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Expression and characterization of the *Plasmodium* translocon of the exported proteins component EXP2

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ABSTRACT

The malaria parasite *Plasmodium falciparum* requires the *Plasmodium* translocon of exported proteins (PTEX) to proliferate in human red blood cells. During the blood stages of malaria, several hundred parasite-encoded proteins are exported from the parasite into the cytosol of red blood cells. PTEX is the translocon for protein export and comprises 5 proteins: EXP2, PTEX150, PTEX88, Hsp101 and TRX2. Among them, EXP2 is thought to constitute the transmembrane pore, whereas the other components seem to play a role in unfolding the luggage proteins or providing a driving force. However, detailed functional and structural characterizations of PTEX proteins have not been performed. In this study, we expressed and characterized the membrane-associated component EXP2. Because expression of EXP2 is lethal to *E. coli*, EXP2 was expressed as a fusion protein with GST, and the recombinant EXP2 was obtained by protease digestion. The recombinant EXP2 formed pores in bilayer lipid membranes. The inner diameter of the pore was estimated to be approximately 3.5 nm based on electron microscopy images and channel currents. From this size and the molecular mass as determined by size exclusion chromatography and blue native polyacrylamide gel electrophoresis, we determined that the pore comprises approximately 10–12 EXP2 subunits. However, there is a possibility that the pore structure is different in the PTEX complex. These results provide important insights in the protein transport mechanism of PTEX, which will aid in developing new drugs targeting PTEX.

1. Introduction

Approximately 3.2 billion people – almost half of the world's population – are at the risk of malaria infection (http://www.who.int/mediacentre/factsheets/fs094/en/). Malaria is a mosquito-borne infectious disease caused by a parasitic protozoan belonging to the *Plasmodium* genus. The life-cycle of *Plasmodium* spp. is considerably complex, comprising of several different stages both in the insect and the vertebrate host [1]. The insect vector injects malaria parasites in the sporozoites stage into the vertebrate host's blood. Sporozoites infect the host liver and produce between 10,000 to more than 30,000 daughter merozoites, which move into the blood where they infect red blood cells. In the red blood cells, the number of asexual parasites can reach over 100 million [1].

During the blood stage, *Plasmodium* spp. parasites extensively modify the host red blood cell (RBC) by exporting hundreds of proteins into the infected cell [2]. The parasite replicates in a parasitophorous vacuole (PV) surrounded by a PV membrane (PVM). Thus, the proteins have to cross two membranes: the parasite plasma membrane (PPM) and the PVM to reach the cytosol of RBC

Abbreviations: RBC, red blood cell; PV, parasitophorous vacuole; PVM, PV membrane; PTEX, Plasmodium translocon of exported proteins; PPM, parasite plasma membrane; PEXEL, Plasmodium export element; HT, host targeting signal; PNEP, PEXEL negative exported protein; rEXP2, recombinant EXP2; BLM, bilayer lipid membrane; IPTG, β-D-1-thiogalactopyranoside; PBS, phosphate-buffered saline; DDM, n-dodecyl-β-D-maltopyranoside; GST, glutathione S-transferase; HRV, human rhinovirus; Blue Native PAGE, blue native polyacrylamide gel electrophoresis; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; SM, Sphingomyelin; HlyE, hemolysin E; TEM, Transmission electron microscopy.

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[3]. The exported proteins are classified into two types. One group contains a five-amino acid motif termed the *Plasmodium* export element (PEXEL) or host targeting signal (HT). The other group comprises PEXEL-negative exported proteins (PNEPs), which are defined by the absence of a PEXEL/HT signal [4,5]. This protein translocation machine, known as '*Plasmodium* translocon of exported proteins' (PTEX), is involved in this export step. PTEX comprises 5 proteins: heat shock protein 101 (HSP101), PTEX150, EXP2, PTEX88, and TRX2 [6]. Among these, Hsp101, PTEX150 and EXP2 are essential for protein transport [7]. EXP2 is thought to be the PTEX membrane pore component [6,8,9]. HSP101 belongs to a group of Hsp104 chaperones that facilitate recovery of aggregated proteins [10]. Thus, Hsp101 seems to be responsible for making the proteins in the unfolded state for crossing the EXP2 pore [6,11]. PTEX150 is thought to cooperate with HSP101 and EXP2 in exporting proteins [8,12], and PTEX88 and TRX2 may regulate this process [7]. The current model for PTEX function is as follows. PEXEL/HT-containing proteins arrive PTEX complex at the PVM, and then are unfolded by Hsp101 in an ATP dependent manner. The unfolded proteins pass through a pore formed by EXP2, and are finally refolded in the erythrocyte cytoplasm by parasite-encoded and/or host cell chaperones [6,8,9].

Due to the recent emergence of *Plasmodium* spp. strains that are resistant to conventional drugs [13], it is urgent to develop novel treatments by investigating the proliferation mechanism of the parasite at the molecular level. PTEX has been proposed as a novel drug target because PTEX genes are indispensable for the growth of the parasites [7,14].

Modeling of the predicted structure of EXP2 has shown its structural similarity with the pore-forming toxin of *Escherichia coli*, Hemolysin E (HlyE) [6], which forms a 12-mer or 13-mer pore within host cell membranes [15,16]. Subsequently, it is of particular interest whether EXP2 can oligomerize to form a protein-translocating pore within the PVM. It has been shown that PTEX is present as a 1230-kDa complex containing a 600–700 kDa EXP2 homo-oligomeric species, of which a dimer is likely to be the core subunit [8,12]. EXP2 is the most strongly membrane-associated PTEX component throughout the intra-erythrocytic life cycle, which is conducive with its potential to form a membrane pore [8].

Currently, studies on PTEX have been performed by using *in vivo* studies. In contrast, the biochemical and functional characterizations of PTEX have not advanced. In this study, we obtained and characterized recombinant EXP2 (rEXP2). The rEXPs formed oligomers and formed pores in bilayer lipid membranes (BLMs). The size of pores was comparable with that of HlyE but was smaller than that of previous estimations. It might be possible that EXP2 has a fixed conformation by interacting with other components in PTEX.

2. Materials and methods

2.1. Expression and purification of EXP2

A synthetic gene encoding the full-length EXP2 from the strain *P. falciparum* 3D7 (PF14_0678) was obtained from Eurofins Genomics (Tokyo, Japan) (Supplementary Fig. S1). The codons were optimized for expression in *E. coli*. To express wild-type EXP2, the full-length EXP2 gene was amplified by PCR using primers (Pfal_OptEXP2_*Nde* I_Fw 5′ - ggaattcCATATGAAAGTCTCTTACATCTTTTC - 3′ and Pfal_OptEXP2_*Xho* I_Rv 5′ - ccgCTCGAGTTAGTGGTGATGG - 3′) and then subcloned into the pET30b plasmid using the restriction enzymes *Nde*I and *Xho*I. To obtain a fusion protein with glutathione S-transferase (GST), the EXP2 gene was amplified with primers (Fw_HRV_EXP2_BamHI 5′-CGggatccccctggaagttctgttc-caggggcccGTGGTGTGTGACAATGGC-3′, Rv_HRV_EXP2_EcoRI 5′ CGgaattcttaCTCTTTATTCTCATCTTTTTTCTC-3′) without including the region encoding the signal sequence (64–864 nt). The resulting PCR product was inserted into the *Bam*HI/*Eco*RI site of the pGEX-3X plasmid (GE Healthcare, Buckinghamshire, UK). The primer was designed to insert a human rhinovirus (HRV) 3C protease recognition site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) between GST and EXP2. The order of the elements in the final product should be (from the N terminus) GST-HRV 3C protease recognition site-EXP2.

The resulting plasmid, pGEX-3X-HRV3C-EXP2, was transformed into the *E. coli* BL21 (DE3) strain. The transformed *E. coli* cells were grown in LB broth supplemented with 100 μg/ml ampicillin at 37 °C to an OD_{600} of approximately 0.5, and then isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. The bacteria were incubated for an additional 24 h at 18 °C. The cells were centrifuged and resuspended in the lysis buffer (phosphate-buffered saline (PBS), 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) supplemented with 1 mM EDTA and 0.15 mM phenylmethylsulfonyl fluoride). The cells were disrupted by sonication using an UD-211 ultrasonic disruptor (TOMY SEIKO, Tokyo, Japan), and the supernatant was collected by centrifugation and filtered through a membrane with 0.45 μm pores (ADVANTEC, Tokyo, Japan). The prepared supernatant was used for purification of recombinant EXP2 (rEXP2).

rEXP2 was obtained as follows. The supernatant was incubated with 0.05% n-dodecyl-β-D-maltopyranoside (DDM) (Dojindo Laboratories, Kumamoto, Japan) at 4 °C for 1 h. The supernatant containing DDM was loaded onto a Glutathione Sepharose 4B column (GE Healthcare, Tokyo, Japan), which was then washed with a 10-fold volume of washing buffer (PBS with 0.02% DDM). To release rEXP2 from its GST tag and the column, HRV 3C protease (Takara Bio, Shiga, Japan) in cleavage buffer (50 mM Tris-HCl, 200 mM NaCl, 0.02% DDM, pH 8.0) was applied to the column after replacing the washing buffer with cleavage buffer. After incubating the column at 4 °C for 16 h, rEXP2 was released from the column. The released rEXP2 contains 2 additional amino acids (Gly-Pro) in the N-terminus. The protein concentration was estimated by measuring the absorbance at 280 nm using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA).

2.2. Hemolytic assay

A hemolytic assay was performed as previously described with slight modifications [17]. Whole blood from sheep (Cosmo Bio, Tokyo, Japan) was centrifuged at 500×*g* for 3 min, and the pellet containing red blood cells was washed 5 times with PBS. The purified rEXP2 was diluted to various concentrations in cleavage buffer to a total volume of 20 μl and mixed with 130 μl of PBS. Afterwards, 50 μl of erythrocytes was added to the rEXP2 solution. H_2O was used as the positive control. The samples were incubated at room temperature for 30 min in a total volume of 200 μl. After the samples were centrifuged at 500×*g* for 3 min, 100 μl of each supernatant was diluted to 1 ml with PBS. The absorbance of hemoglobin released was continuously scanned between 350 nm and 700 nm wavelengths by using a V-660 Spectrophotometer (JASCO, Tokyo, Japan). Hemolytic activity of 100% was defined by the diluted supernatant treated with H_2O.

2.3. Blue native polyacrylamide gel electrophoresis

rEXP2 (0.58 mg/ml) was dialyzed against 1000 vol of SEC buffer (50 mM Tris-HCl (pH 8.0), 200 mM NaCl), diluted 5 times with the same buffer, and either untreated or adjusted to a final concentration of 0.2% DDM. These samples were mixed with non-reducing and non-denaturing sample buffer at a volume ratio of 1:1. Samples were electrophoresed on a 4–16% gradient blue native

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