**Background**

Malaria causes over a half million deaths per year, chiefly among children and pregnant women in sub-Saharan Africa, and most of the cases are caused by *Plasmodium falciparum* [1]. A closely related species, *Plasmodium reichenowi*, infects chimpanzees [2, 3]. Until 2009, only one *P. reichenowi* isolate was genetically characterised [4]. Due to the morphological similarity of these two species, it was initially suggested that *P. falciparum* originated from *P. reichenowi*, most likely by a single transfer from chimpanzees (*Pan troglodytes*) [5, 6] or evolved in bonobos (*Pan paniscus*) [7]. However, it was previously shown that while humans cannot be infected by *P. reichenowi*, *P. falciparum* can infect chimpanzees, albeit without severe symptoms as seen in humans [3, 8].

Recently, the use of molecular tools for species identification to explore the diversity of *Plasmodium* species, have revealed new phylogenetic species in great apes [6, 7, 9, 10]. As a result, in the old subgenus *Laverania*, six *Plasmodium* species were confirmed, of which *P. reichenowi*, *P. gaboni* and *P. billcollinsi* only infect chimpanzees, whereas *P. praefalciparum*, *P. adleri* and *P. blacklocki* only infect gorillas. Indeed, it was proposed that great apes are
natural hosts to diverse Plasmodium species, including P. falciparum previously considered as strictly human-specific [6].

Sequencing of Plasmodium DNA from a large collection of ape fecal samples revealed the closest relative, and likely origin of human P. falciparum, is a clade of parasites found in the western gorillas [11]. This finding suggested a possible gorilla origin of human P. falciparum, in opposition to previous theories proposing chimpanzee-human transmission of P. reichenowi-related parasite. In particular, all known strains of P. falciparum circulating in humans nowadays resulted from a single cross-transmission event from gorilla to human [11]. These data suggested that ape-to-human transmission was possible, but an alternative theory that each parasite species evolved independently along with its natural hosts to diverse Plasmodium species, including P. falciparum previously considered as strictly human-specific [6].

Plasmodium falciparum merozoite ligands that bind glycoporphins show distinct binding behaviors, which result in different invasion pathways. The well-studied P. falciparum EBA-175 recognizes glycoporphin A (GPA) [23–25], while EBA-140 [26–28] was shown to bind glycoporphin C (GPC) [29–33], a minor erythrocyte sialoglycoprotein [32, 34]. Both EBA-140 and EBA-175 bind to erythrocytes in a sialic acid-dependent manner [35], but binding of EBA-140, also, requires that GPA is N-glycosylated [36]. EBA-175 recognizes sialic acids present on clusters of O-linked glycans of glycoporphin A (GPA). Homologs of merozoite EBL and RBL, including the EBA-140 protein, were identified in P. reichenowi [37, 38]. The amino acid sequences of the P. falciparum and P. reichenowi EBA-140 proteins are 81% identical, and the highest degree of similarity is seen within the binding region (Region II). However, the binding specificity of the P. reichenowi EBA-140 is still poorly understood. Both ligands require sialic acid for binding. Using the surface expression of human and chimpanzee EBA ligands on COS 7 cells, it was shown that the P. falciparum EBA-140 requires Neu5Ac, while its P. reichenowi counterpart requires Neu5Gc [8]. Thus, the difference in the binding specificity of these proteins may have arisen in response to the change of host "sialome" during the evolution of the human-specific Plasmodium species [39]. The human lineage lost the ability to turn Neu5Ac into Neu5Gc as a result of a mutation in the CMASH gene, which encodes the CMP-Neu5Ac hydroxylase. Thus, it has been hypothesized that the difference in sialic acid structure between humans and apes is the primary factor determining species-specific binding of malaria parasites [8, 39]. However, it was found that the EBA-175 ortholog from the chimpanzee-restricted parasites binds to human GPA with a similar affinity to that of P. falciparum, which suggests that the EBA 175-GPA interaction is probably not the sole determinant of Plasmodium host specificity [40]. Moreover, it was proposed that the interaction of the P. falciparum Rh5 RBL ligand with basigin on erythrocytes is a major determinant of host species tropism.

There is a general agreement that P. falciparum recognizes GPA on human erythrocytes [29–33]. GPA is encoded in humans by the GYPc gene, which is unique among the glycoporphin genes because it contains two separate translation initiation sites [41, 42]. This leads to the synthesis of (predominantly) GPA and its truncated form, called glycoporphin D (GPD), which lacks the first 21 aa residues of GPA. It was shown that GPA, but not GPD, plays a role in erythrocyte invasion mediated by P. falciparum EBA-140 [29–32]. Also, GPA (but not GPD) contains N-glycan at the Asn8 residue, the presence of which seems to be necessary for receptor recognition [35, 36, 43]. The sequences of the GYPc gene homologs
in six Hominidae species (human, chimpanzee, bonobo, gorilla, orangutan and white-cheeked gibbon) reveal a C to A transversion, which results in the emergence of a new start codon present only in humans [41]. Thus, humans are the only species that produce both GPC and GPD, with GPC being the major gene product. Therefore, it was suggested that GPD might be an ancestral receptor in nonhuman primates for *P. reichenowi*-like parasites, while GPC emerged as a new receptor in humans, targeted by *P. falciparum* [41].

To explain the role of GPC and GPD in *P. reichenowi* binding, we used the recombinant binding region (Region II) of *P. reichenowi* EBA-140 obtained in baculovirus expression vector system [44]. We demonstrate that the receptor for the *P. reichenowi* EBA-140 is probably the homolog of human glycophorin D on chimpanzee erythrocytes.

**Methods**

**Erythrocytes**

Chimpanzee (*Pan troglodytes*) blood was freshly collected on EDTA during non-experimental clinical veterinary practice in the Warsaw Zoological Garden. The blood was drawn from immobilised chimpanzee female during a medical intervention. This sample was used to perform diagnostic tests, and the intact remainder (0.5 ml) was used in our experiments.

**Flow cytometry analysis**

The recombinant *P. reichenowi* EBA-140 Region II was incubated in phosphate buffered saline (PBS), pH 7.4 for 2 h at 4 °C with 3 x 10^5 native and trypsin- and chymotrypsin- (Sigma-Aldrich, St. Louis, MO, USA) treated chimpanzee erythrocytes. The heat-denatured Region II was used as the negative binding control. The cells were washed three times with PBS and incubated for 1 h at 4 °C with rabbit serum (diluted 1:200) against the whole *P. falciparum* EBA-140 Region II [45]. The cells were washed three times with PBS and incubated for 45 min at 4 °C with FITC-conjugated swine anti-rabbit Ig antibody (DakoCytomation, Glostrup, Denmark) and analyzed for fluorescence intensity using flow cytometry (FACSCalibur, BD Biosciences, San Jose, USA). Mouse monoclonal antibody (MoAb) (clone 2G11 [46], diluted 1:500) recognizing human GPC and the chimpanzee monoclonal antibody (MoAb) (clone 2G11 [46], diluted 1:500) recognizing human GPC/GPD, respectively. The PageRuler Prestained Protein Ladder (Fermentas, Vilnius, Lithuania) was used as a protein standard.

**Results**

**Binding of the *P. reichenowi* EBA-140 region II to chimpanzee erythrocytes**

Treatment of erythrocytes with proteolytic enzymes may influence binding of antibodies or other ligands in two ways: they can either degrade the receptor, thwarting the binding, or trim off only the proteins that shield the receptor, thus exposing its binding sites. We found that treatment of chimpanzee erythrocytes with chymotrypsin causes a slight decrease of the EBA-140 Region II binding (Fig. 1a). In contrast, the binding after trypsin treatment was markedly increased. We suggest that trypsin digestion removes proteins that sterically hinder the ligand-receptor interaction. MoAb 2G11, which specifically binds to human GPC/GPD showed a similar binding profile to chimpanzee erythrocytes (Fig. 1b), although the drop in binding after chymotrypsin treatment was more evident.

**Binding of EBA-140 region II to glycophorin D**

The binding of the *P. reichenowi* EBA-140 ligand Region II to chimpanzee erythrocyte proteins was evaluated by Western blotting (Fig. 2). The protein recognized by the Region II showed the apparent molecular weight of 35 kDa, which is a similar value to GPD recognized by MoAb 2G11. The binding of the EBA-140 Region II to the erythrocyte receptor was decreased after chymotrypsin treatment, but augmented by trypsin. These data suggest that the chimpanzee erythrocyte receptor for the *P. reichenowi* EBA-140 is the GPD homolog. The bands of higher molecular weight are GPC/GPD aggregates with other erythrocyte glycophorins, mostly GPA [22].

Western blotting corroborated the specificity of the *P. reichenowi* EBA-140 Region II binding to GPD with human erythrocyte membranes (Fig. 3). We found that the *P. reichenowi* Region II binds to human GPC and its truncated form, GPD, while the homologous *P. falciparum* EBA-140 Region II binds only to GPC, as it was previously shown [32].
Discussion
While genomes of *P. falciparum* and *P. reichenowi* are remarkably similar, large differences in the genes involved in red cell invasion (which determine host specificity), stand out against that conserved background [16]. It was shown that these genomes are essentially colinear in the core central regions with a small number of significant differences. The most striking of these involve genes associated with red cell invasion. Within the five-member EBL family, EBA-165 is a pseudogene in *P. falciparum* but not in *P. reichenowi*, while EBL-1 has a substantial deletion in *P. reichenowi*. Similarly, of six RBLs in the *P. falciparum* genome, only three (Rh2b, Rh4, Rh5) have orthologues in *P. reichenowi*. The Rh2 locus seems to be the most different between these two species. Notable differences can also be seen between the *rif* and *stevor* multigene families where the numbers are much lower in the human parasite [16].

Moreover, genes encoding other erythrocytic malaria antigens: MSP2 [50] and *var2CSA* (which is associated with malaria in pregnancy [51]), have shown recently extended polymorphism in *P. falciparum* that likely originated after the *P. reichenowi*-*P. falciparum* split [9]. Thus, it was hypothesized that changes in the sequence and arrangement...
of genes encoding erythrocytic stage antigens, especially the EBL and RBL proteins, may be directly associated with Plasmodium human adaptation. Additional support for this hypothesis comes from the evaluation of dimorphism of P. falciparum EBA-175 alleles [17] and human specificity of Rh5 ligand in receptor-binding preferences [40]. Explaining how this host-switch occurred in the evolution of P. falciparum lineage remains still one of the greatest challenges.

There is general agreement that GPC is the sialylated receptor on human erythrocytes for the P. falciparum EBA-140 [29–32]. Since EBA-140 ligand does not recognize GPA, which is also a highly glycosylated protein, it may be presumed that the GPC protein backbone is involved in the binding. Location of the binding site of EBA-140 close to N- and O-glycans at the N-terminal portion of GPC and lack thereof in its truncated form, GPD, may explain why the P. falciparum EBA-140 Region II does not bind to GPD.

Until now, the erythrocyte receptor for the P. reichenowi EBA-140 remained unknown. To identify it, we used a soluble, recombinant Region II obtained in insect cells. We showed previously that the P. reichenowi Region II of EBA-140 binds specifically to chimpanzee erythrocytes in a sialic acid-dependent manner [44]. We found that binding of the EBA-140 Region II to GPD is markedly decreased by treatment of erythrocytes with chymotrypsin. Conversely, trypsin treatment enhances the binding, probably by removing proteins that sterically hinder access to GPD, while leaving GPD intact. This is in stark contrast to the effects of trypsin on human erythrocytes, which fail to bind the P. falciparum EBA-140 after digestion [32]. In our opinion, this discrepancy results from the presence of arginine (R27) in the human GPD (R48 in GPC), which introduces a trypsin digestion site, while the chimpanzee GPD contains tryptophan (W) at this position. The difference makes the chimpanzee GPD resistant to trypsin, but susceptible to chymotrypsin (Fig. 4). However, since tryptophan in chimpanzee GPD is followed by methionine, which is not a preferred residue at P1’ position, GPD may undergo only a partial digestion by chymotrypsin. As a result, residual binding of the P. reichenowi EBA-140 RII to erythrocytes after chymotrypsin treatment is detected by both flow cytometry and Western blotting experiments.

It was shown before that GPC, but not GPD plays a role in human erythrocyte invasion mediated by P. falciparum EBA-140 ligand [29–32]. Also, only GPC is N-glycosylated at the Asn8 residue, and the N-glycan seems to be necessary for receptor recognition [35, 36, 43]. It is presumed that P. falciparum EBA-140 ligand does not bind to human GPD [31], due to the lack of N-glycan. Thus, it may be speculated that the P. falciparum EBA-140 ligand lost the ability of its ancestor to bind GPD, but developed the specificity for GPC, involving its N-glycan and O-glycans.

Conclusions
Our results show that the chimpanzee GPD is the receptor for the P. reichenowi EBA-140 on chimpanzee erythrocytes. Also, these results hint that development of EBA-140 GPC specificity may have helped P. falciparum to thrive in human erythrocytes when the human and chimpanzee lineages diverged from their ancestor.

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Availability data and materials
All data generated or analysed during this study are included in this published article.

Authors’ contributions
AZ performed the experiments and drafted the paper. RK proofed the manuscript. MCZ and EJ conceived the experiments and analysed the data. EJ helped write the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Chimpanzee erythrocytes were freshly collected on EDTA during non-experimental routine veterinary practice in the Warsaw Zoological Garden. The blood was taken from an immobilized female chimpanzee during non-experimental routine veterinary practice in the Warsaw Zoological Garden. The blood was taken from an immobilized female chimpanzee during non-experimental routine veterinary practice in the Warsaw Zoological Garden.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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