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*P. falciparum* ligand binding to erythrocytes induce alterations in
deformability essential for invasion

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Abstract

The most lethal form of malaria in humans is caused by *Plasmodium falciparum*. These parasites invade erythrocytes, a complex process involving multiple ligand-receptor interactions. The parasite makes initial contact with the erythrocyte followed by dramatic deformations linked to the function of the Erythrocyte binding antigen family and *P. falciparum* reticulocyte binding-like families. We show EBA-175 mediates substantial changes in deformability of erythrocytes by binding to glycophorin A and activating a phosphorylation cascade that includes erythrocyte cytoskeletal proteins resulting in changes in the viscoelastic properties of the host cell. TRPM7 kinase inhibitors FTY720 and waixenicin A block the changes in deformability of erythrocytes and inhibit merozoite invasion by directly inhibiting the phosphorylation cascade. Therefore, binding of *P. falciparum* parasites to the erythrocyte directly activate a signaling pathway through a phosphorylation cascade and this alters the viscoelastic properties of the host membrane conditioning it for successful invasion.
Introduction

Malaria is a major global disease of humans and the most severe form is caused by *Plasmodium falciparum*. This protozoan parasite has a complex life cycle, however, the symptoms of malaria are mediated by the asexual blood stage that is initiated by entry of the merozoite form into the host erythrocyte. During the initial steps of invasion, the merozoite intermittently contacts the erythrocyte until it attaches and initiates internalization. Attachment and internalization involve a series of dramatic changes that include deformation of the erythrocyte (Fig. 1 A) and calcium ($\text{Ca}^{2+}$) influx after which a tight junction is formed and invasion mediated using force generated by the parasite actomyosin motor [1, 2]. The mechanical alterations of the erythrocyte that occur during merozoite invasion have been described using video-microscopy [1, 3]. Merozoite invasion involves the interaction of multiple parasite ligands with specific erythrocyte receptors that include merozoite surface proteins (MSPs) [4], erythrocyte binding antigens (EBAs) [5] and reticulocyte binding-like homologs (PfRhs) [6-9], and the functional requirement of these during specific steps of invasion has been defined [1, 2]. This includes binding of the merozoite surface protein Duffy Binding-Like 1 and 2 (MSPDBL1 and 2) with unknown receptors [10], EBA-175 with glycoporphin A (GPA) [11], EBA-140 (also known as BAEBL) with glycoporphin C (GPC) [12], PfRh4 with Complement Receptor 1 (CR1) [13] and PfRh5 with basigin [14]. Nothing is known with respect to alterations that these ligand-receptor interactions mediate on the host cell during these initial stages of merozoite invasion.

Erythrocytes are very flexible and dynamic cells that are able to flow smoothly through the microvasculature and pass swiftly through the spleen. The shear elastic properties of the erythrocyte are predominantly determined by the underlying spectrin network as well as connection of integral membrane proteins with this cytoskeleton. The erythrocyte can undergo repeated large deformations to facilitate movement through microcapillaries, and these deformations involve dynamic remodeling of the spectrin network [15]. Additionally, under normal physiological conditions, calcium ($\text{Ca}^{2+}$) influx or treatment with certain amphipathic drugs can induce membrane budding [16-18]. Also, active ATP-dependent cytoskeleton forces that are uncorrelated with Brownian noise have been detected in erythrocytes [19]. Environmental factors can trigger post-translational modifications and change the erythrocyte membrane properties, and antibody ligation of CR1 increases erythrocyte membrane
deformability [20]. Phosphorylation and dephosphorylation of membrane and cytoskeletal proteins is a likely mechanism by which properties of the erythrocyte membrane are regulated [21], and increased phosphorylation of erythrocyte proteins occurs on attachment of *P. falciparum* merozoites suggesting changes to the host cell cytoskeleton may be important for parasite entry [22].

In this study, we show that *P. falciparum* ligand-receptor interactions affect the deformability of the erythrocyte. In particular, EBA-175 binding to GPA causes substantial changes in deformability of erythrocytes and activates a phosphorylation cascade that alters the viscoelastic properties of the host membrane, a process that is essential for successful parasite invasion.

**Results**

**Binding of *P. falciparum* ligands to human erythrocytes affects deformability**

*P. falciparum* merozoites significantly deform the erythrocyte during invasion and potentially alter the visco-elastic properties of the host cell (Fig. 1 A). The effect of *P. falciparum* ligand-receptor interactions on the visco-elastic properties of the erythrocyte was determined with recombinant proteins that bind to specific receptors. MSPDBL1, MSPDBL2 [10], EBA-175 region II (EBA-175 RII), PfRh4 [13], PfRh5 [23], EBA-140 region II (EBA140 RII) [12] were incubated with human erythrocytes and the Young’s modulus (E) calculated from the atomic force microscopy (AFM) cantilever deflection as a measure of cell stiffness (Fig. 1 B) [24]. The recombinant proteins MSPDBL1 [25, 26], MSPDBL2 [25, 26], PfRh4, PfRh5, EBA-140 RII and EBA-175 RII specifically bind to erythrocytes. Erythrocyte stiffness was generally reduced by binding of MSPDBL1, MSPDBL2, PfRh4, PfRh5 and EBA-140 RII (P < 0.0001) with EBA-175 RII binding showing a dramatic reduction of Young’s modulus (Fig. 1 B; Figure 1–figure supplement 1 and Figure 1–figure supplement 2). The erythrocyte-binding domain of EBA-175 is region II, a 616 amino acid fragment consisting of two cysteine-rich Duffy binding-like (DBL) domains (F1 and F2) [27], that binds GPA (Fig. 1 C) [28-30]. EBA-175 dimerizes upon receptor engagement [27], and neutralizing antibodies block the dimer interface and receptor binding residues of EBA-175 RII [31]. EBA-175 and EBA-140 contain the conserved domain architecture of the EBL family and bind similarly to GPA and GPC respectively, although the latter binds as a monomer [32, 33].
As controls we tested the effect of the intrinsically disordered EBA-175 III-V domain [34], which does not bind erythrocytes [35], and it did not significantly influence erythrocyte deformability ($P < 0.05$, Fig. 1 C). Erythrocytes incubated with EBA-175 RII together with anti-EBA-175 RII antibodies, that block binding to GPA and merozoite invasion [31, 35, 36], showed no detectable change in host cell stiffness ($P > 0.05$) consistent with them blocking ligand binding (Fig. 1 C). EBA-175 and EBA-140 binding are both dependent on sialic acid moieties on glycophorin receptors [5] and thus sensitive to neuraminidase-treatment of erythrocytes. EBA-175 RII did not affect the stiffness of neuraminidase-treated erythrocytes (Fig. 1 D) indicating a direct link between EBA-175 binding to GPA and changes in erythrocyte deformability. EBA-175 RII binding showed an expected dose response on deformability, which was confirmed using ektacytometry [37], consistent with the quantification of EBA-175 RII binding to erythrocytes (Fig. 1 E; Figure 1–figure supplement 1). Bulk rheology measures of the elongation index showed an increase in erythrocyte deformability with EBA-175 RII reaching a plateau after 35 nM (Fig. 1 E), which was close to saturation of GPA binding sites [38]. Therefore, binding of EBA-175 RII to glycophorin A on the erythrocyte surface was responsible for the changes observed in deformability of the host cell.

**Binding of EBA-175 to GPA on the erythrocyte increases phosphorylation of the cytoskeleton**

We next determined if the mechanism of EBA-175 RII induced deformability of erythrocytes was a result of changes in phosphorylation of cytoskeleton components. The EBA-175 RII-treated erythrocytes were radiolabeled with $^{32}$P inorganic phosphate, and ghosts containing membrane and cytoskeleton proteins extracted and analyzed by 2-dimensional (2-D) gel electrophoresis (Fig. 2 A and B). An altered intensity of $^{32}$P labeling in the EBA-175 RII treated erythrocytes indicated an overall increase of phosphorylation, and false-color overlays of aligned autoradiographs showed a shift of particular 2-D spots, revealing some proteins were multiply phosphorylated (Fig. 2 A and B; Figure 2–figure supplement 3). Twenty eight of the most prominent $^{32}$P-labeled protein spots were excised from an aligned preparative 2-D gel and analyzed by LC-MS/MS. This identified a subset of phosphorylated proteins corresponding to erythrocyte membrane skeleton proteins including tropomodulin-1, adducin-2, tropomyosin,
beta-actin, ankyrin-1, protein 4.1 and coflin-1. The identity of these proteins was validated by immuno-blots (Fig. 2; Figure 2 – figure supplement 3). 2-D image analysis of $^{32}$P autoradiographs and immuno-bLOTS of the same membranes indicated that $^{32}$P labeled 2-D phospho-spots matched accurately with bands for tropomodulin-1 and adducin-2, with clear changes in their phosphorylation state (Fig. 2 C-F).

To provide a more quantitative approach we used dimethyl labeling and quantitative liquid chromatography-tandem mass spectrometry (LC-MSMS) to enable identification of phosphopeptides at a global level [39] (Fig. 2 G-H). EBA-175 RII treated and untreated erythrocytes were digested prior to labeling of the lysine and N-terminal residues with either a light or a heavy dimethyl isotope. Global phosphoproteomics analysis of the phosphopeptide enriched Heavy (H) + Light (L) mixture revealed a high number (982) of unique phosphopeptides, and the H/L ratio of unique peptides singly, doubly or triply phosphorylated indicated an overall increase in phosphorylation in the EBA-175 RII treated samples (Fig. 2 G; Figure 2–figure supplement 4). Quantitative LC-MSMS analysis identified approximately 400 erythrocyte phosphoproteins and revealed a significant ($P < 0.05$) enrichment of phosphopeptides corresponding to trans-membrane spanning glycoproteins and cytoskeletal proteins that include GPA and GPC, adducin, ankyrin and dematin, with many of them multiply phosphorylated (Fig. 2 H; Figure 2–figure supplement 4; Supplementary File 1 and 2). Interestingly, both GPA and GPC phosphorylation sites were detected in the cytosolic domains of these proteins (Fig. 2 I; Figure 2–figure supplement 4). Therefore EBA-175 RII binding to erythrocytes induced an overall change in phosphorylation of the cells cytoskeletal proteins. These observations suggested an induced modification of the cytoskeleton and consequent increase deformability upon specific interaction of merozoite ligands with the host erythrocyte.

**EBA-175 binding to GPA triggers a phosphorylation cascade of erythrocyte membrane glycoproteins and cytoskeleton proteins by TRPM7.**

To identify the kinase(s) involved in erythrocyte phosphorylation triggered by EBA-175 RII binding to GPA we tested erythrocyte kinase inhibitors that influence erythrocyte deformability. A protein kinase C (PKC) inhibitor, Gö9676 [40], four transient receptor potential cation channel (TRPM7) inhibitors, FTY720, sphingosine [41], waixenicin A [42] and NS8593 [43], three Rho-
kinase inhibitors, Y-27632 [44], simvastatin [45] and HA1077-fasudil [46], an adenylyl cyclase and protein kinase A (PKA) stimulator, forskolin [47], an AMP-activated PK inhibitor, dorsomorphin [48], a phosphodiesterase inhibitor, isobutyl-methyl-xantine (IBMX, [47]), a spleen tyrosine kinase (syk) inhibitor, BAY-3606 [49], a Ca\(^{2+}\) channel inhibitor, verapamil [44] and three inhibitors of mechanically activated currents through channels such as TRPM7 or Piezo1, gadolinium (III), ruthenium red [50] and GsMTx-4 [51]. Some inhibitors had no effect on overall growth of *P. falciparum* whilst others reduced it to less than 50% compared to controls (sphingosine, Y-27632, IBMX, BAY61-3606, gadolinium (III) and verapamil) (Fig. 3 A). Strikingly, FTY720, waixenicin A and NS8593, which are TRPM7 inhibitors, blocked parasite growth completely. TRPM7 is a two-domain protein expressed in most tissues containing both a TRP ion channel and an alpha-kinase domain [52]. TRPM7 phosphorylates tropomodulin-1 at the N-terminal Ser2 and Thr54 residues [53], and Ser2 is phosphorylated in erythrocytes [54]. These data along with the phosphorylation of tropomodulin-1, induced by EBA-175 binding to glycophorin A (Fig. 2 C and 2 D), suggested that TRPM7 may be involved in this phosphorylation cascade and that TRPM7 inhibitors could block parasite growth by impairing invasion.

**Increased deformability of the host erythrocyte is required for merozoite invasion**

To determine if TRPM7 inhibitors, that blocked *P. falciparum* growth, directly impaired merozoite invasion we used the reversible TRPM7 inhibitor FTY720 [41]. Purified merozoites were added to FTY720 or sphingosine treated erythrocytes, and invasion quantified (Fig. 3 B). FTY720 inhibited merozoite invasion (IC\(_{50}\) of 34.1 ± 1.1 µM) whereas sphingosine had no effect. Additionally, we performed growth inhibition assays to confirm the activity of FTY720. Synchronized 3D7 trophozoites were treated with different concentrations of each compound and the parasitemia determined to obtain an inhibitory dose response. FTY720 (IC\(_{50}\) 107.3 ± 1.0 µM) showed potent inhibition (Fig. 3 C) whilst HA1077 and sphingosine showed little to none. To further test whether FTY720 was inhibiting invasion rather than interfering with parasite development we tested the inhibitory activity of waixenicin A (Fig. 3 D). This compound is a slow acting inhibitor of TRPM7 and therefore could be incubated with erythrocytes and washed out before testing merozoite invasion [42]. When waixenicin A-treated erythrocytes were tested a similar result was obtained for either washed (IC\(_{50}\) 59.4 ± 1.2 µM) or unwashed (IC\(_{50}\) 58.8 ±
1.1 µM) host cells (Fig. 3 D). Therefore, FTY720 and waixenicin A block invasion of *P. falciparum* merozoites by inhibiting an essential erythrocyte function(s).

Interestingly, the same results were observed for 3D7ΔEBA-175 parasites that lack expression of EBA-175, where FTY720 inhibited merozoite invasion (IC$_{50}$ 20.8 ± 1.2) (Fig. 3 B) and parasite growth (IC$_{50}$ 95.2 ± 1.0 µM) (Fig. 3 C). Similarly, waixenicin A also inhibited 3D7ΔEBA-175 growth (IC$_{50}$ 43.8 ± 1.3 µM) (Fig. 3 D). The ability of FTY720 and waixenicin A to inhibit invasion of EBA-175 deficient parasites were consistent with this ligand not being essential for merozoite invasion as other members of the EBA and PfRh family of proteins have overlapping functions [55-57]. EBA-175 function is redundant but the overall function of the EBA and PfRh families is essential [57]. Whilst EBA-175 RII binding had a significant effect on erythrocyte deformability other ligands such as EBA-140 RII, which binds to GPC [12], and PfRh4, which binds to CR1 [13], also had an effect although not to the same magnitude as EBA-175 RII (Fig. 1 B). GPA is the most abundant integral protein on the erythrocyte surface, present at 10$^6$ copies per cell [38]. In contrast, there are approximately 225,000 molecules of GPC per erythrocyte [58], and the normal level of CR1 in Europeans is between 50-1200 molecules per cell [59]. The higher abundance of GPA explains the lesser effect of EBA-140 RII and PfRh4 on the deformability of treated erythrocytes. Therefore, it is likely that in the absence of EBA-175 expression other members of the EBA and PfRh protein families perform an identical function that alters the deformability of the erythrocyte through the TRPM7 pathway, and this is essential for merozoite invasion.

Binding of EBA-175 to GPA on erythrocytes altered their deformability and, in addition, induced phosphorylation of host trans-membrane spanning glycoproteins and cytoskeletal proteins. Inhibitors of TRPM7, a divalent cation channel with a kinase domain, blocked merozoite invasion. The hypothesis that TRPM7 blocked invasion by interfering with the phosphorylation cascade induced by EBA-175 RII was tested by performing a global phosphoproteomic analysis. Erythrocytes were treated with EBA-175 RII in the absence or presence of FTY720, labeled at protein primary amine residues with a heavy (H) or an intermediate (M) dimethyl isotope respectively, and mixed with light (L) dimethyl labeled untreated erythrocytes (1:1:1). Average H/L ratios of unique peptides singly, doubly or triply
phosphorylated of EBA-175 RII-treated erythrocytes were higher than average M/L ratios in EBA-175 RII/FTY720 host cells, indicating that FTY720 inhibited the phosphorylation cascade activated by EBA-175 RII binding to GPA (Fig. 4 A). Importantly, quantitative analysis of confidently identified phosphoproteins (peptide FDR < 1%) revealed a significant downregulation of phosphorylation of host cytoskeletal proteins when treated with EBA-175 RII in the presence of FTY720 ($P < 0.05$), in particular GPA (Fig. 4 B). Therefore, FTY720, a TRPM7 inhibitor, blocked phosphorylation of erythrocyte proteins induced by EBA-175 RII binding to GPA. Consequently, FTY720 inhibits merozoite invasion by blocking the phosphorylation cascade activated by EBA-175 RII binding to GPA in the erythrocyte.

Physiological shear stress in the circulation causes a reversible increase in erythrocyte $Ca^{2+}$ permeability [60]. In a similar manner, the EBA-175 RII induced increase in deformability could increase erythrocyte calcium permeability. A high content fluorescence imaging method was established and used to test $Ca^{2+}$ uptake into Fluo-4-AM (a $Ca^{2+}$ indicator) loaded erythrocytes incubated with EBA-175 RII (Fig. 4 C). Imaging showed an increment in the number of fluorescent cells when incubated with EBA-175 RII suggesting either a decrease in integrity of the cell membrane due to increased deformability or a directly induced $Ca^{2+}$ uptake (Fig. 4 D). Additionally, inclusion of FTY720 with EBA-175 RII decreased the number of fluorescent cells detected ($P < 0.03$) whereas HA1077 and sphingosine did not show a significant effect ($P > 0.05$). Furthermore, FTY720 and waixenicin A inhibited the increased deformability caused by binding of EBA-175 RII to GPA as measured by AFM ($P < 0.0001$) (Fig. 4 E), confirming causal connection between deformability and the changes in phosphorylation upon EBA-175 RII binding. AFM also showed that FTY720 inhibited the changes in erythrocyte stiffness caused by binding of EBA-140 RII and PfRh4 ($P < 0.001$) (Fig. 4 E) consistent with invasion ligands other than EBA-175 RII having the same effect on the TRPM7 dependent phosphorylation pathway.

To determine if FTY720 decreased the ability of merozoites to deform the erythrocyte during invasion, schizonts were purified and added to erythrocytes stained with Bodipy TR Ceramide, a viable membrane dye that allows invasion [2]. Live imaging of merozoites in the process of interaction with an erythrocyte showed that, in the presence of FTY720, the parasite was able to contact and attach, but could not invade (Fig. 4 F and G, Video 1 and 2). Furthermore, they were
only able to weakly deform the erythrocytes with deformation scores [1] of 0-1, as compared to merozoites in the absence of inhibitor, which were able to dramatically deform the host cell (Fig. 4 G and 4 H). Overall, merozoites in the presence of FTY720 were able to contact and attach to erythrocytes as well as untreated parasites but showed either weak or no deformation of the cell they attempted to invade (Fig. 4 H). The decrease or lack of deformation by treated parasites confirmed that FTY720 blocked merozoite invasion by decreasing the deformability of the erythrocyte as a result of inhibition of the phosphorylation cascade induced by interaction of parasite ligands with host receptors.

Discussion

Our results showed that interaction of \textit{P. falciparum} merozoites with the outside of the erythrocyte activates a phosphorylation cascade resulting in increased deformability and that this activation is essential for successful invasion. Whilst we have used soluble EBA-175 RII to bind to GPA over the surface of the erythrocyte the merozoite only interacts with a small portion of the surface, and it is likely that the increased deformability is more local and that interaction of the EBA and PfRh proteins families conditions this area so that the parasite can more easily deform the host cell, a step that is required for subsequent tight junction formation and entry [1]. Whilst this step appears to be essential EBA-175 function is generally redundant [55, 57] but the fact that FTY720 and waixenicin A block invasion in the absence of this ligand suggests phosphorylation is a required step in invasion. The ability of other members of the EBA and PfRh family of proteins to render the erythrocyte more deformable suggests they are capable of performing this function to condition areas of the host cell as well as signaling downstream events in the invasion pathway [61].

Interaction of EBA-175 with the erythrocyte surface compromises the mechanical stability of the membrane and its skeleton. Many constituent proteins of the erythrocyte membrane skeleton can be phosphorylated by various kinases, and phosphorylation of β-spectrin by casein kinase and protein 4.1R by PKC has been documented to modulate erythrocyte membrane mechanical stability [62]. A recent study showed that activation of endogenous PKA by cAMP decreases membrane mechanical stability and that this effect is mediated primarily by phosphorylation of dematin [63]. Our data shows that EBA-175 binding to GPA triggers the phosphorylation of
trans-membrane spanning and cytoskeletal proteins, including GPA, a mechanism that weakens 
the erythrocyte membrane and increases cytosolic calcium concentration, as a result of 
permeability rather than receptor mediated store release as erythrocytes lack intracellular stores 
[64]. FTY720, a transient receptor potential cation channel (TRPM7) inhibitor, reduces 
quantitatively the level of phosphorylation on the erythrocyte membrane and cytoskeleton, 
induced by EBA-175. TRPM7 plays a key role in the regulation of calcium and magnesium 
homeostasis [65], and although the function of TRPM7 kinase domain [52] in human cells is still 
unclear, it autophosphorylates, autoregulates and it can also phosphorylate other proteins such as 
tropomodulin-1, β-actin [53], annexin I [66] and myosin IIA [67]. FTY720 has been shown to 
suppress TRPM7-dependent motility of HEK293 cells [68] and to abrogate erythrocyte rigidity 
in trauma/haemorrhagic shock [69].

This study shows that inhibitors of TRPM7 block parasite invasion by interfering with host 
phosphorylation mechanisms making the area around which the merozoite is interacting less 
defeormable. Therefore the initial interaction of parasite ligands with erythrocyte receptors 
activates host cell changes that are essential for successful invasion and infection.

Materials and methods

Reagents.

3D7 is a cloned line derived from NF54, supplied by David Walliker, Edinburgh University. 
Disruption of EBA-175 was previously published [57]. Purified recombinant EBA-175 RII 
antigen was kindly provided by Science Applications International Corporation (SAIC). 
Sequence encoding EBA-175 RIII-V was codon-optimized for expression in E. coli and 
synthesized by Genscript. Antibodies against EBA-175 RII were obtained as described before 
[35]. Recombinant PfRh4 containing the full erythrocyte-binding domain (Rh4.9) was obtained 
as described [70]. MSPDBL1 and MSPDBL2 was expressed recombinantly in E. coli as 
previously described [26]. EBA-140 RII was expressed and purified as described previously [71, 
72]. Briefly, EBA-140 RII was expressed in E. coli and recovered from inclusion bodies. The 
denatured protein (100mg/L) was refolded by rapid dilution for 48 hr at 4 °C in 50 mM Tris (pH 
8.0), 10 mM EDTA, 200 mM arginine, 0.1 mm PMSF, 2 mM reduced glutathione and 0.2 mM
oxidized glutathione. After refolding, EBA-140 RII was purified by ion-exchange and size exclusion chromatography into the final buffer of 10 mM HEPES pH 7.4 150 mM NaCl. Kinase inhibitors were obtained commercially. FTY720, NS8593, Y-27632, simvastatin, HA1077-fasudil, forskolin, dorsomorphin, IBMX, BAY-3606, verapamil and gadolinium (III) from Sigma. Sphingosine, ruthenium red and GsMTx-4 from Abcam and Gö9676 from Cell Signalling. Waixenicin A was extracted and purified from soft coral tissue as described previously [42].

Erythrocyte binding assays.

Erythrocyte binding assays prior to AFM indentation and rheology were performed as follows. 10 μL washed packed blood (Australian Red Cross) was mixed with different amounts of protein (1-4 μg), resuspended in 500 μL RPMI-HEPES (2% haematocrit) and incubated at room temperature for 1 hr. For controls and inhibitor tests, antibodies (10 μg/mL) and inhibitors (50 μM) were added together with EBA-175 RII. Waixenicin A was incubated and washed prior to adding EBA-175 RII. Erythrocyte binding assays to generate binding curves using EBA175 RII, Rh4.9 and Rh5 were performed as above. Erythrocytes and proteins were centrifuged for 30 sec at 13000 rpm through 400 µl dibutyl phthalate (Sigma) to remove unbound protein. After aspiration of dibutyl phthalate and buffer, 10 μl 1.6M NaCl solution was added to the erythrocyte pellet to elute bound protein. Samples were vortexed for 5 seconds at 3 min intervals over 10 min to ensure equal mixing of 1.6M NaCl solution. Samples were spun at 13000 rpm, 4 min and the supernatant, containing eluted protein, was suspended in 2X SDS sample buffer. In all binding assays, a buffer control was included containing RPMI wash buffer + amount of protein suspension buffer equivalent to the highest concentration of protein used. In one repeat experiment binding was performed in PBS instead of RPMI wash buffer.

Western blotting

Samples were denatured in 2X SDS reducing sample buffer and loaded on 4-12% or 10% Bis-Tris or 3-8% Tris-Acetate gels (Invitrogen). Gels were run at 150V in MOPS or Tris-Acetate for one hour, and electrophoresed proteins were transferred to nitrocellulose membrane (Protran, Whatman) via wet transfer protocol. Blocking solution used was skim milk solution or Odyssey blocking buffer (LiCor). Protein bands after incubation with HRP-conjugated secondary
antibody were detected by ECL detection reagent (GE Healthcare) and exposure to X-ray film (FUJIFILM). In the case of fluorescent WB, visualisation and quantitation was carried out with the LiCor Odyssey® scanner (800nm and 700nm wavelength channels) and Odyssey® software.

Densitometry quantitation
Fluorescently probed membranes were scanned and analysed by LiCor Odyssey® imaging software. An extra-sum-of-squares F test was used to compare the linear regression of the Log (protein levels) and Log (adhesin amount) to a line where y-intercept and slope = 0.

Flow cytometry quantitation
Erythrocyte binding assay was adapted from Salinas et al. 2014 (add reference 27). Briefly, 1uM RII-140-6xhis, purified identically as untagged RII-140, was added to the RBCs for 1 hour at room temperature and then washed three times with DMEM 10% FCS. Anti-his antibody conjugated to FITC was then incubated with the samples for 1 hour at room temperature and then washed three times with DMEM 10% FCS. Finally, RBCs were diluted to 2mL in DMEM 10% FCS. Samples, in triplicate, were run using a BD FACSCanto machine to collect 30,000 counts each and analyzed with FlowJo software.

Neuraminidase treatment of erythrocytes
50 μL washed packed blood (Australian Red Cross) was mixed with 10 μL Neuraminidase from Vibrio cholerae (Sigma), 90 μL RPMI-Hepes/NaHCO3 and incubated in a waterbath at 37°C for 1 h. Treated cells were spun down and washed 5 times with RPMI-Hepes/NaHCO3 before incubation for binding assays and AFM measurements.

Atomic force microscopy indentation of erythrocytes
Erythrocyte solutions were deposited onto freshly cleaved mica and an MFP3D-BIO instrument (Asylum Research) used. Samples were nanoindented under RPMI-HEPES with MLCT silicon nitride probes (nominal spring constant 0.1 N/m, resonant frequency 38 kHz, Bruker AFM Probes). To calculate erythrocyte modulus a total of 1000 force curves were registered distributed in at least 20 different points for each condition. Analysis was made by NanomechPro software (Asylum Research).
Rheology measurements.

Samples were centrifuged at 6000 rpm for 1 min and the 10 μL blood pellet resuspended in 600 μL polyvinylpyrrolidone (PVP) solution at 25 mPa second viscosity (Rheo Meditech). Elongation index (EI) over 1-20 Pa shear stress was measured 3 times for each sample in a RheoScan rheometer according to manufacturer’s instructions (RheoMeditech, Seoul, Korea). EI at low shear stress (3 Pa) was plotted for every concentration of EBA-175 RII. At low shear stress rheology presents the highest sensitivity to RBC membrane properties, and erythrocytes exhibit maximal deformability [73].

Ghost preparation and radiolabeling.

Erythrocytes were washed and resuspended (50% haematocrit) with calcium-free Krebs-Ringer buffer (Sigma). 125 μL of cell suspension was incubated for 2 hr with 125 μCi of $^{32}$P (Perkin Elmer) and either 18 μg of EBA-175 RII or the equivalent volume of Krebs-Ringer buffer. Suspensions were spun down at 3000 g for 4 min at room temperature and the supernatant was discarded. Erythrocyte pellets were lysed with ice cold 5 mM sodium phosphate pH 8 buffer with protease (cOmplete, Sigma) and phosphatase (Halt, Thermo Fisher) inhibitors, spun down at 100000 g and washed 3 times with the same buffer. The resulting ghost pellets were snap-frozen in liquid Nitrogen and stored at -80°C until further use.

2D Gel Electrophoresis.

Ghost pellets were subjected to a series of freezing/thawing cycles to remove remaining haemoglobin and resuspended in 300 μL of 2-DE sample buffer (7 M Urea, 2M Thiourea, 4% CHAPS, 50 mM DTT, 1% ampholytes), loaded onto 13 cm pI 4-7 IPG strips by passive rehydration and focused at a current limit of 50 mA/IPG strip using a fast voltage gradient (8000V max, 24,000 Vh) at 15°C. The second dimension was carried out on pre-cast 4-12% Bis-Tris Midi Protein Gels using a NuPAGE® Novex® system (Life Technologies) at 75 V constant voltage and 10°C. Analytical 2-D gels were transferred to PVDF membranes using an iBlot® Dry Blotting System (Life Technologies) and imaged using BAS-IP SR 2040 E phosphor storage plates (GE Healthcare) for 48 hr. High-resolution imaging and accurate quantitation of $^{32}$P-labeled protein spots was achieved using a Typhoon FLA 7000 laser-scanning detection system.
(GE Healthcare). Preparative 2-DE gels were stained using Colloidal Coomassie Brilliant Blue (Sigma) and spots matching $^{32}$P-labeled protein spots manually excised and subjected to LC-MS/MS analysis.

Gel excision, in-gel digestion and nano-LC-MS/MS.

Protein spots were manually excised from preparative 2-D gels and subjected to manual in-gel reduction, alkylation and tryptic digestion. All gel samples were reduced with 10mM DTT (Sigma) for 30 min, alkylated for 30 min with 50 mM iodoacetamide (Sigma) and digested with 375 ng trypsin gold (Promega) for 16 hr at 37°C. The extracted peptide solutions were then acidified (0.1% formic acid) and concentrated to 10 mL by centrifugal lyophilisation using a SpeedVac AES 1010 (Savant). Extracted peptides were injected and fractionated by reversed-phase liquid chromatography on a nanoACQUITY UHPLC system (Waters, USA) using a nanoACQUITY C18 150 mm × 0.15 mm I.D. column (Waters, USA) developed with a linear 60 min gradient with a flow rate of 250 nL/min from 100% solvent A (0.1% formic acid in Milli-Q water) to 60 % solvent B (0.1% formic acid, 60% acetonitrile (Thermo Fisher, USA), 40% Milli-Q water). The nano-UHPLC was coupled on-line to a Q-Exactive Orbitrap mass spectrometer equipped with a Proxeon nano-electron spray ionization source (Thermo Fisher, USA) for automated MS/MS. High mass-accuracy MS data was obtained in a data-dependent acquisition mode with the Orbitrap resolution set at 75,000 and the top-ten multiply charged species selected for fragmentation by HCD (single and doubly charged species were ignored). The ion threshold was set to 15,000 counts for MS/MS. The CE voltage was set to 27.

Mass spectra database searching.

For protein identification of protein spots LC-MS/MS data were searched against a non-redundant protein decoy database comprising sequences from \textit{P. falciparum}, as well as their reverse sequences and common contaminants. Mass spectra peak lists were extracted using extract-msn as part of Bioworks 3.3.1 (Thermo Fisher Scientific) linked into Mascot Daemon (Matrix Science, UK). Peak lists for each nano-LC-MS/MS run were searched using MASCOT v2.2.04 (Matrix Science, UK), provided by the Australian Proteomics Computational Facility (www.apcf.edu.au). The search parameters consisted of carbamidomethylation of cysteine as a fixed modification (+57 Da), with variable modifications set for NH$_2$-terminal acetylation (+42 Da), oxidation (+15 Da) and deamidation (+1.01 Da).
Da) and oxidation of methionine (+16 Da). A precursor mass tolerance of 20 ppm, fragment ion mass tolerance of ±0.04 Da and an allowance for up to three missed cleavages for tryptic searches was used. Scaffold v4.4.3 (Proteome Software, Inc., USA) was used to validate protein identifications derived from MS/MS sequencing results (FDR <0.5 %).

Mass spectrometry sample preparation.

For global phosphoproteomics analyses, ~200 μG of frozen ghost pellets were extracted in 200 μL lysis buffer (1% SDS, 2 x Complete Protease Inhibitor Cocktail-EDTA (Roche), in 10 mM HEPES pH 8.5), reduced with 20 mM TCEP (Sigma) for 15 min, alkylated for 30 min with 50 mM iodoacetamide (Sigma) and digested with 4 μG trypsin gold (Promega) for 16 h at 37°C, using the SP3 on-bead digestion method [74]. The extracted peptide solutions were then desalted using C18 MacroSpin columns and on-column stable isotope dimethyl labeling of differentially treated samples was performed using light (12CH2O), medium (12CD2O) or heavy (13CD2O) formaldehyde (Sigma), as previously described [75]. Differentially labelled peptide samples were then mixed 1:1 and phosphopeptide enrichment was achieved using sequential elution from IMAC or TiO2 columns, as detailed elsewhere [76].

Quantitative proteomics analysis.

Relative quantitation of differentially labeled RBC phosphopeptide preparations was performed using the MaxQuant software package [77]. High-resolution MS data were searched using a tolerance of 10 ppm for precursor ions and 20 mmu for product ions. Enzyme specificity was tryptic and allowed for up to 2 missed cleavages per peptide. Carbamidomethylation of cysteines (+57) was specified as a constant modification with oxidation of methionine (+16), protein N-terminal acetylation (+52) and light (L; Δ=+28), intermediate (M; Δ=+32) or heavy (H; Δ=+36) dimethyl labels on the peptide N termini and lysine residues set as variable modifications. The MS data were searched against human, bovine, or P. falciparum proteins in the non-redundant MSPnr protein database at a 1% false discovery rate (FDR). A robust permutation test was used to analyze MaxQuant data and evaluate statistically significant differences in the relative abundance of RBC phosphopeptides [78].

Inhibitor screen.
Highly synchronous mature schizonts (from a 30 mL culture of >5% parasitemia) were magnet purified (MACS; Miltenyi Biotec) and plated in triplicates (50 μL) at 1% parasitemia and 1% haematocrit. Parasites were allowed to egress and reinvade fresh erythrocytes for a period of 12-16 hr at 37°C in the presence of inhibitors (50 μM) or equivalent volumes of buffer (water, DMSO or KR buffer). Parasitemia was determined by GIEMSA staining.

Merozoite invasion inhibition assays.
Merozoites were purified based on an established method [79]. Highly synchronous mature schizonts were isolated from uninfected erythrocytes with a MACS magnet separation column (Miltenyi Biotec). Purified schizonts were incubated with 10 μM of the cysteine protease inhibitor E64 to prevent schizont ruptures. After 5–6 hr of incubation, schizont pellets were passed through a 1.2 μm syringe filter (Acrodisc; 32 mm; Pall). Filtrate containing purified merozoites was immediately added to fresh erythrocytes (70-80% hematocrit). The mix was transferred to a 96 well round bottom microtitre plates (Falcon) in 50 μL aliquots with doubling dilutions of each inhibitor (5-250 μM) or control buffer and incubated in a shaker at 37°C. After incubation for 24 h each well was fixed at RT for 30 min with 50 μL of 0.25% glutaraldehyde (ProSciTech) diluted in PBS. Following centrifugation at 1200 rpm for 2 min, supernatants were discarded and trophozoite stage parasites were stained with 50 mL of 5X SYBR Green (Invitrogen) diluted in PBS. The parasitemia of each well was determined by counting 50,000 cells by flow cytometry using a Cell Lab Quanta SC – MPL Flow Cytometer (Beckman Coulter). Invasion rate was calculated as %-invaded relative to untreated controls. All samples were tested in triplicate.

Growth inhibition assays.
A Growth Inhibition Assay (GIA) protocol was modified from a previously described method [80]. Trophozoite stage parasites at 0.5% parasitemia were grown in a 50 μL culture at 2% hematocrit in 96 well round bottom microtitre plates (Falcon) with doubling dilutions of each each inhibitor (5-250 μM) or control buffer. After incubation for 48 hr each well was fixed at RT for 30 min with 50 μL of 0.25% glutaraldehyde (ProSciTech) diluted in PBS. Following centrifugation at 1200 rpm for 2 min, supernatants were discarded and trophozoite stage parasites were stained with 50 mL of 5X SYBR Green (Invitrogen) diluted in PBS. The parasitemia of
each well was determined by counting 50,000 cells by flow cytometry using a Cell Lab Quanta SC – MPL Flow Cytometer (Beckman Coulter). Growth was expressed as a percentage of the parasitemia obtained using an inhibitor-free control. All samples were tested in triplicate.

High content fluorescence screen.

Erythrocyte binding assays in this case were performed as follows. Erythrocytes (Australian Red Cross) were previously incubated with Fluo-4 AM (Life Technologies), 1:250 dilution to 1% haematocrit erythrocytes in RPMI-HEPES and washed. 2.5 μL packed RBCs were mixed with EBA-175 RII or RIII-V (2 μg), resuspended in 50 μL RPMI-HEPES (5% haematocrit) and incubated at RT for 1 hr. FTY720 and sphingosine were added together with EBA-175 RII at a 50 μM concentration. After incubation samples were diluted with RPMI-HEPES to 0.05% haematocrit and transferred to 100 μL aliquots into BD Falcon 384 well TC-treated plates. Plates were read in an Opera Phenix high content screening system for brightfield, phase contrast and 488 nm fluorescence. These experiments were repeated three independent times and standard error of the mean calculated.

Live imaging of merozoite invasion.

Fresh erythrocytes (1% hematocrit) were washed in complete RPMI-Hepes culture medium. Bodipy TR Ceramide (Thermo Fisher Scientific) was added at 1:1000 dilution and incubated for >1 hr. Erythrocytes were washed 2-3 times with complete RPMI-Hepes and then resuspended in 2 mL of complete RPMI-Hepes. Highly synchronous schizonts (>5% parasitemia from a ~3% hematocrit 30 mL culture) were magnet purified, then added to the labelled erythrocytes (0.1% haematocrit) and 2 mL of this transferred to a 35 mm Fluorodish (World Precision Instruments). Live imaging was performed at 37°C on a Leica SP8 confocal microscope. A 63x/1.4 NA Oil Immersion objective on the Leica SP8 confocal. Time-lapsed images were collected every second upon schizont rupture (594 filter) with an 8 kHz resonant scanner with 4x line averaging and HyD detectors. Cells were maintained at 37 °C in a low O₂ and CO₂ nitrogen atmosphere. ImageJ Fiji was used to assemble image series and perform image analyses. Deformation scores were determined according to an established method [1].


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Figures and legends

Figure 1. *P. falciparum* EBA-175 RII binding to GPA increases deformability of the erythrocyte. **A.** Live imaging time frames showing a merozoite deforming an erythrocyte. Scale bar 5 μm. **B.** Atomic force microscopy (AFM) screen of the effect of *P. falciparum* invasion ligands on the erythrocyte Young’s modulus (E). **C.** Schematics showing EBA-175 and EBA-140 domain structure (top). Bottom panel is the AFM Young’s modulus of erythrocytes treated with EBA-175 region II, region III-IV and EBA-175 RII in the presence of EBA-175 RII antibodies (EBA-175+Ab). **D.** AFM Young’s modulus of neuraminidase treated erythrocytes in the presence or absence of EBA-175 RII. **E.** EBA-175 titration on erythrocytes and comparison of the AFM Young’s modulus (left) with the elongation index measured by rheology (right). 4 μg (B), 3.5 μg (C), 3.8 μg (D) and 1-4 μg (E) were added to 500 μL erythrocytes in RPMI-HEPES at 2% haematocrit. Error bars represent the mean and SEM for three independent experiments.

Figure 2. EBA-175 RII induces an increase in phosphorylation of trans-membrane and cytoskeletal erythrocyte proteins. **A-B.** MW vs. pl 2D electrophoresis gel autoradiographs of EBA-175 RII treated (B) or untreated (A) $^{32}$P radio-labeled erythrocyte ghosts. **C-F.** Western blot phosphorylation validation of tropomodulin 1 (C-D) and adducin-2 (E-F). P0-P10 indicate the number of phosphorylation sites. **G.** Mass spectrometry phosphopeptide heavy (H) to light (L) ratios of EBA-175 (H) mixed with PBS (L) treated ghosts. **H.** Volcano plot showing quantitative analysis of unique phosphopeptides. Red dots indicate significant upregulated unique phosphopeptides corresponding to cytoskeletal protein IDs highlighted in the list. **I.** Amino acid sequence of glycophorin A (top), red square highlights the cytoplasmic domain conserved across different species. Glycophorin A unique phosphopeptides detected (bottom), red circles point out phosphorylation sites all located in the cytoplasmic domain.

Figure 3. FTY720 and Waixenicin A, inhibitors of TRPM7, block *P. falciparum* erythrocyte invasion. **A.** Parasitemia after 12-16 hr post addition of synchronous schizonts to erythrocytes treated with different inhibitors at 50 μM. Parasitaemia was normalized to the control. Error bars show SEM corresponding to three independent experiments. **B.** Invasion inhibition assays.
Purified merozoites from either 3D7 (left) or 3D7ΔEBA-175 (right) parasite lines were added to erythrocytes treated with varied concentrations of FTY720 or sphingosine. Parasitaemia was counted after 24 hr, normalized to the control and expressed as % inhibition. C. Growth inhibition assays (GIAs) showing inhibitory activity of FTY720, HA1077 and sphingosine for 3D7 (left) and 3D7ΔEBA-175 (right) parasite lines. D. GIAs after washing or not washing waixenicin A treated 3D7 (left) and 3D7ΔEBA-175 parasites (right). All trophozoite-synchronized cultures were treated with serial inhibitor concentrations and parasitaemia was counted after 24 hr. Error bars show SEM corresponding to three independent experiments.

Figure 4. FTY720 inhibits \textit{P. falciparum} invasion by interfering with a post-translational pathway triggered by EBA-175 binding to GPA. A. Phosphopeptide heavy (H) to light (L) ratios of EBA-175 (H) with PBS (L) treated ghosts (left). Phosphopeptide intermediate (M) to light (L) ratios of EBA-175 and FTY720 treated (M) with untreated (L) ghosts (right). B. Equivalent quantitative analysis of unique phosphopeptides represented in volcano plots showing that GPA phosphorylation sites are not phosphorylated in the presence of FTY720. C. Fluorescence microscopy showing of erythrocytes in the presence of calcium indicator Fluo-4-AM. EBA-175 treated erythrocytes (bottom) are more permeable to Ca^{2+}. Scale bar 40 μm. D. High content screen analysis of the number of Fluo-4-AM bright cells untreated or treated with EBA-175 RIII-IV and EBA-175 RII in the presence of FTY720, HA1077 and sphingosine (50 μM). E. AFM Young’s modulus measurements. Erythrocytes treated with PBS or EBA-175 RII in the presence of FTY720 or waixenicin A (left). Erythrocytes treated with EBA-140 RII (middle) or PfRh4 (right) in the absence or presence of FTY720. F. Frames of live imaging experiments for 3D7 merozoites and erythrocytes in the absence or presence of FTY720 (6 μM). In each condition, bright field is shown in top panels and 594 channel in the bottom ones. Scale bars are 5 μm. G. Table detailing the number of merozoites (Mero) that attach, contact (number of contacted erythrocytes), deform, invade and undergo echinocytosis (Echinocyt). H. Stacked graphs showing the deformation score percentage of total interactions for 3D7 merozoites in the absence or presence of FTY720. Deformation scores are according to a simplified four-point deformation scale [1]. Error bars show SEM corresponding to three independent experiments.
Figure supplement legends:

**Figure 1 - figure supplement 1.** A. Schematics showing EBA-175, PfRh4 and PfRh5 domain structure. The black bars represent the recombinant fragments spanning the binding domains used in this study. B. Coomassie gel of the recombinant proteins EBA-175 RII, Rh4.9 and Rh5 used for erythrocyte binding assays. C. Erythrocyte binding assays showing that EBA-175 RII, Rh4.9 and Rh5 bind erythrocytes as detected by immuno-blot (molecular weight markers are labeled on the left). D. Erythrocyte binding of EBA-175 RII, Rh4.9 and Rh5. Erythrocyte binding profiles with bound protein analysed by immuno-blotting using specific primary antibodies. Bound protein was quantified using fluorescent secondary antibodies and densitometry. The curves show that EBA175 RII and Rh5 binding is saturable as the slope of the curve decreases with increasing amounts of input protein. Binding of Rh4.9 increases linearly between 0 and 20 μg of input protein. An immuno-blot is shown below the graph, with molecular weight markers on the right and lanes labeled as the amount of protein added to binding assays (μg).

**Figure 1 - figure supplement 2.** A. Quantitative binding assay of a 6x-His tagged recombinant EBA-140 RII. Erythrocytes were labeled with recombinant RII-140 and stained with a FITC conjugated anti-6x-His antibody. B. Representative flow cytometry profile for untreated erythrocytes (gray, left) and erythrocytes treated with RII-140 (red, right).

**Figure 2 – figure supplement 3.** A. Autoradiographs overlay of MW vs. pl 2D electrophoresis gel of EBA-175 treated (red) or untreated (green) ^32^P radio-labeled RBC ghosts. B. Coomassie blue stained preparative 2-D gel of untreated erythrocyte ghosts. C. Ponceau stains (top) of the 2-D electrophoresis gels of untreated (left) and EBA-175 treated (right) ghosts with the corresponding autoradiographs (bottom). D. Overlap of western blots of the 2-D preparative gel using antibodies against adducin-2, tropomodulin-1, beta-actin, tropomyosin, ankyrin-1 and coflin-1. Top panel corresponds to untreated red blood cell ghosts and bottom panel to EBA-175 RII ghosts. Notice the increase in band intensity and acid shift for adducing (1), tropomodulin (2) and β-actin (3) in the bottom panel.
**Figure 2 – figure supplement 4.** A. Stacked bar graph (left) of the total number of unique peptides detected (982) in the H + L mix plotting the percentage of no phosphorylated peptides (0P) and with single (1P), double (2P) and triple (yellow) phosphorylation. Scatter plot (right) showing the intensity of the different H/L ratio groups detected (0P, pink; 1P, blue; 2P, green; 3P, yellow). B. Number of unique phosphopeptides with multiple phosphorylations (1P, 2P, 3P) for a number of erythrocyte transmembrane and cytoskeletal protein IDs. C. Amino acid sequence of glycophorin C (top), red square highlights the cytoplasmic domain conserved across different species. Glycophorin C unique phosphopeptides detected (bottom), red circles point out phosphorylation sites all located in the cytoplasmic domain.

**Supplementary File 1.** Table of LC-MSMS evidence and quantitative statistics for the double labelling experiment in which EBA-175 treated erythrocytes proteins were labelled with heavy isotope (H) versus untreated that were labelled with light isotope (L).

**Supplementary File 2.** Table of LC-MSMS evidence and quantitative statistics for the triple labelling experiment in which EBA-175 treated erythrocytes were labelled with heavy isotope (H), EBA-175+FTY720 treated erythrocytes proteins were labelled with medium isotope (M) versus untreated erythrocytes proteins labelled with light isotope (L).

**Video 1.** This video is linked to Fig. 4 F which shows the still time lapse images of this videomicroscopy of *P. falciparum* merozoites invading Bodipy TR Ceramide labelled human erythrocytes. This shows the deformation caused by the merozoites during the initial interaction before invasion is activated.

**Video 2.** This video is linked to Fig. 4 F which shows the still time lapse images of this videomicroscopy of *P. falciparum* merozoites attempting to invade Bodipy TR Ceramide labelled human erythrocytes in the presence of FTY720. This shows the lack of severe deformation caused by the merozoites during the initial interaction and their inability to invade. This is linked to Fig. 4 F which shows the still time lapse images of the movie.