Plasmodium falciparum erythrocyte-binding antigen 175 triggers a biophysical change in the red blood cell that facilitates invasion

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Invasion of the red blood cell (RBC) by the *Plasmodium* parasite defines the start of malaria disease pathogenesis. To date, experimental investigations into invasion have focused predominantly on the role of parasite adhesins or signaling pathways and the identity of binding receptors on the red cell surface. A potential role for signaling pathways within the erythrocyte, which might alter red cell biophysical properties to facilitate invasion, has largely been ignored. The parasite erythrocyte-binding antigen 175 (EBA175), a protein required for entry in most parasite strains, plays a key role by binding to glycophorin A (GPA) on the red cell surface, although the function of this binding interaction is unknown. Here, using real-time deformability cytometry and flicker spectroscopy to define biophysical properties of the erythrocyte, we show that EBA175 binding to GPA leads to an increase in the cytoskeletal tension of the red cell and a reduction in the bending modulus of the cell's membrane. We isolate the changes in the cytoskeleton and membrane and show that reduction in the bending modulus is directly correlated with parasite invasion efficiency. These data strongly imply that the malaria parasite primes the erythrocyte surface through its binding antigens, altering the biophysical nature of the target cell and thus reducing a critical energy barrier to invasion. This finding would constitute a major change in our concept of malaria parasite invasion, suggesting it is, in fact, a balance between parasite and host cell physical forces working together to facilitate entry.

erythrocyte | malaria | real-time deformability cytometry | flicker spectroscopy | merozoite

Malaria infections cause ~438,000 deaths per year, most of which are due to the protozoan parasite *Plasmodium falciparum* (1). Although extensive eradication efforts have helped to reduce the incidence of malaria, the spread of drug resistance is a growing concern and novel treatments are urgently needed (2). Throughout the *Plasmodium* life cycle, parasites shuttle between replicative and motile life-cycle stages, with the motile forms or "zoites" highly adapted to the invasion of host cells. During the blood stages of infection, the merozoite targets and invades the red blood cell (RBC) rapidly (< 30 s) in a process that involves numerous parasite ligands and host cell receptors (3). Merozoite entry is a multistep process commencing with initial attachment and ending with parasite actomyosin-driven invasion (4). Although extensive cellular and molecular details of each step have been elucidated (5, 6), there is still little mechanistic understanding of the role of each protein involved or the signaling events within the erythrocyte that accompany entry (7).

Initial attachment to the RBC surface is likely mediated by merozoite surface proteins. Subsequently, the key signaling and strong attachment interactions between host and parasite membranes are thought to be mediated by two major classes of adhesins released either before or concomitant with invasion at the merozoite apex. These adhesins are the erythrocyte binding-like [EBL, or erythrocyte-binding antigen (EBA)] and reticulocyte binding-like (RBL, or Rh) protein families, composed in *P. falciparum* of the EBA175, EBA140, EBA181, and EBL1 proteins and the Rh1, Rh2a, Rh2b, Rh4, and Rh5 proteins, respectively (8, 9). Although the binding between Rh5 and its receptor, Basigin, is the only ligand–receptor interaction identified to date that is essential for all *P. falciparum* strains (10), EBA175 and its interaction with glycophorin A (GPA) is nonetheless functionally almost universally important (11). When EBA175 function is ablated (e.g., genetically), some strains are able to switch to an alternative invasion pathway using the interaction of Rh4 and its receptor CR1 (12). The precise function of each ligand–receptor complex during invasion is largely unknown; however, in recent years, it has become clearer that these interactions do not merely serve for adhesion but, instead, may be involved in transducing signals into either the merozoite (13, 14) or potentially the RBC (7).

Recently, we attempted to model invasion in terms of the biophysical forces required to enwrap a merozoite toward full RBC entry. Of particular focus were the properties of membrane tension (which, in the RBC, is primarily provided by the cytoskeleton) and bending modulus (the energy required to cause bending of the lipid bilayer) (15). Although the actin-myosin motor provides a critical force to push the merozoite into the host cell, other factors could significantly contribute to lowering the

Significance

The blood-stage malaria parasite, the merozoite, invades the human red blood cell (RBC) using receptor–ligand interactions between the parasite and host cell surface, yet the function of these interactions to invasion is not known. We have analyzed the binding between one key merozoite invasion ligand, called erythrocyte-binding antigen 175 (EBA175), and glycophorin A on the RBC surface (the most dominant surface antigen) and explored how this interaction affects the biophysical properties of the red cell. Using a combination of imaging techniques, we demonstrate that the malaria parasite changes the biophysical nature of the red cell, facilitating its own entry by effectively reducing the energy barrier to invasion. This study demonstrates a red cell biophysical contribution to merozoite entry.

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Conflict of interest statement: O.O. is a cofounder of a company commercializing real-time deformability cytometry.

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energy barrier for invasion. These factors include energy gains from tight adhesion to the host cell (via receptor–ligand interactions) or manipulation of the host cell cytoskeletal or membrane properties, each of which could facilitate wrapping of the merozoite. Beyond malaria invasion biology, binding of two critical red cell receptors used by the merozoite during invasion (CR1 and GPA) have been shown to transmit biophysical changes to the RBC when bound by certain ligands (16, 17). To date, it has not been assessed whether parasite ligands during invasion transmit similar changes, potentially influencing invasion efficiency.

Here, we explore the hypothesis that parasite ligands prime the RBC by changing its biophysical properties, reducing the energy barriers for invasion. Using two separate techniques, we demonstrate that binding of the merozoite ligand EBA175 to its receptor GPA causes distinct biophysical changes to the RBC. EBA175 binding causes an increase in cytoskeletal tension, which is dependent on the cytoplasmic tail of the GPA protein. EBA175 binding also causes a reduction in the bending modulus of the membrane. Chemically induced reduction of the bending modulus significantly increases invasion efficiency, supporting our hypothesis that parasite-dependent RBC changes reduce the energy barrier for successful malaria parasite entry into the human erythrocyte.

Results

EBA175 Region II Binding to GPA Alters RBC Deformability. Theoretical models and phosphoproteomic studies suggest that the *P. falciparum* merozoite triggers host changes to facilitate its invasion on binding to the RBC (7, 15). To explore whether these changes are mediated by EBAs, we investigated the effect of EBA175 binding to its RBC receptor, GPA, on overall erythrocyte biophysical properties. This receptor–ligand interaction is specific; it is mediated by glycosylated residues on GPA that are absent in other glycophorin molecules (18). Using a recombinantly expressed receptor-binding region (region II) of EBA175 (rEBA175-RII) (19, 20), we first validated receptor specificity of the protein incubated with untreated or neuraminidase (Neu)-treated human RBCs (Fig. 1*A*), with the latter ablating sialic acid residues on GPA, which are known to be crucial for binding (21).

Having demonstrated the Neu-binding specificity of the rEBA175-RII ligand, we next incubated RBCs with different concentrations of the protein before analyzing cells using real-time deformability cytometry (RT-DC) (22). This flow cytometry-based technique permits the label-free analysis of ∼1,000 cells per second, making it particularly useful for analyzing cells that show natural variability in their biophysical characteristics, such as RBCs (23). For untreated cells, analysis of deformation versus cell size (cross-sectional area) found that most cells were contained within one population, with an average cross-sectional area of 36.5 μm^2 (Fig. 1*B*). A second population of larger cells (>50 μm^2), when manually inspected, was found to contain cell clumps, predominantly doublets (Fig. 1*B*). Because RT-DC analysis is based on extracting cell contours to quantify cell deformation, and contours of doublets could potentially lead to a skew in the data dependent on the shape of the cell clump, such clumps were removed from downstream analyses by creating a gate containing the singlet RBC population (Fig. 1*B*). Incubation of RBCs with rEBA175-RII resulted in many of the cells forming larger clumps (Fig. 1*C*). These aggregates were removed from our analysis as described above. RT-DC measurements of RBCs treated with 25–50 μg/mL rEBA175-RII, a concentration similar to the concentration previously used for antibody studies (24), revealed a significant reduction in RBC deformability (Fig. 1*D*). At higher concentrations of rEBA175-RII, a significant proportion of RBCs were present in cell doublets or clumps, complicating analysis using this technique. To test the specificity of these identified biophysical changes, rEBA175-RII was also incubated with Neu-treated RBCs (Fig. 1*E*). These cells do not show a decrease in deformability, confirming this effect to be specific to rEBA175-RII binding.

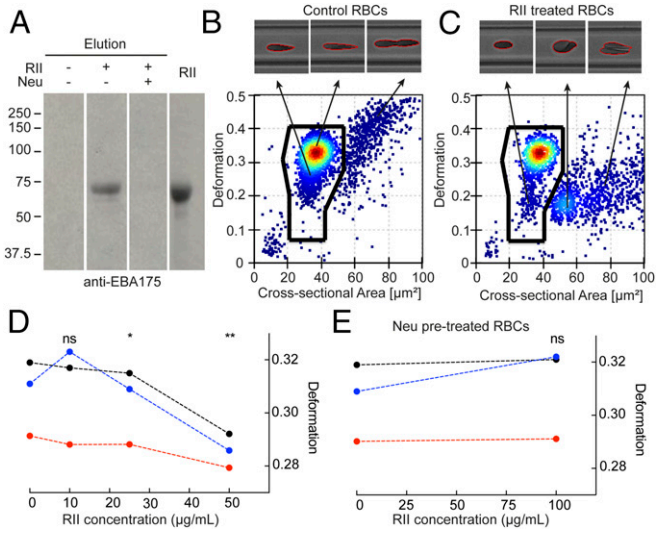

Fig. 1. rEBA175-RII–mediated RBC biophysical changes. (*A*) Erythrocyte-binding assay with rEBA175-RII (labeled as RII). Untreated human RBCs and Neu-treated RBCs were incubated with rEBA175-RII. Bound protein was eluted with NaCl, and the presence of rEBA175-RII was evaluated by Western blotting. (*B*) Scatter plot of deformation versus cross-sectional area of control RBCs. Secondary populations of "larger cells" contains cell doublets or clumps. (Scale, channel width 20 μm.) (*C*) rEBA175-RII–treated RBCs caused a much higher percentage of cells to clump. These cells were removed from analysis of all samples. (Scale, channel width 20 μm.) (*D*) RBCs from three different donors (red, blue, and black lines) were exposed to a range of rEBA175-RII concentrations and show a concentration-dependent reduction in RBC deformation. (*E*) Deformation of RBCs pretreated with Neu was not significantly affected by treatment with rEBA175-RII. *P* values comparing the treatment versus control were calculated using linear mixed models (*$P < 0.05$; **$P < 0.01$). ns, nonsignificant.

Flicker Spectroscopy of RBC Biophysical Parameters. To determine the cause of biophysical changes seen by RT-DC, we sought to use a method that would permit independent measurement of the tension and bending modulus of the RBC surface on EBA175 binding. Membrane fluctuation analysis (flicker spectroscopy) has been used extensively as a method for mechanical characterization of vesicles (25, 26) and erythrocytes (27–29) at the single-cell level. Critically for our purposes, the cytoskeletal and lipid membrane properties dominate different ranges of the fluctuation spectrum. It is well established that tension in RBCs largely arises from the cytoskeleton underlying the erythrocyte bilayer and that changes in tension are primarily reflected in the lower fluctuation mode numbers (Fig. S1). Conversely, variations in the RBC bending modulus are largely expected to come from changes to the cell membrane, and this parameter is reflected in the higher mode numbers (27) (Fig. S1).

Although many chemicals known to induce cellular biophysical changes have wide-ranging effects causing both cytoskeletal and membrane alterations, we sought to validate the assumptions outlined above by inducing several controlled biophysical changes in RBCs to confirm the reliability of flicker spectroscopy in detecting erythrocyte cytoskeletal and membrane changes. Glutaraldehyde preferentially cross-links proteins, and is therefore expected to affect cytoskeletal properties (i.e., tension), although it can interact with and cross-link amino groups on phospholipids (30). In agreement with this expectation, at 0.01% glutaraldehyde, RBC tension increased significantly (Fig. 2*A*), with no significant effect on bending modulus (Fig. 2*B*). Fig. S2*A* shows representative spectra of individual RBCs from each treatment sample. At higher concentrations, membrane oscillations were reduced to negligible levels (Fig. S2*A*), suggesting that all protein and membrane constituents had been cross-linked. As a further test, we explored diamide, an oxidizing reagent that has previously been shown to cause tightening of the RBC cytoskeleton and reduce RBC deformability (31) (Fig. 2

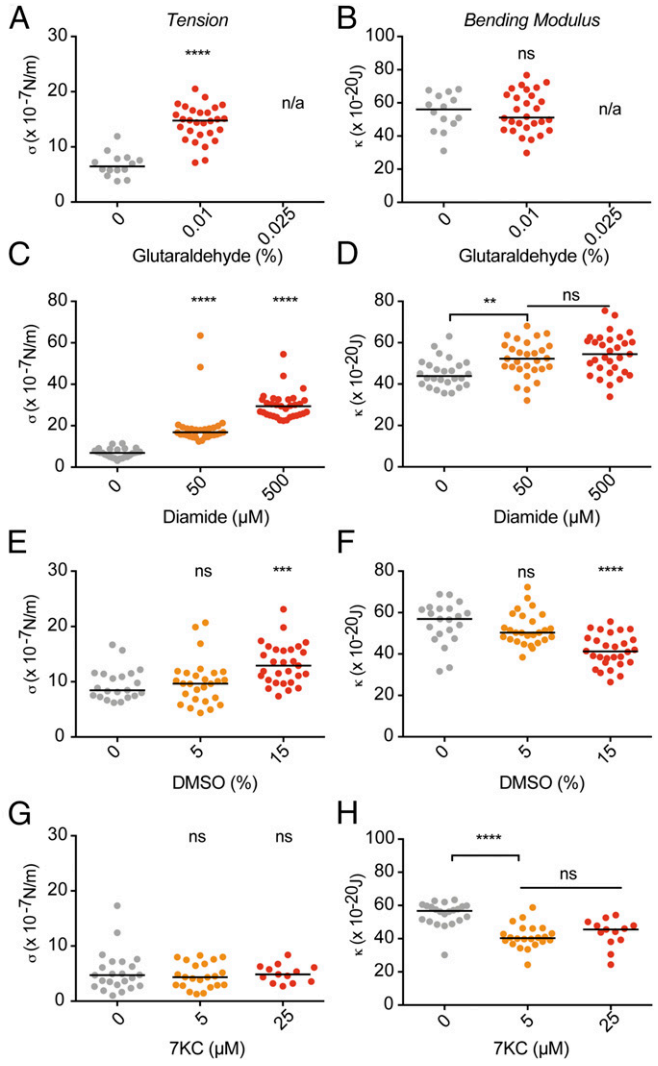

Fig. 2. Chemically induced tension and bending modulus changes in RBCs measured by flicker spectroscopy. Tension (σ) and bending modulus (κ) values of RBCs treated with glutaraldehyde (*A* and *B*), diamide (*C* and *D*), DMSO (*E* and *F*), and 7-KC (*G* and *H*) are summarized. Each circle represents data from a single cell, and the solid line represents the median. The mean square amplitude $[h(q_x)^2\langle m\rangle^2]$ of contour fluctuation modes versus the wave vector (q_x) of representative RBCs is shown in Fig. S2. *P* values comparing the treatment versus control were calculated using the Mann–Whitney test (**$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

C and *D* and Fig. S2*B*). Treatment of RBCs with 50 μM and 500 μM diamide resulted in a concentration-dependent increase in tension (Fig. 2*C*) in line with previously published work (31). Although the exact effect of RBC treatment with diamide on its bending modulus has not previously been tested, the bending modulus change (Fig. 2*D*) is comparable to that seen with other oxidative reagents, which cause lipid peroxidation resulting in higher bending modulus (32) as well as reduced membrane fluidity (33).

We next sought to isolate the effect of changing bending modulus, specifically targeting RBC lipid properties. X-ray diffraction measurements and molecular dynamic simulations suggest that DMSO disrupts interactions between the headgroups of different lipids, decreasing bilayer thickness and increasing membrane fluidity (34, 35). These simulations suggest that at a DMSO concentration of 5%, the average area between the inner and outer membrane layers would be reduced by ∼10%. Similarly, at 15% DMSO, the area reduction would be expected to be >55%. Tests at both concentrations by flicker spectroscopy (Fig. 2 *E* and *F* and Fig. S2*C*) demonstrated that the bending modulus was indeed reduced at both 5% and 15% DMSO (Fig. 2*F*); however, this reduction was only statistically significant at 15%. Of note, at this concentration, some RBCs (omitted from analysis) undergo morphological changes wherein tension (Fig. 2*E*) is also affected, likely due to the stress caused by the high percentage of DMSO used.

As a final way of experimentally validating our flicker approach, we investigated use of 7-ketocholesterol (7-KC), a probe that reduces lipid packing and lipid order by integrating into the RBC membrane (36). We found that 7-KC significantly and specifically reduces bending modulus without affecting tension properties (Fig. 2 *G* and *H* and Fig. S2*D*). Collectively, these data demonstrate our ability to decouple membrane and cytoskeletal changes in the RBC using flicker spectroscopy.

EBA175 Binding to GPA Triggers Specific RBC Cytoskeletal and Membrane Changes. Having established adequate controls, we next investigated how EBA175 binding to the RBC affects cytoskeletal and membrane properties of the target cell. In agreement with the RT-DC data as well as previous biophysical data examining GPA binding by antibody moieties (24), we found a clear concentration-dependent increase in RBC tension on addition of rEBA175-RII (Fig. 3*A* and Fig. S3*A*). At very low concentrations, we noticed a small but reproducible reduction in tension at low rEBA175-RII concentrations, suggesting the RBC biophysical response to rEBA175-RII might be a nonlinear concentration-dependent effect at low concentrations (Fig. 3*A*). Noticeably, this reduction in tension was also present using RBCs pretreated with Neu, supporting the notion that a small number of binding sites are still present following enzyme treatment, giving rise to the small effect alone. For bending modulus, rEBA175-RII binding significantly reduced values across a wide range of concentrations (Fig. 3*B* and Fig. S3*B*). This finding was dependent on sialic acid residues, confirming that the change is

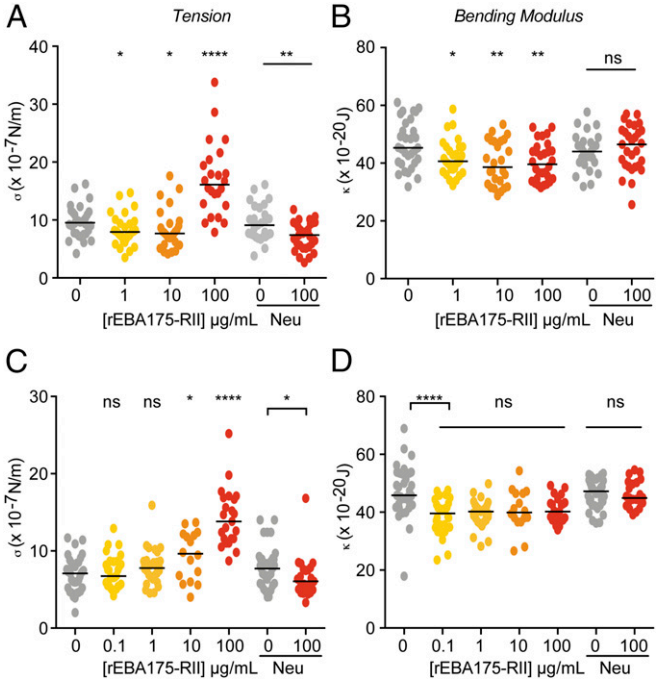

Fig. 3. rEBA175-RII–mediated tension and bending modulus changes measured by flicker spectroscopy. (*A–D*) Summary of tension (σ) and bending modulus (κ) values of RBCs from different blood donors treated with a range of rEBA175-RII concentrations and with Neu. Each circle represents data from a single cell, and the solid line represents the median. Mean square amplitude $[h(q_x)^2\langle m\rangle^2]$ of contour fluctuation modes versus wave vector (q_x) of representative RBCs is shown in Fig. S3. *P* values comparing the treatment versus control were calculated using the Mann–Whitney test (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

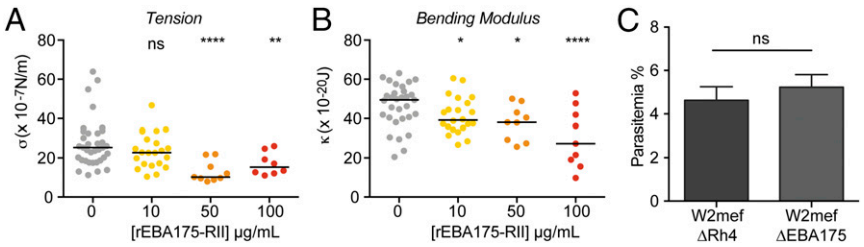

Fig. 4. rEBA175-RII mediated increase in tension is dependent on the GPA cytoplasmic tail. (*A* and *B*) Summary of tension and bending modulus changes in MiV cells following rEBA175-RII incubation. Each circle represents data from a single cell, and the solid line represents the median. Mean square amplitude $[h(q_x)^2(m)^2]$ of contour fluctuation modes versus wave vector (q_x) of representative RBCs is shown in Fig. S4. (*C*) Parasite invasion into MiV cells was measured by flow cytometry. Parasites used were a *P. falciparum* strain that invades using GPA (W2mefΔRh4) and a strain invading independent of this receptor (W2mefΔEBA175). *P* values comparing the treatment versus control were calculated using the Student's *t* test (*$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$).

binding-specific (Fig. 3*B*). The effects of rEBA175-RII on RBC biophysical properties were reproducible across different RBC samples (Fig. 3 *C* and *D* and Fig. S3*B*).

Significance of RBC Tension for Parasite Invasion. To investigate the relevance of the rEBA175-RII–mediated tension and bending modulus changes to parasite invasion, each component was isolated to test its importance independently. Previous studies using anti-GPA antibodies have shown that the binding-mediated decrease in deformability occurs through signaling involving the GPA cytoplasmic tail (24). We therefore hypothesized that in the absence of this region, rEBA175-RII would not initiate an increase in tension. To test this hypothesis, we used RBCs isolated from an individual with an extremely rare human glycophorin variant, the Miltenberger class V (MiV) condition, in which GPA and GPB are replaced with a hybrid GPA-GPB protein. This protein contains the extracellular regions of GPA, but the shortened intracellular tail of GPB (18, 37), which is not predicted to interact with the underlying cytoskeleton as wild-type GPA does. Entirely in line with predictions, rEBA175-RII did not cause an increase in tension in MiV RBCs (Fig. 4*A* and Fig. S4). Indeed, in the absence of the cytoplasmic GPA tail, EBA175 binding caused a small but noticeable decrease in tension instead. Unexpectedly, the bending modulus decrease still occurred in MiV cells (Fig. 4*B* and Fig. S4), suggesting that this biophysical change is independent of the interaction between the cytoplasmic tail of GPA and the RBC interior.

Although the importance of the glycophorins for erythrocyte invasion by *P. falciparum* is indisputable (12, 18, 38), some parasite strains have evolved to invade independent of GPA. If the GPA tail-mediated tension change following EBA175 binding was required for invasion, parasites that relied on GPA for invasion would be expected to invade MiV RBCs less efficiently than parasites invading via a GPA-independent pathway. To test this hypothesis, we purified mature schizonts from two strains, W2mefΔRh4 and W2mefΔEBA175, and incubated these schizonts with either MiV cells or normal RBCs. W2mefΔRh4 parasites, lacking Rh4, rely on EBA175–GPA interaction for invasion (12). W2mefΔEBA175 parasites are, in contrast, able to invade RBCs independent of EBA175–GPA interaction. No significant differences were found in the ability of either parasite strain to invade MiV RBCs (Fig. 4*C*). This finding suggests that, contrary to expectations, the tension increase triggered by the cytoskeletal tail of GPA is not required for invasion.

Significance of RBC Bending Modulus for Parasite Invasion. Having assessed the role of tension in invasion, we next sought to assess the relationship between bending modulus and *P. falciparum* invasion directly. Because bending modulus describes the amount of energy required to bend a lipid bilayer, a reduction in this property would also decrease the energy barrier to invasion and, as such, would be predicted to increase parasite invasion efficiency (15). This bending modulus reduction might derive from changes in lipid composition [e.g., the percentage of cholesterol (39)], asymmetry between inner and outer membrane leaflets (25), or the extent of lipid chain saturation or modification (40), each of which has been described to affect membrane thickness and bending modulus. To begin to assess whether the decrease in bending modulus following GPA binding resulted from changes in membrane asymmetry, we tested whether binding of rEBA175-RII to its RBC receptor triggered calcium changes (associated with scramblase activation in the RBC) or externalization of lipid moieties normally limited to the internal membrane leaflet. Fluro-4 acetoxymethyl ester (Fluo-4 AM) and Annexin V labeling of RBCs in the presence or absence of rEBA175-RII found no evidence for calcium signaling (marked by Fluo-4 AM) (Fig. 5*A*) or for a differential externalization of phosphatidylserine (marked by Annexin V) (Fig. 5*B*), as opposed to incubation with the calcium channel activator Yoda 1 (used here as a positive control) (41). This result suggests asymmetry is not the direct cause of the reduction in bending modulus. At present, therefore, the direct cause of the reduction in bending modulus remains to be elucidated.

To explore the significance of a reduction in bending modulus, and given the essential role of the GPA invasion route for sialic acid-dependent invasion, which is not attributable to the changes in tension, we investigated whether an experimentally induced reduction in this parameter improved invasion efficiency. Pretreatment with 7-KC has been shown to reduce lipid packing/order in membranes (42), and as demonstrated above (Fig. 2 *G* and *H* and Fig. S2*D*), it leads to a reduction in bending modulus. Pretreatment of RBCs with 7-KC would therefore be predicted to increase invasion efficiency if parasite-induced reduction of bending modulus is a key parameter for invasion. RBCs pretreated with 7-KC to decrease the bending modulus were more readily invaded by *P. falciparum* merozoites (Fig. 5 *C* and *D*). These results demonstrate a clear linkage between parasite-induced changes in the RBC surface and invasion ligand binding, the direct consequence of which is to change RBC biophysical properties to increase invasion efficiency. Combined, these data strongly imply that *P. falciparum* merozoites alter the target erythrocyte's biophysical properties before invasion to facilitate their own entry.

Discussion

Here, complementary biophysical approaches demonstrate that binding of the malaria parasite invasion ligand EBA175 to its host cell receptor GPA triggers a biophysical response in the RBC. Exploration of this change identified a reduction in the RBC bending modulus on binding, which itself significantly increases invasion efficiency. Thus, consistent with biophysical predictions of the energetics involved in invasion (15), the malaria parasite does appear to alter the RBC surface to facilitate its own entry.

One of the key challenges with biophysical approaches that explore changes in RBC properties is differentiating between contributions arising from cytoskeletal and membrane changes. By using a number of chemical reagents previously described to affect cytoskeletal and lipid properties (i.e., glutaraldehyde, diamide, DMSO, 7-KC), we confirm that we can distinguish between changes occurring within the cytoskeletal and lipid membrane

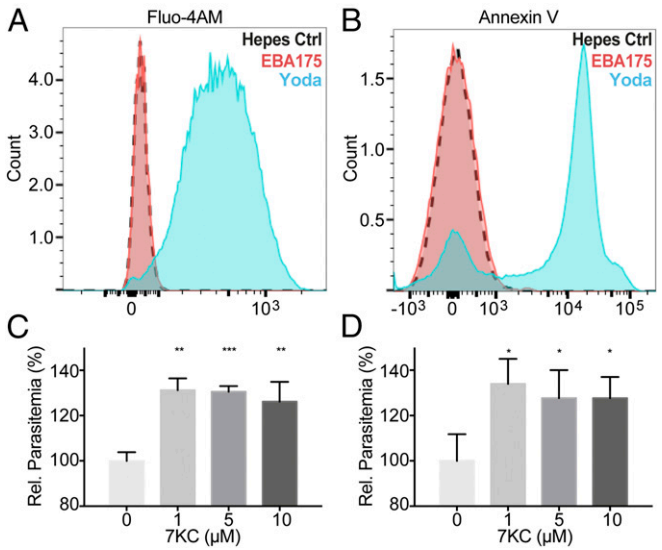

Fig. 5. RBCs treated with 7-KC to decrease bending modulus are more readily invaded than control RBCs. RBCs pretreated with Fluo-4 AM (*A*) were incubated with rEBA175-RII and then stained with annexin V (*B*). Calcium influx (*A*) and phosphatidylserine exposure (*B*) were assessed by flow cytometry. Ctrl, control. (*C* and *D*) RBCs were treated with varying concentrations of 7-KC for 30 min and then washed and resuspended in fresh media before parasite schizonts were added. Parasite invasion [Rel. Parasitemia (%)] was quantified by flow cytometry. Strains used were w2mefΔEBA175 (*C*) and w2mefΔRh4 (*D*). Rel., relative. *P* values comparing the treatment versus control were calculated using the Student's *t* test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

components described by tension and bending modulus. This ability provides a key foundation for investigating the RBC biophysical response to binding by the invasion ligand EBA175.

Both biophysical techniques used in this study identified an increased rigidification (reduction in RBC deformability/increase in tension) at the higher concentrations tested (≥10 μg/mL rEBA175-RII), which is in agreement with previous work using GPA antibodies (24, 43, 44). Of interest, we measured a small, but repeatable, reduction in tension at low concentrations by flicker spectroscopy. This result would suggest that the interaction between EBA175 and GPA, and resulting changes in the RBC, is not a linear relationship. In the context of merozoite invasion, where the absolute number of EBA175 molecules at the RBC interface is unknown, several different scenarios could arise that have an impact on the invasion process. At the invasion site, a higher local EBA175 concentration could significantly change the local tension and bending modulus conducive to invasion (model in Fig. S5). Additionally, diffuse EBA175 on the RBC surface [or shed from the parasite surface (45)] may affect the wider RBC, either triggering further changes conducive to invasion or potentially binding to bystander RBCs, thereby lowering their energy barrier for invasion events.

Of the two key changes in tension and bending modulus, it is surprising that the cytoskeletal changes, as mediated by the cytoplasmic tail of GPA, were not found to be essential for invasion in vitro. This finding contradicted our expectation that increased cross-linking of the GPA tail upon EBA175 binding might be functional for invasion, for instance, serving as a focal point where the RBC cytoskeleton could be opened up around the invading parasite or providing stability for the tight junction (46). This result suggests that direct cross-linking of GPA to the cytoskeleton via its cytoplasmic tail does not significantly impact invasion efficiency.

In contrast, a reduction in bending modulus, which still occurred in MiV cells, appears to be one of the critical roles of EBA175 binding. Bending modulus is an intrinsic property of the lipid bilayer that describes the amount of energy required to bend it and is affected by lipid composition, membrane thickness, and membrane asymmetry. These properties regulate numerous cellular functions through membrane curvature (47), endocytosis (48), opening of protein channels (49), and initiation of signaling processes (50), and, unsurprisingly, they are often exploited by intracellular pathogens (40, 51). We were therefore intrigued by the possibility that the architecture of the lipid bilayer might be exploited by the merozoite during invasion. Invasion into RBCs pretreated with 7-KC, mimicking the bending modulus decrease caused by GPA binding, demonstrated that invasion efficiency for both sialic acid-dependent (W2mefΔRh4) and -independent (W2mefΔEBA175) strains significantly increased. This result strongly suggests that parasite–host interactions trigger RBC biophysical responses that contribute to invasion efficiency. What is the nature of this change? Because the decrease in bending modulus is not dependent on the cytoplasmic tail of GPA, this change might arise through a reduction in the lateral pressure within the membrane caused by a conformational change in GPA. Just as transmembrane proteins (i.e., mechanosensors) can be regulated by forces exerted on them by the lipid bilayer, transmembrane- and membrane-associated proteins are also capable of affecting membrane properties by modulating the bilayer structure (52). Dimerization of EBA175 around GPA could lead to a conformational change in GPA within the membrane, effectively freeing up space in the bilayer and reducing lateral pressure (and hence bending modulus) (25). The fact that EBA175 binding causes a signaling cascade in the RBC is not surprising, given that the strong RBC deformations that occur during invasion are dependent on the EBA175–GPA interactions (6, 13). Future efforts should focus on identifying molecular and/or signaling events that lead to these biophysical changes.

Materials and Methods

Methods for parasite culture, measuring invasion efficiency, erythrocyte binding, and flow cytometry followed standard approaches. A full description of these methods and details of statistical analysis are provided in *SI Materials and Methods*.

RT-DC. Fifty microliters of packed RBCs were incubated with appropriate concentrations of EBA175-RII at room temperature for 1 h before being drawn into a 1-mL syringe and connected to a custom-made polydimethylsiloxane (PDMS) microfluidic chip. Cells were recorded at ∼2,000 frames per second (fps) using a complementary metal oxide semiconductor (CMOS) based camera (MC1362; Mikrotron) and an NI-1433 Frame Grabber (National Instruments) as they travel through the PDMS chip at flow rates of 0.4–0.12 $\mu L \cdot s^{-1}$. The extent of deformation is extracted from the cross-sectional area calculated using a custom-derived contour-tracking algorithm. A full discussion of the technique and analysis is provided by Otto et al. (22).

Flicker Spectroscopy. RBCs were diluted into RPMI-1640 (Sigma–Aldrich) at 0.5% hematocrit and incubated at room temperature for 30 min. Fluctuation videotapes were recorded on a Nikon Ti Microscope (objective lens: Nikon Plan Apo 100× 1.4-N.A. oil immersion) using an OrcaFlash4.0 CMOS camera. Approximately 4,500 frames were recorded at a frame rate of 150 fps and an exposure time of 1 ms. Data analysis was carried out using a custom-built LabVIEW (National Instruments) program that detects and extracts membrane contours from each frame with subpixel resolution. Full details of membrane fluctuation analysis are given elsewhere (27). Briefly, the deviation of each contour from the mean membrane position was decomposed into fluctuation modes by Fourier transforming to give a fluctuation power spectrum of mean square mode amplitudes at the cell equator [$h^2(q_x, y = 0)$] as a function of mode wavenumber (q_x). From these data, the bending modulus (κ) and tension (σ) can be fitted using the following equation:

$$\langle h(q_x, y=0)^2 \rangle = \frac{1}{L}\frac{k_B T}{2\sigma}\left(\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{k_c} + q_x^2}}\right),$$

where k_B is the Boltzmann constant, T is temperature, and L is mean circumference of the cell contour. This model assumes that the cell surface behaves as a flat sheet and that we image the equator of the cell. When fitting the fluctuation data, mode numbers 4 and below were excluded due

to significant influence of the cell shape (breakdown of the flat sheet assumption) and mode numbers above 20 were removed because these fluctuations lie outside the spatial and temporal resolution of the experiment. The tension and bending modulus terms dominate different parts of the fluctuation power spectrum (and can be attributed to distinct cellular properties as described below), and so can be resolved independently using a double-parameter nonlinear fit to the model above.

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