Acquisition of human plasminogen facilitates complement evasion by the malaria parasite Plasmodium falciparum

The tropical disease malaria claims more than 400,000 victims every year. Most deaths are due to infections with Plasmodium falciparum, the causative agent of malignant malaria [1]. Affected people particularly suffer from high fever, anemia, hemoglobinuria, and neurological dysfunctions, caused by the red blood cell (RBC)-infecting stages of the unicellular parasite.

To avoid destruction by human complement, the parasites acquire complement regulators like factor H, which bind to the surface of the infected RBC [2,3]. In addition, the factor H-related protein FHR-1 is able to bind to infected RBCs, where it counteracts complement evasion [4]. Plasmodium falciparum is also able to acquire the plasma zymogen plasminogen (Plg) [5,6]. Mediated by the urokinase-type and tissue-type plasminogen activators tPA and tPA, Plg can be converted into the serine protease plasmin (Plm), which is involved in fibrin degradation but can also process complement factors such as C3 and C5 [7,8].

We here investigated the potential role of Plg acquisition during complement evasion by the P. falciparum blood stages. Schizonts cultivated with normal human serum were subjected to Western blotting, using a polyclonal Ab (pAb) against Plg, which detected a specific ∼92 kDa band, while a much fainter band was detected in noninfected RBCs (Fig. 1A). Immunoblotting with a mAb against the RBC-specific α1-spectrin and a pAb against the ER protein Pf39 of P. falciparum served as loading controls. ELISA and immunoblot quantification confirmed significantly increased binding of Plg to schizonts compared to noninfected RBCs (Fig. 1B; Supporting Information Fig. S1A). Subsequent immunolabeling demonstrated that Plg strongly binds to the surface of RBCs infected with rings, trophozoites, or schizonts, while Plg binding to noninfected RBCs is weak (Fig. 1C). An accumulation of Plg was frequently observed at the probable entry site of the invading merozoite.

To study Plg-to-Plm conversion, RBCs infected with trophozoites or schizonts, devalized paraformaldehyde-fixed schizonts and noninfected RBCs were incubated with Plg for 2 h in the presence or absence of tPA and lyses were immunoblotted with pAb Plg to detect Plg (92 kDa) and Plm (83 kDa). In the absence of tPA, Plg was partially processed into Plm by live trophozoites, while Plm levels increased in schizonts. Contrary, no Plm bands were detected in devalized schizonts or noninfected RBCs (Fig. 1D and E). Addition of tPA resulted in complete Plg processing in all samples. Purified Plg and immunoblotting with mAb α1-spectrin and pAb Pf39 served as controls.

Plm can inactivate C3b by different pathways; cleavage of the C3b α′-chain either leads to two fragments of 68 and 46 kDa or of 27 and 87 kDa (Fig. 1F; Supporting Information Fig. S1B), while further processing can result in additional fragments of approximately 40 kDa [7]. To study C3b processing, schizonts were incubated with Plg, tPA or both for 2 h, followed by incubation with C3b for 1 h. Lysates were immunoblotted with pAb C3 to detect C3b α′ (114 kDa) and C3b β (75 kDa) as well as additional C3b degradation products. In schizonts incubated with C3b alone, the C3b α′- and β-chains were detected. Pretreatment with Plg resulted in an additional band, representing C3b α′87 (87 kDa) (Fig. 1G). When Plg was added together with tPA, a strong C3b α′87 band was detected; in addition, a 40-kDa band was visible, indicating further processing of the α′-chain. Incubation with tPA alone had no effect on C3b inactivation.

To study formation of the terminal complement complex (TCC), schizonts were incubated with normal human serum in the presence or absence of additional Plg. Supplementation resulted in decreased TCC band intensities (∼330 kDa) compared to parasite samples lacking additional Plg, when a mAb directed against the TCC was used for blotting (Supporting Information Fig. S1C). ELISA and immunoblot quantification confirmed significantly decreased TCC levels, when Plg and/or tPA was added to the parasite cultures (Fig. 2A, Supporting Information Fig. S1D).

TCC formation was further investigated for cultures incubated with Plg-depleted plasma (Supporting Information Fig. S2A). Immunoblotting with
Figure 1. *Plasmodium falciparum* blood stage parasites bind Plg to process C3b. (A) Schizonts (SZs) and noninfected RBCs (niRBCs) were incubated with NHS for 1 h. Lysates were immunoblotted with pAb Plg (Plg, 92 kDa). Immunoblotting with mAb a1-spectrin (Spect, 280 kDa) and pAb Pf39 (Pf39, 39 kDa) served as loading controls. (B) SZs and niRBCs were treated as described in (A) and subjected to ELISA, using pAb Plg (technical triplicate; mean ± SD; niRBCs set to 1). Student’s t-test, **p < 0.01. (C) Rings (RS), trophozoites (TZs), SZs, and niRBCs were treated as described in (A) and subjected to immunofluorescence assay, using pAb Plg (green). Incubation with PBS served as control. RBCs were labeled with Evans Blue (EB, red) and nuclei were stained with Hoechst 33342 (blue). Bar, 5 μm and 1000× magnification. (D) TZs and niRBCs were incubated with Plg in the presence (+) or absence (−) of tPA in PBS for 2 h. Lysates were immunoblotted with pAb Plg (Plg, 92 kDa; Plm, 83 kDa). Immunoblotting with mAb Spect served as loading control. (E) Live and PFA-fixed SZs were treated and immunoblotted with pAb Plg as described in (D). Immunoblotting with pAb Pf39 served as loading control. (F) Schematic of C3b cleavage by Plm. iC3b, inactivated C3b. (G) SZs were incubated with Plg, tPA or both for 2 h, followed by incubation with C3b for 1 h. Lysates were immunoblotted with pAb C3 to detect C3b α (114 kDa), C3b α87 (87 kDa), C3b β (75 kDa), and additional C3b degradation products (asterisk). Results are representative of three independent biological experiments with individual parasite samples (A-E, G).
Figure 2. Plg binding impairs TCC formation and promotes *P. falciparum* blood stage growth. (A) SZs were incubated with NHS with/without Plg and/or tPA for 1 h and subjected to ELISA, using mAb TCC (technical triplicate; mean ± SD; NHS set to 1). (B) SZs were incubated with Plg-depleted plasma (ΔPlg) with/without Plg and/or tPA for 1 h and subjected to ELISA, using mAb TCC (technical triplicate; mean ± SD; ΔPlg set to 1). (C) Ring stages were cultivated with HIS or NHS with/without Plg for 96 h and the parasitemia was calculated (technical triplicate; mean ± SD; HIS set to 1). (D) Ring stages were cultivated with plasma or ΔPlg with/without Plg and tPA for 96 h and the parasitemia was calculated (technical triplicate; mean ± SD; plasma set to 1). One-way ANOVA, *p* < 0.05, **p** < 0.01, ***p*** < 0.001. Results are representative of three independent biological experiments with individual parasite samples (A–D).

mAb TCC demonstrated lower TCC levels in parasites incubated with Plg-depleted plasma, when this was supplemented with exogenous Plg (Supporting Information Fig. S2B). ELISA and immunoblot quantification confirmed a significant decrease of TCC levels, when Plg was added to the cultures (Fig. 2B, Fig. Supporting Information S2C). The TCC levels were even lower, when both Plg and tPA were present, while addition of tPA alone had no effect on TCC formation. In addition,
a concentration-dependent decrease of TCC formation was observed by ELISA, when either Plg-depleted plasma or normal human serum was supplemented with Plg (Supporting Information Fig. S2D and E).

Plg further affected parasite growth in vitro. When ring stage parasites were cultured in normal human serum for 96 h, the parasitemia increased in the presence of additional Plg with or without tPA (Fig. 2C, Supporting Information Fig. S2F). Heat-inactivated serum was used for control. Furthermore, the parasitemia significantly decreased when the parasites were cultivated in Plg-depleted plasma instead of plasma, while the addition of Plg and tPA could revert this effect (Fig. 2D).

In summary, we show that Plg acquisition enhances the viability of the \textit{P. falciparum} blood stages by promoting C3b inactivation and, in consequence, preventing TCC formation. Our data further suggest that a protease present on the surface of the infected RBC facilitates Plg-to-Plm conversion required for C3b inactivation. In accord with our findings, Plg internalization and processing by plasmodial proteases was reported [6]. Furthermore, acquisition of uPA by malaria parasites has previously been assigned to functions in RBC egress by merozoites, while Plm and uPA/tPA inhibitors impaired growth of blood stage parasites [9,10]. Future studies need to investigate the detailed mechanism of Plg-mediated complement evasion by malaria parasites.

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