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The *Plasmodium* alveolin IMC1a is stabilised by its terminal cysteine motifs and facilitates sporozoite morphogenesis and infectivity in a dose-dependent manner

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ABSTRACT

Apicomplexan parasites possess a unique cortical cytoskeleton structure composed of intermediate filaments. Its building blocks are provided by a conserved family of proteins named alveolins. The core alveolin structure is made up of tandem repeat sequences, thought to be responsible for the filamentous properties of these proteins. A subset of alveolins also possess conserved motifs composed of three closely spaced cysteine residues situated near the ends of the polypeptides. The roles of these cysteine motifs and their contribution to alveolin function remains poorly understood. The sporozoite-expressed IMC1a is unique within the *Plasmodium* alveolin family in having conserved cysteine motifs at both termini. Using transgenic *Plasmodium berghei* parasites, we show in this structure-function analysis that mutagenesis of the amino- or carboxy-terminal cysteine motif causes marked reductions in IMC1a protein levels in the parasite, which are accompanied by partial losses of sporozoite shape and infectivity. Our findings give new insight into alveolin function, identifying a dose-dependent effect of alveolin depletion on sporozoite size and infectivity, and vital roles of the terminal cysteine motifs in maintaining alveolin stability in the parasite.

1. Introduction

Plasmodium species, the causative agents of malaria, have a complex life cycle in vertebrate host and mosquito vector. Among the many different developmental forms of the parasite feature three motile and invasive stages (also known as 'zoites'): the ookinete, sporozoite and merozoite. The zoites of *Plasmodium*, as well as those of related apicomplexan parasites, possess an unusual cortical structure termed the pellicle. The pellicle is defined by a double membrane structure termed the inner membrane complex (IMC) situated directly underneath the plasma membrane, which is equivalent to a system of flattened sacs or alveoli [1–3]. On the cytoplasmic face of the IMC is anchored a network of intermediate filaments termed the subpellicular network (SPN), the function of which is to support the pellicle membranes and give the cell mechanical strength [4].

A family of proteins now termed alveolins have been identified as components of the SPN [4,5]. The alveolin superfamily includes structurally related proteins from apicomplexan parasites, ciliates and dinoflagellate algae, the three phyla comprising the Alveolata superphylum [6]. In the genus *Plasmodium*, 13 conserved and syntenic alveolin family members have been identified that are differentially expressed among the three different zoites stages of malaria parasites [7,8]. It has been shown in the rodent malaria species *P. berghei* that disruption of alveolins gives rise to morphological aberrations that are accompanied by reduced tensile strength of the zoite stages in which they are found [5,8–11]. In *Tetrahymena thermophila*, knockdown of the alveolin *Tt*ALV2 was also reported to affect cell morphology [12], indicating that alveolin functions, like their structures, are evolutionary conserved. *Plasmodium* alveolins also have roles in parasite gliding motility [5,9–11] most likely by tethering glideosome associated proteins that reside in the IMC.

The alveolins identified in *Plasmodium* are characterised by having one or more highly conserved domains separated by regions of variable length and amino acid composition. These conserved 'alveolin domains' are composed of tandem repeat sequences [7,12]. This has revealed an interesting parallel with metazoan intermediate filament proteins such as lamins and keratins, whose underlying architectures include a helical rod domain that can form coiled-

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A

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IMC1a amino-terminus (4X)C(5X)CC.........CC(3X)C(4X) carboxy-terminus
IMC1c amino-terminus ...................CC(3X)C(1X) carboxy-terminus
IMC1g amino-terminus (1X)C(7X)CC..................... carboxy-terminus
IMC1i amino terminus ...................C(5X)CC(3X) carcoxy-terminus
IMC1j amino-terminus (5X)C(6X)CC..................... carboxy-terminus
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B

alveolin	Position	Peptide	Score	Cutoff
IMC1a	5	***MFDACKINSNCC	39.45	4.222
IMC1a	11	ACKINSNCCHDELGE	13.588	3.419
IMC1a	12	CKINSNCCHDELGED	36.543	4.222
IMC1a	775	SDNSEDKCCNYFCNQ	15.285	3.419
IMC1a	776	DNSEDKCCNYFCNQD	8.229	4.222
IMC1c	272	EEAKPVGCCTGTCR*	9.35	3.419
IMC1c	273	EAKPVGCCTGTCR**	5.043	4.222
IMC1g	2	******MCSTPNKLA	40.756	4.222
IMC1g	10	STPNKLACCSGDNVF	6.81	3.419
IMC1g	11	TPNKLACCSGDNVFD	38.998	4.222
IMC1i	490	FCNIMNKCCGGE***	14.765	3.419
IMC1j	6	**MENKQCKLIFSDC	34.614	4.222
IMC1j	13	CKLIFSDCCKGRENV	10.098	3.419
IMC1j	14	KLIFSDCCKGRENVA	29.829	4.222

Fig. 1. The *Plasmodium* alveolin cysteine motifs. A: Conserved cysteine motifs at the amino- and carboxy-terminal ends of *Plasmodium berghei* alveolins IMC1a (PbANKA_0402600), IMC1c (PbANKA_1202000), IMC1g (PbANKA_1240600), IMC1i (PbANKA_0707100) and IMC1j (PbANKA_1120400). The number of non-cysteine residues (X) adjacent to the conserved terminal cysteines (C) are indicated. Cysteines in red are predicted to be palmitoylated. B: Prediction scores of palmitoylated cysteine residues (red) using CSS-Palm 4.0 software (http://csspalm.biocuckoo.org/) and high threshold settings (95% specificity, 90% accuracy). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coils by virtue of a seven amino acid tandem repeat structure [13]. These coiled-coil domains are thought to be fundamental for the filament-forming properties of these molecules. Apart from the conserved alveolin domains, a subset of the alveolins also possess conserved cysteine motifs close to their amino- or carboxy-terminus (Fig. 1). These motifs are made up of a single cysteine and a double cysteine that are separated by a small number of other amino acids (Fig. 1). With the exception of IMC1i, The N- and C-terminal motifs are inverted, with the single cysteine located nearest the end of the polypeptide (Fig. 1). The function of these cysteine motifs is largely unknown, although they have been suggested to provide sites for post-translational S-palmitoylation [14] (Fig. 1). A subset of alveolins in *Toxoplasma* (IMC1, IMC4, IMC14 and IMC15) possess similar conserved terminal cysteine motifs [14]. Because these conserved cysteine motifs have not been identified in alveolins from dinoflagellates or ciliates, their function could be related to the unique motility and/or cytokinesis associated with the Apicomplexa [6]. IMC1a is the only *Plasmodium* alveolin with conserved cysteine motifs at both ends, and in this study we employ site-directed mutagenesis and allelic replacement in *P. berghei* to investigate the contribution of these motifs to the function of the protein and the SPN as a whole. We also describe a new method for accurate size measurements of sporozoite populations, providing a valuable new tool for assessing sporozoite phenotypes.

2. Materials and methods

2.1. Animal use

All laboratory animal work is subject to regular ethical review by the London School of Hygiene and Tropical Medicine, and has approval from the United Kingdom Home Office. Work was carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 implementing European Directive 2010/63 for the protection of animals used for experimental purposes. Experiments were conducted in 6–8 weeks old female CD1 mice, specific pathogen free and maintained in filter cages. Animal welfare was assessed daily and animals were humanely killed upon reaching experimental or humane endpoints. Mice were infected with parasites by intraperitoneal injection, or by infected mosquito bite on anaesthetized animals. Parasitemia was monitored regularly by collecting of a small drop of blood from a superficial tail vein. Drugs were administered by intraperitoneal injection or where possible were supplied in drinking water. Parasitized blood was harvested by cardiac bleed under general anaesthesia without recovery.

2.2. Parasite maintenance, transmission, culture and purification

P. berghei ANKA clone 2.34 parasites were maintained as cryopreserved stabilates or by mechanical blood passage and regular mosquito transmission. Mosquito infection and transmission assays were as previously described using *Anopheles stephensi* [5,15] and infected insects were maintained at 20 °C at approximately 70% relative humidity.

2.3. Construction of gene targeting vectors

To allow mCherry tagging of IMC1a, an approximately 3.5 kb fragment corresponding to the entire *imc1a* gene (introns included) plus 5′-UTR was PCR amplified from *P. berghei* gDNA using primers pDNR-imc1a-F (ACGAAGTTATCAGTCGAGGTACCTTTCATGATTCTATCTATTGTTAATTTTAATTG) and pDNR-imc1a-R (ATGAGGGCCCCTAAGCTTTTATCTTGATTACAAAAATAATTACAACATTTG) and introduced into SalI/HindIII-digested pDNR-mCherry [16] by in-fusion to give plasmid pDNR-IMC1a/mCherry (Fig. S1).

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