Molecular & Biochemical Parasitology 167 (2009) 48–53

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

Recruitment of human aquaporin 3 to internal membranes in the *Plasmodium falciparum* infected erythrocyte

Sven Bietz, Irine Montilla, Simone Külzer, Jude M. Przyborski, Klaus Lingelbach*

Department of Parasitology, Faculty of Biology, Philipps Universität, 35032 Marburg, Germany

ARTICLE INFO

Article history:
Received 19 January 2009
Received in revised form 15 April 2009
Accepted 16 April 2009
Available online 23 April 2009

Keywords:
Aquaglyceroporin
Aquaporin 3
Parasitophorous vacuolar membrane
Plasmodium falciparum

ABSTRACT

The molecular mechanisms underlying the formation of the parasitophorous vacuolar membrane in *Plasmodium falciparum* infected erythrocytes are incompletely understood, and the protein composition of this membrane is still enigmatic. Although the differentiated mammalian erythrocyte lacks the machinery required for endocytosis, some reports have described a localisation of host cell membrane proteins at the parasitophorous vacuolar membrane. Aquaporin 3 is an abundant plasma membrane protein of various cells, including mammalian erythrocytes where it is found in distinct oligomeric states. Here we show that human aquaporin 3 is internalized into infected erythrocytes, presumably during or soon after invasion. It is integrated into the PVM where it is organized in novel oligomeric states which are not found in non-infected cells.

1. Introduction

Inside its host erythrocyte the malaria parasite *Plasmodium falciparum* resides within a parasitophorous vacuole (PV), the membrane of which (PVM) separates the parasite surface and the erythrocyte cytosol and thus acts as an interface between parasite and the host cell [1]. Studies have revealed that the PVM is formed during or immediately after invasion, but its biochemical composition and the molecular processes that lead to its expansion during parasite growth are largely unknown. During parasite development, parasite encoded proteins such as the exported proteins 1 and 2 (PfEXP-1, PfEXP-2), and the early transcribed membrane proteins (ETRAMPs) become integrated into, or associated with, the PVM [2–4]. It was long believed that, while the PVM contains lipids of the red blood cell plasma membrane (RBCM), host cell membrane proteins are excluded when the PVM is formed [5,6]. This view was later challenged when it was observed that transmembrane and glycosylphosphatidylinositol (GPI) anchored proteins of the RBCM were internalized and associated with the PVM [7]. Analysis of detergent resistant microdomains (DRM) obtained from a total membrane fraction of infected erythrocytes (iRBC) revealed an association of several host proteins with these biochemically distinct membrane domains. Immunofluorescence microscopy demonstrated a localisation of some of these proteins at the PVM [8]. Notably, proteins of high abundance, such as band 3, were excluded from DRMs suggesting a selective uptake of RBCM resident proteins, possibly based on their association with DRMs. While some of the identified proteins such as flotillins may be required for the formation of the microdomains [8], the cell biological functions of other proteins that were found to be internalized remains enigmatic. It is unclear whether internalized proteins are required for parasite survival, or whether such proteins are internalized fortuitously, merely due to their DRM association.

In its function as a host–parasite interface, the PVM is a barrier which restricts the exchange of macromolecules between the parasite and its host cell. Nevertheless, acquisition of nutrients from the iRBC and from the extracellular milieu, as well as the release of metabolic waste products, is essential for parasite viability [9]. In order to identify proteins within the PVM that may contribute to nutrient acquisition and the export of unwanted metabolic products, we have begun a systematic search for such candidates. Aquaporins constitute a group of multispanning plasma membrane proteins which are largely required to maintain homeostasis in eukaryotic cells. AQP1 belongs to the group classical aquaporins and its main function is to act as a water selective pore [10]. Interestingly, in iRBC AQP1 has been identified as a tentative DRM protein which, by immunofluorescence, appears to be associated with the PVM [8]. Aquaporin 3 (AQP3), in contrast to AQP1, is an aquaglyceroporin with an only moderate permeability for water, but with a permeability for glycerol. In human erythrocytes it is

Abbreviations: AQP1, aquaporin 1; AQP3, aquaporin 3; DRM, detergent resistant microdomains; ETRAMPS, early transcribed membrane proteins; IFA, immunofluorescence assay; iRBC, infected red blood cell; PfEXP-1, *Plasmodium falciparum* exported protein 1; PfEXP-2, *Plasmodium falciparum* exported protein 2; PfSERP, *Plasmodium falciparum* serine-rich protein; PV, parasitophorous vacuole; PVM, parasitophorous vacuolar membrane; RBC, red blood cell; RBCM, red blood cell plasma membrane; SLO, streptolysin O.

* Corresponding author. Tel.: +49 6421 2823404; fax: +49 6421 2821531.
E-mail address: lingelba@staff.uni-marburg.de (K. Lingelbach).

0166-6851/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.molbiopara.2009.04.006

present in different oligomeric states [11]. Here we have investigated the cell biological and biochemical properties of AQP3 in iRBC. Our data provide evidence that AQP3 is internalized and locates to the PVM where it is found in an oligomeric state, distinct from that found in non-infected cells.

2. Materials and methods

2.1. Cell culture

P. falciparum (isolate FCBR) was cultivated in human red blood cells of blood group A+ (Marburg Blood Bank), at a haematocrit of 2%. The culture medium was RPMI 1640, supplemented with 10% heat inactivated human plasma of the same blood group. Cell cultivation and enrichment of infected erythrocytes were performed using standard procedures [12]. Enrichment of ring stage infected RBC was performed as previously described [13].

2.2. Protease treatment of intact RBC and iRBC

Erythrocytes (1×10^8 cells) were washed three times in Dulbecco's PBS (PAA) and subsequently incubated in PBS containing either 1 mg mL^{-1} trypsin (Sigma) or chymotrypsin (Applichem). Incubation was performed at 37 °C for 30 min. The cells were then washed three times in Dulbecco's PBS containing 1 mM of PMSF to inactivate and remove the proteases.

2.3. Ghost preparation

Infected erythrocytes were washed three times in Dulbecco's PBS, then resuspended in 5 mM KH_2PO_4 containing protease inhibitor cocktail set III (Calbiochem) and incubated on ice for 10 min. Ghost and non-ghost fractions were sedimented at $10{,}000 \times g$ for 10 min at 4 °C. The top layer (ghosts) of the pellet was carefully separated from the non-ghost fraction. Each fraction was washed three times with PBS containing protease inhibitor cocktail. The fractions were lysed in 5 mM KH_2PO_4 containing protease inhibitor cocktail, applying three cycles of freezing and thawing in liquid nitrogen.

2.4. Membrane preparation and immunoblotting

Erythrocytes (RBC or iRBC) were lysed by three cycles of freezing and thawing in 5 mM KH_2PO_4 containing a protease inhibitor cocktail set III. A membrane fraction was sedimented by centrifugation at $36{,}000 \times g$ for 20 min at 4 °C. Membrane proteins were solubilized in SDS-PAGE sample buffer for 10 min at 95 °C, separated by 12% SDS-PAGE and transferred to nitrocellulose membrane using standard procedures. For comparative purposes, two rabbit polyclonal antibodies, both raised against a synthetic peptide corresponding to amino acids 275–292 of rat AQP3, were obtained from different suppliers (Calbiochem or Sigma). The antibodies were used at a dilution of 1:200 and the proteins were detected after incubation with horseradish peroxidase-conjugated anti-rabbit IgG (DAKO) at a dilution of 1:2000. To absorb antibodies reacting with the synthetic peptide, 1 μg of the antibody was pre-absorbed with 1 μg of the peptide for 1 h at room temperature, according to the suppliers' manual. Antibodies against the PVM resident protein PfExp-1 and the band 3 protein have been described elsewhere [2].

2.5. Deglycosylation of membrane proteins

Membrane fractions were prepared as described above. The membrane proteins were solubilized by boiling in sample buffer for 15 min. Remaining membrane debris was removed after centrifugation of the samples. Solubilized membrane proteins were deglycosylated overnight at 37 °C using the N-glycosidase F Deglycosylation kit (Roche).

2.6. Protease treatment of permeabilised infected erythrocytes

Permeabilisation of iRBC with streptolysin O (SLO) was performed as described previously [14]. Permeabilised cells were centrifuged, the supernatant (containing the erythrocyte cytosol) was kept for further analysis and the pellet (containing intact parasites within the intact PVM) was washed three times in PBS. The pellet fraction was incubated in the presence or absence of trypsin or chymotrypsin as described above and subsequently lysed in 5 mM KH_2PO_4 containing protease inhibitor cocktail as described above. The soluble fraction was separated from the membrane fraction by centrifugation at $36{,}000 \times g$. Antisera to PfSERP and PfAldolase were used as previously described [2,14].

2.7. Indirect immunofluorescence assay (IFA)

IFA was performed on mixed stage infected erythrocytes as previously described [15] except that fixation was carried out at 37 °C for 30 min. Anti-AQP3 (1:100 dilution) and monoclonal mouse anti-Band3 (Sigma, dilution 1:1000) were used. Parasite nuclei were stained with Hoechst 33258 (Invitrogen, 1:100,000). Images were acquired on a Zeiss Cell observer inverse epifluorescence microscope system and processed with Image J v 1.39 (http://rsb.info.nih.gov/ij/).

3. Results and discussion

AQP3 is an N-glycosylated protein located in the membrane of human and other mammalian erythrocytes where it can be detected in several oligomeric states which resist dissociation

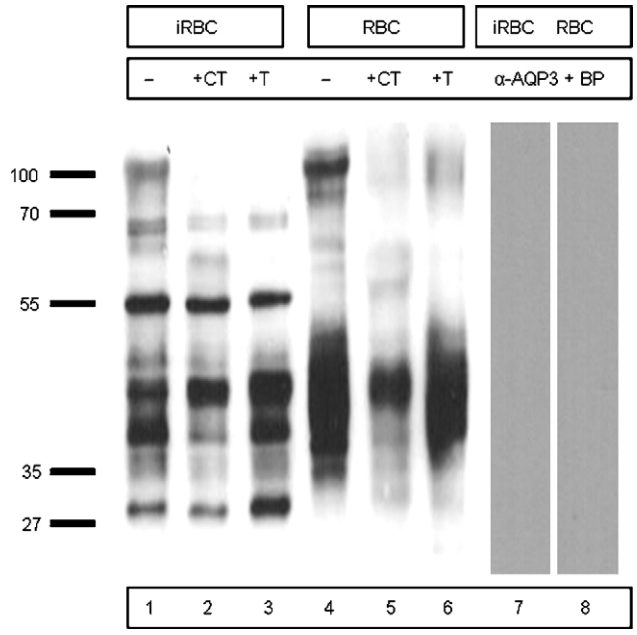

Fig. 1. Protease treatment of intact erythrocytes. Intact infected RBC (lanes 1–3) and non-infected RBC (lanes 4–6) were mock-treated (lanes 1 and 4) or treated with either chymotrypsin (CT) or with trypsin (T). A total membrane fraction was prepared, and equivalents corresponding to 5×10^7 cells separated through 12% SDS-PAGE, blotted onto nitrocellulose and probed with affinity purified antibodies raised against the C-terminal peptide of rat aquaporin 3. One sample each of the membrane fractions from iRBC (lane 7) and from RBC (lane 8) was reacted with the antibodies that were pre-absorbed with a blocking peptide (BP) which corresponds to the peptide used for antibody generation. The molecular size markers in kDa are indicated on the left.