International Journal of Medical Microbiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Identification of novel parasitophorous vacuole proteins in *P. falciparum* parasites using BioID

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ARTICLE INFO

Keywords:
Malaria
Plasmodium falciparum
Parasitophorous vacuole
BioID
Proteome

ABSTRACT

Malaria blood stage parasites develop within red blood cells where they are contained in a vacuolar compartment known as the parasitophorous vacuole (PV). This compartment holds a key role in the interaction of the parasite with its host cell. However, the proteome of this compartment has so far not been comprehensively analysed. Here we used BioID in asexual blood stages of the most virulent human malaria parasite *Plasmodium falciparum* to identify new proteins of the PV. The resulting proteome contained many of the already known PV proteins and validation by GFP-knock-in of 10 previously in *P. falciparum* uncharacterised hits revealed 5 new PV proteins and two with a partial PV localisation. This included proteins peripherally attached to the inner face of the PV membrane as well as proteins anchored in the parasite plasma membrane that protrude into the PV. Using selectable targeted gene disruption we generated mutants for 2 of the 10 candidates. In contrast we could not select parasites with disruptions for another 3 candidates, strongly suggesting that they are important for parasite growth. Interestingly, one of these included the orthologue of UIS2, a protein previously proposed to regulate protein translation in the parasite cytoplasm but here shown to be an essential PV protein. This work extends the number of known PV proteins and provides a starting point for further functional analyses of this compartment.

1. Introduction

In 2015 more than 200 million cases of human malaria and over 400′000 deaths were estimated to have occurred worldwide (WHO Report 2016). The deaths were primarily due to infection with *Plasmodium falciparum*, the most virulent of human malaria parasites. The pathology of malaria is caused by the asexual development of the parasite within red blood cells (RBC). In this life cycle stage the parasite invades red blood cells (RBCs) wherein it multiplies to produce new invasive forms (termed merozoites) that are released under destruction of the host cell. The released merozoites infect new RBCs, leading to an exponential increase of parasite numbers in the blood. In the RBC the parasite resides in a tightly fitting vacuolar compartment termed the parasitophorous vacuole (PV) (Lingelbach and Joiner, 1998; Spielmann et al., 2012). The PV is formed during active invasion of the parasite into the RBC and is defined by the parasitophorous vacuole membrane (PVM) facing the host cell cytosol and by the parasite plasma membrane (PPM) on the parasite side.

As an interface between the parasite and its host cell the PV controls the passage of nutrients (such as monosaccharides, amino acids, nucleotides, ions, and vitamins), the disposal of waste products (such as lactate), and the export of parasite proteins into the host cell (Kirk and Lehane, 2014). While a number of PV and PVM proteins are known, PV1 is the so far only confirmed PV protein identified from systematic attempts to characterise the PV proteome (Chu et al., 2011; Nyalwidhe and Lingelbach, 2006). Other prominent PV proteins are SERAs and SUB1, proteases that are essential for parasite egress (Knapp et al., 1989; Miller et al., 2002; Putrianti et al., 2010; Sajid et al., 2000; Silmon de Monerri et al., 2011), the PTEX components HSP101, PTEX150, PTEX88, EXP2 and TRX2, which are found at the PV luminal side of the PVM and mediate the transfer of exported proteins into the host cell (Beck et al., 2014; de Koning-Ward et al., 2009; Elsworth et al., 2014; Mesen-Ramirez et al., 2016) and integral PVM proteins that have a domain reaching into the PV such as EXP1 and ETRAMPs (Kara et al.,

Abbreviations: LC–MSMS, liquid-chromatography coupled to tandem mass spectrometry; PPM, parasite plasma membrane; PV, parasitophorous vacuole; PVM, parasitophorous vacuole membrane; RBC, red blood cell; SLI, selection-linked integration; SP, signal peptide; TGD, targeted-gene-disruption; TM, transmembrane domain

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http://dx.doi.org/10.1016/j.ijmm.2017.07.007
Received 29 June 2017; Received in revised form 14 July 2017; Accepted 17 July 2017

1988; Spielmann et al., 2003; Spielmann et al., 2006; Stenzel and Kara, 1989). There are also a considerable number of proteins at the parasite plasma membrane (PPM) that have domains reaching into the PV. This includes multi-transmembrane spanning transporters such as PfFNT (Marchetti et al., 2015; Rajendran et al., 2017; Wu et al., 2015), or cationic amino acid transporters (Rajendran et al., 2017) and proteins anchored on the parasite surface such as MSP1, MSP8, MSP10 or Pf113 (Drew et al., 2005; Sanders et al., 2005).

In order to reach the PV, proteins are trafficked through the canonical secretory pathway of the parasite followed by fusion of vesicles with the PPM and release into the PV, or in the case of transmembrane proteins, the PPM (Deponte et al., 2012). This is considered the default destination of secretory proteins lacking any other trafficking signals (Deponte et al., 2012; Waller et al., 2000). Examples of signals that prevent a PV location are for instance the ER retention motif (C-terminal S/KDEL) (Denecke et al., 1992) or export signals such as the PEXEL/HT (Hiller et al., 2004; Marti et al., 2004). Hence, it can be generally assumed that proteins that enter the endoplasmic reticulum via an N-terminal signal peptide (SP), but lack other trafficking signals, will end up in the PV. It is less clear how transmembrane proteins reach the PVM, but there is evidence that this does not include insertion by PTEX (Tribensky et al., 2017).

A previous attempt to identify PV proteins relied on selective permeabilization of infected RBCs to generate access to the PV followed by biotinylation by sulfo NHS-LC-biotin and purification of these proteins (Nyalwidhe and Lingelbach, 2006). BioID (Roux et al., 2012) now enables to biotinylate proteins of a compartment *in vivo* without the need of selective lysis. We here took advantage of this technique to identify new PV and PPM/PVM proteins.

2. Materials and methods

2.1. Parasite culture and transfection

P. falciparum parasites were cultured in human O+ erythrocytes according to standard procedures (Trager and Jensen, 1976) using RPMI 1640 medium containing 0.5% AlbuMAX (Invitrogen). Synchronized segmenter stage parasites were transfected with 50 µg of purified plasmid DNA (Qiagen) as described in (Moon et al., 2013). Alternatively transfectants were obtained by transfecting ring stage parasites as described (Wu et al., 1995). Positive selection was done with 4 nM WR99210 (Jacobus Pharmaceuticals).

2.2. Endogenous GFP tagging of candidates

Initially integration was done conventionally via on- and off-drug cycling with parasites harboring the episomal plasmid (for genes PF3D7_1464600, PF3D7_1123500, PF3D7_1013300, PF3D7_1226900). The corresponding plasmids were obtained by cloning of the 737–1184 bp long C-terminal region of these targets into pARL-GFP (Crabb et al., 2004) without a promoter using NotI and AvrII (see Table S1 for all primers). All other integrants were obtained using selection-linked integration (SLI) as described (Birnbaum et al., 2017). The corresponding plasmids were generated by cloning of the 599–1027 bp long C-terminal region of these target genes into NotI and AvrII digested pSLI-2×FKBP-GFP (Birnbaum et al., 2017) via Gibson assembly. For targeted-gene-disruption (TGD) 300–363 bp long regions (starting with a stop codon) in the N-terminal part of the gene of interest were PCR amplified (see Table S1 for primers) and cloned NotI/MluI into pSLI-TGD (Birnbaum et al., 2017) via Gibson assembly. Correct integration of the plasmid into the genome was checked for all integrants via PCR using two vector and two candidate specific primers as previously described (Birnbaum et al., 2017).

2.3. Immunofluorescence assays (IFA)

Ten-well, 6,7 mm slides (Thermo) were Concanavalin A (Sigma) coated as described in (Spielmann et al., 2003). Infected RBC were applied to each well and incubated for 15 min at room temperature. The cells were fixed using the 'wet acetone' procedure (De Niz et al., 2016). For this, Concanavalin A-unattached cells were washed off from the slide with PBS, excess liquid was aspirated and the slides immediately (to avoid drying) placed into 100% acetone for 30 min. The slides were briefly dried and rehydrated in 1 x PBS and the IFA carried out according to standard procedures (Spielmann et al., 2003). Primary antibodies were mouse anti-GFP (Roche) diluted 1:400, mouse anti-ETRAMP5 (Spielmann et al., 2006) diluted 1:200, both applied in PBS/3%BSA for 1 h. Streptavidin was used 1:1000 (Life Technologies) and secondary antibodies were goat anti-mouse and donkey anti-rabbit conjugated with Alexa Fluor-488 used 1:2000 (Life Technologies) that were all applied for 1 h in PBS/3%BSA containing 1 µg/ml DAPI (Roche). Slides were sealed with a coverslip after mounting medium (Dako) had been applied.

2.4. Imaging

IFA as well as live cell images were taken with a Hamamatsu Orca C4742-95 camera mounted on a Zeiss Axio Scope M1 microscope (controlled by Zeiss AxioVision software) using a 100×/1,4 numerical aperture oil immersion lens. Images were processed in Corel PHOTO-PAINT. For live cell imaging 500 µl of parasite culture was stained with 50 µl of 5 µM Bodipy-TR-C5-ceramide (Molecular Probes) and with 1 µg/ml DAPI (Roche) as described (Gruring and Spielmann, 2012). On Slide saponin lysis was performed with 0.3–0.03% saponin in PBS added between slide and coverslip at the periphery of the sample. The outcome (absence or presence of GFP signal remaining at the parasite periphery) reflected the result obtained when imaging infected RBCs lysed in bulk using 0.015% saponin in PBS.

2.5. Parasite extracts

Infected RBCs were purified from the culture using a Percoll gradient (Aley et al., 1984). To obtain the supernatant fraction of the infected RBCs, the Percoll-collected cells were treated with 0.03% saponin (Sigma) in PBS. Protease inhibitors (Roche) and SDS sample buffer were added to the supernatant. The pellet containing the parasite within the permeabilised RBC and PVM, was washed twice in PBS and proteins were extracted with 4% SDS, 0.5% Triton X-114, 0.5× PBS in presence of protease inhibitors (Roche). Before loading, the extracts were centrifuged at 16,000*g* for 5 min, SDS-PAGE loading buffer was added and the extracts were incubated at 85 °C for 5 min. Volumes of supernatants and pellets were equivalent and equal volumes were loaded onto SDS-PAGE.

2.6. Western analysis

Protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (Protran) in a tankblot device (Bio-Rad) using transfer buffer (0.192 M Glycine, 0.1% SDS, 25 mM Tris) with 20% methanol. Five percent skim milk in PBS was used for blocking and dilution of antibodies. As primary antibody mouse anti-GFP (Roche) 1:1000 was used and as secondary antibody horseradish peroxidase-conjugated goat anti-mouse (Dianova) diluted 1:3000. For Streptavidin blots membranes were blocked with 5% skim milk in TBS. Horseradish peroxidase-conjugated streptavidin (Invitrogen) was diluted 1:5000 in 1% skim milk in TBST. The Clarity™ Western ECL Substrate (Bio Rad) kit was used for detection and the chemiluminiscence signal recorded using a Chemi Doc XRS imaging system (Bio-Rad) and processed with Image Lab Software 5.2 (Bio-Rad).