gives it an important function in maintaining the structural integrity of the cytoskeleton (51). Elliptocytes also have been reported to display decreased membrane deformability and rigidity, and in both ovalocytosis and elliptocytosis, the altered structural integrity of the cytoskeleton impairs the invasion process.

There is conflicting evidence on the role of erythrocyte-binding antigen 175 (EBA-175) in the invasion process. It has been shown that binding of EBA-175 to glycophorin A induces phosphorylation of cytoskeletal proteins tropomodulin, adducin, ankyrin, and band 4.1, leading to increased deformability of the erythrocyte, which is important for merozoite invasion (31, 52). In contrast, Koch et al. reported that binding of EBA-175 to glycophorin A increased erythrocyte stiffness, which seemed to enhance invasion, probably as a result of cross-linking of glycophorin A to the membrane skeleton (40, 53). Although the first observation seems more likely since blocking of this phosphorylation prevented the increase of deformability and thus parasite invasion, more evidence is needed to verify either hypothesis.

It was also reported that elevated Ca^{2+} concentrations cause membrane budding or intake of vesicles into erythrocytes that normally do not phagocytose (54–56). This and the depletion of cytoskeletal proteins from the site of invasion, resulting in loosening of the membrane from the cytoskeleton, could promote membrane wrapping, which had been described to occur during invasion and could contribute to the energy needed for this process (37, 57). Membrane wrapping and subsequent budding are part of the same processes in merozoite invasion and lead to parasite intake. At the same time, the parasite becomes enclosed within the parasitophorous vacuole membrane (PVM).

Most of the cytoskeleton remodeling occurs at the very initial phase of contact between the merozoite and the erythrocyte, with the purpose of facilitating entry and PVM formation. After successful invasion, the membrane is resealed, and the cytoskeleton is most likely restored (57). The RBC membrane and cytoskeleton pose an obstacle to merozoite invasion, but these barriers must be crossed without causing permanent damage to the host cell.

Ring Stage

The first half of the intraerythrocytic developmental cycle is referred to as the ring stage, a name inspired by its typical shape or morphology as seen by Giemsa staining (58). Within minutes after invasion, the parasite starts exporting proteins into the host cell (5), a continuous process until the end of the 48-h developmental cycle. One of the earliest exported proteins is the *Plasmodium* helical interspersed subtelomeric (PHIST) protein ring-infected erythrocyte surface antigen (RESA), which is discharged from dense granules into the parasitophorous vacuole and then exported into the host cell (59). Malaria induces fever episodes upon infected-erythrocyte rupture (60), and the stability of the spectrin network decreases as temperature increases (61). Hence, binding of phosphorylated RESA to repeat 16 of β-spectrin conveys protection against thermally induced denaturation of the iRBC (59, 61–65). This stabilizing effect might be mediated by the DnaJ chaperone domain of RESA, which could prevent unfolding of spectrin (66).

Members of the high-molecular-weight protein family (RhopH) play a dual role. They are discharged from the rhoptries during invasion and are then found throughout the intraerythrocytic cycle at the iRBC periphery, playing a role in nutrient uptake (67–69). Multiple host cytoskeleton and exported parasite proteins have been identified as potential interaction partners, suggesting that RhopH proteins might be involved in host cell remodeling. While no functional analyses supporting this conclusion have been provided (69, 70), this clearly indicates that parasite proteins are present in the host cell from the moment of invasion.

While little phosphorylation is found during the ring stage, band 3 was one of the few proteins shown to be phosphorylated at tyrosine residues (71), suggesting that proteins phosphorylated during invasion are actively dephosphorylated during the ring stage; otherwise, they still would be detected. This also supports the notion that cytoskeleton modifications observed during invasion are reversible and nondestructive. Little is known about further modifications of the iRBC cytoskeleton during the next hours until the transition from ring to trophozoite stage, which is accompanied by major remodeling processes. It is unclear whether there are slowly progressing modifications, but the rest of this part of the cycle seems to be quite uneventful. As the host's fever episode lasts throughout almost the entire ring stage, cytoskeletal modifications other than protection against thermally induced stress would be detrimental and probably are not possible. Any modification which would further increase the deformability of the host cell could lead to the collapse of the iRBC cytoskeleton. Similarly, increasing rigidity too much would cause the circulating iRBCs to be cleared out by the spleen. No further interaction with the cytoskeleton has been observed at this stage. Transcriptome analysis of the early stage shows upregulated genes that are involved in transcription translation and in metabolic processes (72). Structures such as the PTEX (14), MCs (15), J dots (16, 17), and other components of the protein export and trafficking machinery are generated at this stage. Many proteins are synthesized and exported to MCs, where they are stored until being trafficked to their final destination. MCs seem to be larger during the ring stage than in trophozoites (73), suggesting a possible function as storage organelles. Many exported proteins accumulate in MCs but do not yet interact with the host cell cytoskeleton, but they are present in large quantities and in close proximity to their destination, from where they may be discharged when needed. The reduced size of MCs in trophozoites (73) could imply that transiently stored cargo has been discharged and could explain the rapid changes occurring during the transition to trophozoites.

Transition from Ring Stage to Trophozoite Stage

During the first hours of infection, *P. falciparum* establishes a fully functional protein-trafficking machinery enabling transport of proteins to various subcellular localizations. The transition from ring to trophozoite stage at around 16 to 24 h postinvasion is marked by multiple changes in the iRBC. The parasite exports proteins that target the cytoskeleton, changing its properties and structure; knobs appear on the surface of the host cell; and the mobile MCs are tethered to the cytoskeleton. All of these changes, described in detail below, occur to facilitate cytoadherence in order to avoid splenic clearance and to prepare for future growth, replication, and formation of daughter cells.

Reorganization of the iRBC cytoskeleton. During this transition phase, RESA disappears from the cytoskeleton and seems to be replaced by mature parasite-infected erythrocyte surface antigen (MESA), although they do not share the same binding partner or site (61, 74). As described above, RESA seems to protect the host cell cytoskeleton against thermal damage during the parasite ring stage and might not be needed any longer. While RESA stabilizes the cytoskeleton, subsequent binding of MESA alters its stability by competing with the host protein p55 for binding to band 4.1, a protein involved in stabilizing the spectrin-actin network. A 19-amino-acid (aa) motif of phosphorylated MESA interacts with a 51-aa motif encoded by exon 10 of band 4.1 (75–78). The interaction at the ternary complex between band 4.1, actin, and spectrin

seems to be regulated through the level of phosphorylation. In iRBCs, band 4.1 shows an increased level of phosphorylation, which weakens its interaction with the cytoskeleton (79). MESA and band 4.1 are phosphorylated independently, but this modification is important for their interaction (75, 80, 81). The competition between MESA and p55 (77) could weaken the spectrin-actin interaction, providing a possible explanation for the generation of free spectrin ends which are then used to anchor knobs to the cytoskeleton. At the same time, free actin would become available to be used to grow filaments which connect MCs with the cytoskeleton. It is unclear whether MESA competes with every single p55 molecule or whether this competition takes place only in focal spots where knobs are being formed. Actin is absent from knobs but is still found in close proximity (82–84), suggesting that the sites of actin mining and knob formation are identical. The presence of a band 4.1-binding motif in 13 other exported parasite proteins (78) suggests that MESA is most likely not the only protein involved in restructuring the iRBC cytoskeleton.

Knob formation and cytoadhesion. Knobs are protrusions on the iRBC surface formed by an electron-dense layer underneath the iRBC membrane, consisting of a protein complex dominated by knob-associated histidine-rich protein (KAHRP) (18, 85) and an underlying spiral scaffold (84). Knobs were reported in association with cytoskeletal junctions, although not all junctions showed presence of knobs (18, 86, 87). KAHRP self-assembles underneath the iRBC membrane, is essential for knob formation (85, 88), and binds spectrin, actin, and band 4.1 (89–91). A 72-amino-acid stretch of KAHRP binds α-spectrin at repeat 4 (91), while the 5′ repeat region of KAHRP binds β-spectrin at repeats 10 to 14. This binding is strengthened through complementary isoelectric charges and takes place adjacent to the spectrin-ankyrin interaction site at repeats 14 to 15 of β-spectrin (85, 92, 93). This interaction takes place close to ankyrin, and KAHRP also interacts with ankyrin. As a result, each knob is connected to four to eight spectrin tetramers, leading to a higher spectrin density in knob areas than anywhere else in the skeleton (87). One possible explanation of the origin of the spectrin ends connected to the knobs is that they were generated during actin mining. Neither the composition of the knob spiral scaffold (spectrin was excluded as a component) nor the exact interactions to link this spiral to the erythrocyte cytoskeleton are known, but it was proposed that the spiral would give knobs their shape and provide mechanical stability (84). A detailed model of knobs has been described by Cutts et al. (93).

Probably the most important function of knobs is to anchor *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1), which accumulates at the iRBC surface at around 16 to 20 h postinfection (hpi) (94). PfEMP1 mediates cytoadhesion to the endothelial lining of the capillaries (95–99), and iRBC sequestration is linked to severe malaria (100, 101), making PfEMP1 the major virulence factor of *P. falciparum*. A large number of proteins seem to be exported to build knobs and remodel the host cytoskeleton (102), conferring these adherence properties which allow the parasite to massively grow and replicate.

A number of other exported proteins localize close to the knobs and might cross-link or anchor them to the cytoskeleton and play a role in the structural integrity and shape of knobs. PHIST proteins have been implicated as linkers between cytoskeletal and exported parasite proteins (65, 103). PFE1605w (LyMP), a member of the PHIST family, has been shown to interact with band 3 (104) and a number of acidic terminal segment (ATS) domains of PfEMP1 (104–106). PFI1780w, another PHIST protein, also has been shown to bind the ATS domains of some PfEMP1 molecules (103). The same ATS domain of PfEMP1 was shown to interact also with α-spectrin via its repeat 17 (93). There is controversial evidence that KAHRP anchors PfEMP1 to the knobs (90, 107), while an interaction with actin potentially provides another link to the cytoskeleton (108). Some of these interactions have been observed only *in vitro* and have not been confirmed otherwise.

Anchoring of MCs. In ring-stage parasites, Maurer's clefts (MCs) are mobile and become arrested during the transition to trophozoites at around 20 to 24 hpi (109, 110).

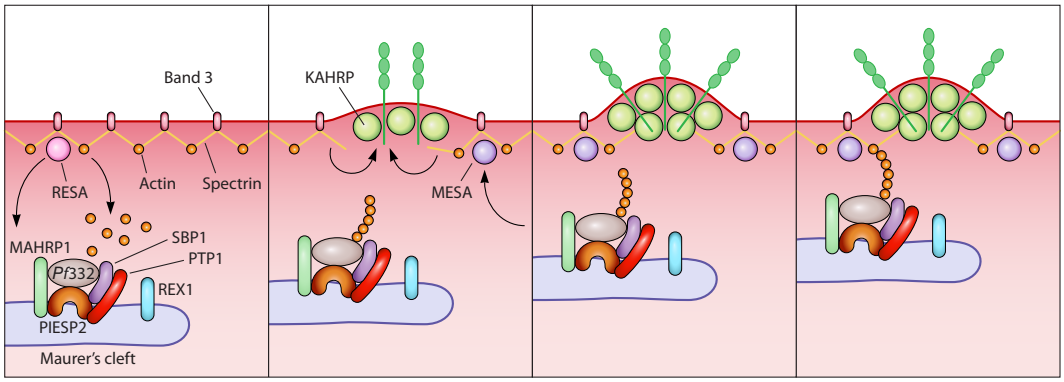

FIG 3 Hypothetical changes during the transition phase. This figure summarizes and chronologically orders the events occurring during the transition from the ring stage to the trophozoite stage. RESA disappears from the cytoskeleton, and actin mining starts (box 1). Knobs are formed, PfEMP1 is found on the surface, and MESA targets the cytoskeleton. Spectrin ends are connected to knobs, and growing actin filaments connect Maurer's clefts to the iRBC cytoskeleton (boxes 2 and 3). MCs are now arrested and in closer proximity to the iRBC membrane (box 4). The figure in part was inspired by reference 122 and additional information from references 83 and 199.

The tethering to the cytoskeleton was already proposed a decade ago, but no mechanism could be shown (94, 111). Recently, two links of MCs to the cytoskeleton have been described, one being mediated by actin filaments. In the erythrocyte cytoskeleton actin filaments are quite short, only 35 to 37 nm in length, and connect multiple ends of spectrin tetramers in the junctional complexes (25). Actin mining and remodeling in the maturing parasite might explain the source of actin used to generate the 40- to 950-nm-long filaments which extend inwards into the iRBC and which make the skeleton three dimensional (82, 111, 112). These new actin filaments show branching points normally not seen in uninfected erythrocytes (25, 82), and cryo-electron tomography showed that these remodeled actin filaments often start close to knobs and connect to MCs (82, 83, 112). The capacity of PfEMP1 and KAHRP to bind actin could indicate their involvement in anchoring the remodeled actin filaments to the iRBC cytoskeleton (85, 89, 108). At the MC, the two proteins PfEMP1 transport protein 1 (PfPTP1) and Pf332 have been found to be essential for the attachment of the remodeled actin filament (83, 113–116). Both proteins show peak expression during the transition phase and have been detected in MCs at transition until egress (83, 116, 117). PfPTP1 not only links MCs to actin filaments but also seems to play a role in remodeling and organizing these filaments (83). Pf332 has been shown to bind actin in a noncompeting way with PfEMP3, which additionally also binds spectrin (115, 118). The MC-resident protein skeleton-binding protein 1 (SBP1) shares its expression pattern and localization with PfPTP1 and Pf332 (114, 119), and it was proposed that these three proteins form a complex (83). Phosphorylated SBP1 further interacts with LANCL1, a human protein that is recruited to MCs (120, 121). Another MC protein, PFE60, also known as parasite-infected erythrocyte surface protein 2 (PIESP2), interacts with MC-associated histidine-rich protein 1 (MAHRP1), SBP1, and Pf332 but was shown not to colocalize to PfEMP3 in immunofluorescence assays, indicating that PfEMP3 localizes to another subcellular location (122), most probably the iRBC cytoskeleton. The role of PfEMP3 remains elusive, but it possibly could bind native actin filaments, or it could provide the anchoring point of remodeled actin filaments to the cytoskeleton. Figure 3 presents a possible scenario of how the actin filaments are anchored to the MCs and the cytoskeleton. The questions of how newly growing actin filaments are directed toward MCs and how they are stabilized remain. Although disputed, one potential candidate could be *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2), which has the capacity to stabilize and bind to actin filaments at acidic pH and has been shown to localize to the iRBC periphery (123).

The importance of actin remodeling and the link to MCs is supported by observations with hemoglobinopathic erythrocytes. Oxidative stress on hemoglobinopathic

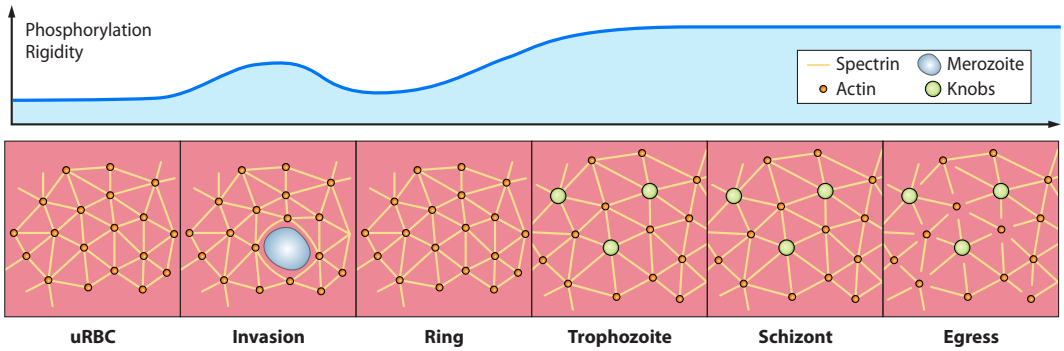

FIG 4 Cytoskeleton time course in asexual stages. (Upper panel) Changes in phosphorylation level and cellular rigidity over the course of the intraerythrocytic asexual development of *P. falciparum* (approximations). (Lower panel) Changes occurring in the spectrin network over the course of development, as described in references 40, 87, and 188.

iRBCs impaired the growth of actin filaments and caused MCs to be distorted and to retain their mobility during mature stages. This coincides with a decreased replication rate and reduced levels of protein export to the iRBC skeleton and membrane, and no PfEMP1 was found on the iRBC surface. It has been proposed that cargo vesicles would be moved along actin filaments toward the iRBC membrane by actin treadmilling (73, 82, 83, 112, 124, 125).

Another connection of MCs to the cytoskeleton is mediated by tethers consisting mainly of the exported small MAHRP2 protein (7, 126, 127), but no anchor point at MCs or at the cytoskeleton is known, and no further function has been assigned to these structures.

Both events, i.e., linking the MCs to the cytoskeleton and knob formation, seem to occur at the same time, and we propose that knob formation and MC arrest require free spectrin ends for stabilization and anchoring, which in turn locally frees up actin, which is repurposed into filaments responsible for vesicular cargo transport to the cytoskeleton. Because this would weaken the skeletal stability, exported parasite proteins must interact with cytoskeletal proteins to enhance stability. The number of exported proteins targeting the iRBC skeleton at this life cycle stage is consistent with the model. In this process of refurbishing of the iRBC, a number of questions remain, such as what triggers the process, how it is orchestrated, and whether it would be possible to interfere with it.

Further changes during the ring-to-trophozoite transition. Once MCs are arrested, knobs are formed, the iRBC cytoadheres, and the parasite starts its rapid growth. The completion of these host cell modifications is seen as the end of the transition phase (73, 109). The cytoadhesive properties of iRBCs seem to be in a gradual process under flow conditions, with the still-rather-biconcave trophozoite-stage-infected cells flipping on the endothelium, while the more spherical schizont-stage-infected cells turn to a rolling adhesion (128).

Trophozoite Stage

As a result of the cytoskeletal modifications, the spectrin network size increases in trophozoites (129). Computer modeling suggested that additional linkages between the cytoskeleton and the membrane which are caused by knob structures can account for the observed increased rigidity (Fig. 4) (130).

Similar to the case during the ring stage, during the trophozoite stage few major changes to the host cell seem to occur. It is, rather, the parasite's most metabolically active phase during the intraerythrocytic cycle (131) and prepares the parasite for replication and formation of daughter cells. Although most cytoskeleton remodeling occurs during the transition from ring to trophozoite, some of these changes gradually continue throughout the trophozoite stage and probably even until egress. The first knobs appear at around 20 hpi, and their numbers increase linearly while their size decreases until 36 hpi (132, 133), which marks the end of the trophozoite stage (58).

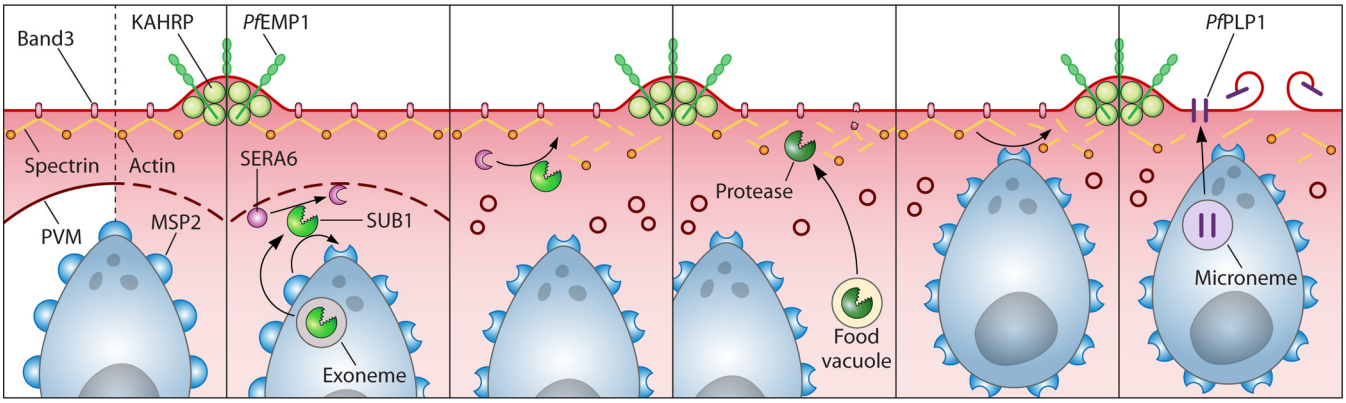

FIG 5 Overview of the events occurring during merozoite egress About 10 to 30 min before egress, the PVM is permeabilized (box 1). Triggered by PfPKG, the parasite protease SUB1 is activated and discharged from exonemes. SUB1 then cleaves and activates SERA6 and the merozoite surface protein MSP1 (box 2). The permeabilized PVM eventually forms multilamellar vesicles. SERA6 and SUB1 degrade cytoskeletal proteins (box 3). Other parasite proteases previously found in the food vacuole (box 4) as well as matured MSP1 also assist in cytoskeleton breakdown (box 5). The pore-forming protein PfPLP1 is secreted from the micronemes and lyses the iRBC membrane, which eventually curls (box 6). The arrows indicate movement of proteases as well as their substrate-processing activity.

This constant remodeling indicates that protein export still occurs and that those proteins accumulate within the iRBC or at its cytoskeleton.

Schizont Stage

During the schizont stage, daughter cells are produced, which subsequently will reinvade new host cells. A series of genome replications and nuclear divisions occur, and individual merozoites are formed by segmentation (134). Protein synthesis during this phase is focused on merozoite proteins and proteins that are required for invasion (72). At this time, the number of knobs decreases (132, 133), and there is some evidence that the erythrocyte cytoskeleton is already being dismantled up to 15 h prior to egress, which corresponds to onset of the schizont stage. Proteins associated with junctional complexes such as adducin and tropomyosin also are lost from the cytoskeleton, indicating that some remodeling or dismantling occurs. This is accompanied by an increased spectrin mesh size and the temporal appearance of holes in the cytoskeleton (135, 136). This might be the gradual process of dissolving the iRBC cytoskeleton described previously (112), but it is unclear why egress would start so early, since the structural integrity must still be maintained until the merozoites are fully developed. Most probably the final, complete destruction of the cytoskeleton before egress is a fast and well-orchestrated process, as recent evidence suggests (137, 138).

Egress

Parasite egress has been described by different models, but irrespective of the model, the actual processes all require degradation of the PVM and the iRBC cytoskeleton and membrane to release the newly formed merozoites. In contrast to invasion, egress is a rather fast, well-orchestrated, destructive, protease-mediated cascade of events which leads to the breakdown of the iRBC cytoskeleton and membrane to release the merozoites. Although not all details are known, we have summarized and temporally ordered these events, leading to a model describing egress (Fig. 5).

Approximately 10 to 30 min before egress, the PVM is permeabilized, subsequently ruptures, and forms multilamellar vesicles, allowing passage of effector proteins that are secreted from the merozoite to reach the iRBC periphery (138). Minutes before egress, *P. falciparum* cGMP-dependent protein kinase (PfPKG) mediates activation of subtilisin-like protease 1 (SUB1), which is then discharged from the merozoite exonemes into the parasitophorous vacuole (PV) (134, 139). Further, SUB1 cleaves and thus activates several substrates, such as MSP1/6/7, serine-repeat antigens 4, 5, and 6 (SERA4/5/6), and others (140–142).

SERA6 cleaves β-spectrin at its actin-binding site, leading to disruption of the connection between the spectrin tetramers and the junctional complex. This is essential for the breakdown of the iRBC cytoskeleton and the final release of merozoites (137, 138). Interestingly, SERA8, another member of the SERA family, was found to be essential in egress of sporozoites from oocysts, indicating that SERA proteases might play a general role during egress of infective *P. falciparum* stages (143). SERA5 has also recently been implicated in egress, although its exact mechanism has not yet been deciphered. An increase in intracellular Ca^{2+} was demonstrated to activate the parasite kinase *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1), which in turn phosphorylates SERA5 (144).

Besides SERA6, other proteases have been shown to assist in iRBC cytoskeleton and membrane breakdown during egress. SUB1 cleaves spectrin and probably band 3 (31, 137). Additional proteases, such as calpain-1, falcipain, and plasmepsins (50, 135, 145–147), have also been found to be involved in this process, and some of these proteases usually localize in the food vacuole (148). Hence, the same processes that perforate the PVM might also perforate the food vacuole and thus release these proteases into the iRBC cytosol. At the same time, SUB1 cleaves surface-bound MSP1, which assists in spectrin breakdown due to its spectrin-binding capacity and potentially interferes with spectrin tetramer stability (140, 149). Other proteins degraded during egress are MESA, ankyrin, and band 4.1 (135, 146, 150).

Through an increase of intracellular Ca^{2+}, perforin-like proteins PfPLP1 and -2 are discharged from the micronemes. PfPLP1 was shown to possess membranolytic abilities, potentially forming pores in the iRBC membrane (151) and leading to membrane curling, which has been described as part of egress. Mechano-physical models suggested that the degradation and restructuring of the cytoskeleton contribute to membrane curling observed *in vivo* (152–154).

As reviewed in references 155, 156, and 157, other models of egress have been suggested, but the model presented here summarizes the most current information and is likely correct because all effector proteins involved in egress are stored in merozoite organelles and can be rapidly discharged to start the cascade of events that result in the release of merozoites. This ensures that degradation of the host cytoskeleton occurs only when merozoites have been formed, making egress a fast and regulated process.

Gametocytes

During each intraerythrocytic developmental cycle, a few *P. falciparum* parasites commit to sexual development and develop into gametocytes. In contrast to the case for all other human malaria parasites, *P. falciparum* gametocytes become sequestered in the bone marrow while they are developing through stages I to IV. After gametocytes have completed their development (10 to 12 days), mature gametocytes (stage V) reenter the bloodstream to be transmitted to mosquitos, where they complete sexual development (1, 158). Little is known of the modifications that occur at the host cytoskeleton during the development of gametocytes, but phosphorylation and changes of rigidity seem to be essential for the reentry into the bloodstream.

While host cytoskeleton remodeling causes morphological changes in asexual stages, morphological changes in gametocytes seem to be caused mostly by changes of the parasite's own cytoskeleton and its inner membrane complex (159). Changes of its own skeleton mostly seem to contribute to changes in cellular rigidity during sexual development (129, 159, 160). Also, during gametocyte development many parasite proteins are exported, but their function and potential role in the host cell remain elusive (158, 161). Great morphological differences between mature gametocytes and asexual stages suggest that different remodeling processes and targets might be involved in the generation of those differently shaped intracellular parasites.

Gametocyte stages I to IV. The cytoskeleton of gametocyte-infected erythrocytes (GIEs) is targeted during sexual development, with actin remodeling occurring when stage III and V gametocytes were investigated, but there is no evidence of actin mining

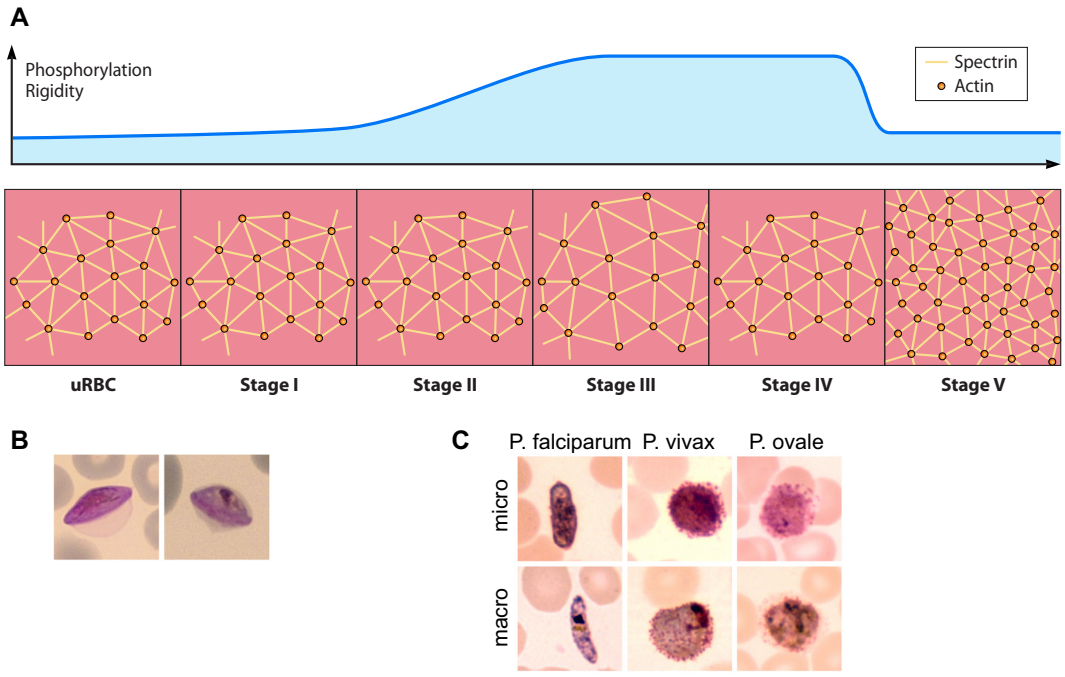

FIG 6 Cytoskeleton time course during gametocyte development. (A) Upper panel, changes in phosphorylation level and cellular rigidity over the course of gametocyte development (approximations). Lower panel, changes occurring in the spectrin network over the course of development, as described in reference 129. (B) Giemsa-stained stage III *P. falciparum* gametocytes (image courtesy of A. Passecker). (C) Giemsa-stained macro- and microgametocytes of several human *Plasmodium* species, showing the unique morphology of sequestering *P. falciparum* gametocytes (image courtesy of Y. Endriss).

as observed in trophozoites, and MCs are not tethered to the cytoskeleton via actin filaments. The number of actin junctions is reduced by 18% in stage III compared to stage I, the size of the spectrin meshwork increases considerably until stage III, and lateral mobility of band 3 is reduced, all leading to decreased deformability of the GIEs (129). The degree of reduced deformability is similar to that in trophozoites (Fig. 6A) (159). It has been shown that the serine residue S_{324} of the subtelomeric variant open reading frame protein (STEVOR), which binds to the host cell cytoskeleton and is present only in *P. falciparum*, is phosphorylated during stages I to IV (162). It remains to be seen whether this protein kinase A (PKA)-mediated phosphorylation is the sole contributor to increased rigidity of gametocytes.

During gametocyte development from stage I to IV, morphological changes are accompanied by a constant increase of rigidity (163), leading to sequestration in the bone marrow and spleen (158). The process of gametocyte sequestration until the end of stage IV is mostly unknown, but PfEMP1 is observed at very low levels in stage I (164–167), and GIEs have no knobs on the surface. Hence, it is likely that the STEVOR protein family might play an essential role in gametocyte sequestration of *P. falciparum* (162, 168). Figure 6B shows micro- and macrogametocytes of several *Plasmodium* species, highlighting the unique morphology of *P. falciparum* gametocytes.

Gametocyte stage V. Stage V gametocytes, however, must reach the blood circulation again to be taken up by mosquitoes during a blood meal to ensure transmission. In order to circulate, GIEs must become flexible again, and previous remodeling steps up to stage III seem to be reversed in stage V. In stage V, the width of the spectrin network decreases (129), deformability suddenly increases (163), and the lateral mobility of band 3 and the number of actin junctions increase again to levels comparable to those in uninfected RBCs (129). Hence, modifications in gametocytes seem to be mostly reversible. Even the previously phosphorylated S_{324} residue of STEVOR becomes dephosphorylated (162) and dissociates from the GIE membrane (163). It is important to understand this process to identify potential targets to block transmission.

TYPES AND MECHANISMS OF CYTOSKELETON REMODELING

Phosphorylation

Phosphorylation and dephosphorylation by a number of human kinases, such as cAMP-dependent kinase (137) or protein kinase C (80), modulate the properties of the erythrocyte cytoskeleton and membrane (79, 169–172). *P. falciparum* hijacks the human system to alter the iRBC skeleton according to its needs (56) in a stage-specific manner (71, 173). In addition, the parasite exports some of its own kinases into the host cell, such as members of the FIKK family, which are dramatically expanded in *P. falciparum*, or a casein kinase (174, 175).

During merozoite invasion, phosphorylation plays an important role (24, 34, 37, 38, 52, 176) when phosphorylation of cytoskeletal proteins causes the cytoskeleton to locally detach from the membrane at the site of merozoite attachment (Fig. 1, 2, and 4). This promotes membrane wrapping pushing the merozoite inwards (37, 54–57) and facilitates invasion without destruction of the cytoskeleton. Most host cytoskeleton proteins seem to be dephosphorylated during early parasite development (177), suggesting that phosphorylation and further modifications of cytoskeletal proteins do not play a major role during the first half of the life cycle.

As described above, transition from the ring stage to the trophozoite stage is accompanied by extensive remodeling of the cytoskeleton and is mediated by interaction of exported parasite proteins with host proteins. Some of these protein-protein interactions require phosphorylation, and the level of phosphorylation (177), such as serine and tyrosine phosphorylation, increases at this time (71). Accordingly, Treeck et al. identified in a proteomic study hundreds of phosphoproteins, both human and parasite proteins (178), including proteins associated with the cytoskeleton or knobs (71).

Despite the large number of phosphorylated proteins, little is known about the kinases and phosphatases involved in this process. At least 20 parasite kinases are thought to be exported (174), and FIKK4.2, one of the exported kinases, shows peak expression during the late ring and early trophozoite stages (174, 179), coinciding with the transition phase when all structural changes and host cell remodeling occur. Depletion of FIKK4.2 causes increased iRBC rigidity, reduced knob count on the iRBC surface, and impaired host cell remodeling (179). Because phosphorylation is linked to cytoskeleton remodeling and knob formation, it was proposed to influence cytoadhesion (74, 81, 174).

At the end of the intraerythrocytic development, proteins begin to become dephosphorylated (71), most likely partially reversing previous cytoskeleton remodeling steps and thus weakening the cytoskeleton in preparation for merozoite release (71). Phosphorylation plays a central role in modulating host cell alterations at the beginning of the asexual life cycle and dephosphorylation at the end of the cycle. A similar dynamic of phosphorylation is also observed during gametocyte development (180–182).

Altered Rigidity and Deformability

The ability of a cell to change its shape under predefined conditions without hemolysis is defined as deformability (160), but terms such as rigidity and stiffness are used interchangeably in this review. The structural integrity and deformability of the host cell cytoskeleton are important for the survival of *P. falciparum* and are stage-specifically modulated at each stage of the life cycle (Fig. 4 and 6).

During invasion, a temporary increase in iRBC deformability is required (31, 52), and the degree thereof correlates with the success of invasion (33). As phosphorylation is reversed after invasion, deformability is reverted to the original state. There is limited membrane stiffening, mostly attributed to the effect of RESA interaction with the spectrin network (64). However, no further cytoskeleton remodeling during the first half of the asexual life cycle is known, and ring-stage iRBCs circulate and pass through the spleen despite this reduced deformability (183, 184).

In the trophozoite and schizont stages, the shape of the host cell changes, and it sequesters to the endothelial capillary lining. There is an increase in phosphorylation

(177), but in contrast to phosphorylation during invasion, there is no partial dissociation and weakening of the cytoskeleton, but it facilitates protein-protein interactions which contribute to increased rigidity (183, 184). In addition, metabolic products from the parasite exert oxidative stress which also contributes to the rigidification of the iRBC cytoskeleton (112, 185).

Exported proteins. Computer simulations suggested that the stiffening effect during the trophozoite and schizont stage is caused mainly by newly formed knobs providing vertical linkages between the spectrin cytoskeleton and the membrane rather than by direct remodeling of the spectrin network (130). Since several knob-resident proteins interact directly with spectrin, the formation of knobs seems to depend on cytoskeleton remodeling. Knobless parasites also show an increase in rigidity, albeit much less than knob-positive parasites (186), which suggests that other factors are involved in changes of cytoskeletal deformability. Over the years, reverse genetic studies have identified a number of interactions of exported proteins, which in most cases lead to increased rigidity (28, 64, 113, 187, 188).

Chaperones. Although not directly binding to or interacting with the cytoskeleton, chaperones are also of importance to host cytoskeleton remodeling. Among the exported *P. falciparum* proteins are several chaperones (17) and seven PHISTb proteins containing a DnaJ domain (189). DnaJ domains have been shown to interact with or recruit parasite heat shock proteins for use in the remodeling process (190). A parasite cell line deficient in Hsp70-x, an exported parasite chaperone, showed higher retention rates in microsphiltration, indicating increased rigidity (191), which suggests that chaperones might play a role in remodeling the cytoskeleton.

Protein Carbonylation

Reactive oxygen species lead to protein carbonylation (192), and although not controlled by the parasite, carbonylation of host membrane and cytoskeleton proteins can affect the integrity of the cytoskeleton. Carbonylation of iRBCs has been observed at the transition from ring to trophozoite and lasting throughout the trophozoite stage. This correlates in time with hemoglobin metabolism and generation of free radicals. All major cytoskeleton proteins, such as spectrin, actin, ankyrin, band 4.1, and band 4.2, were found to be carbonylated (193). Hence, through hemoglobin metabolism the parasite indirectly influences the rigidification of the host membrane, and some of the remodeling mediated by exported parasite proteins might counteract the effects of carbonylation.

Protein Features

Many exported proteins contain motifs or charge distributions that can target exported parasite proteins to the cytoskeleton. The MEC motif found in MESA is present in a number of other exported proteins, some of which have been shown to localize to the iRBC cytoskeleton or to have an effect on rigidity (78). Similarly, lysine-rich repeats in a group of exported proteins were identified and shown or predicted to target the cytoskeleton (194). In several exported proteins (e.g., Pf332, SURFIN, and PfEMP1), tryptophan-rich domains interact with actin and spectrin (195, 196). A large number of exported proteins share a *Plasmodium* helical interspersed subtelomeric (PHIST) domain, and several PHISTb members with an extended PHIST domain were targeted to the iRBC periphery (197). Finally, other exported parasite proteins were found to contain an amino acid sequence which mediates binding to band 4.1 (198). Overall, many proteins have been identified through molecular or bioinformatics approaches to be potentially involved in cytoskeleton remodeling; however, there is a significant redundancy of interacting proteins, and each might not necessarily be involved in a particular interaction. Thus, many of these proposed interactions need to be confirmed in the future.

CONCLUSION

Host cell remodeling by *P. falciparum* with regard to the cytoskeleton can be divided into three different phases: invasion, the transition phase between ring and trophozoite

stages, and egress. During invasion, the erythrocyte plasma membrane, as a barrier, has to be crossed in a conservative way that restores its properties and allows intracellular growth. This also includes the cytoskeleton stabilization when the cell is exposed to fever-induced thermal stress. At the transition phase of asexual growing parasites, knobs are formed, conveying cytoadhesive properties to the infected cell. In contrast, sequestration of gametocytes must be reversible and hence requires different modifications at the host cell cytoskeleton. During egress, the parasite crosses the cytoskeleton again but this time in a more destructive way.

Cytoskeleton remodeling has been shown to be the key actor for these events, but little is known about events occurring between these steps. The available data suggest that the cytoskeleton is in a dynamic steady state.

Here we have shown that the iRBC cytoskeleton is the interface of most host-parasite protein-protein interactions that are essential for intracellular development of *P. falciparum*. The identification of key players involved in these major remodeling events could potentially provide new targets both to inhibit growth of the malaria parasite and also to inhibit transmission.

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