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AdDLP, a bacterial defensin-like peptide, exhibits anti-*Plasmodium* activity

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ABSTRACT

Antimicrobial defensins with the cysteine-stabilized α-helical and β-sheet (CSαβ) motif are widely distributed in three eukaryotic kingdoms. However, recent work suggests that bacteria could possess defensin-like peptides (DLPs). Here, we report recombinant expression, *in vitro* folding, structural and functional characterization of a DLP from the myxobacterium *Anaeromyxobacter dehalogenans* (AdDLP). Circular dichroism analysis indicates that recombinant AdDLP adopts a typical structural feature of eukaryotic defensins, which is also consistent with an *ab initio* structure model predicted using I-TASSER algorithm. We found that AdDLP is an antimalarial peptide that led to more than 50% growth inhibition on sexual stages of *Plasmodium berghei* at micromolar concentrations and killed 100% intraerythrocytic *Plasmodium falciparum* at 10 μM in a time-dependent manner. These results provide functional evidence for myxobacterial origin of eukaryotic defensins. High-level production of the pure anti-*Plasmodium* peptide without harming mammalian red blood cells in *Escherichia coli* makes AdDLP an interesting candidate for antimalarial drug design.

Introduction

Antimicrobial defensins isolated from plants, fungi and invertebrates constitute a large family of effector polypeptides of innate immunity, which showed strong microbicidal activity against Gram-positive bacteria and fungi [1–3]. Some defensins from insects have also been found to exhibit antiparasitic activity [4–6]. Mechanically, these molecules could form voltage-dependent channels in microbial membranes [7]. Their protective roles have been well documented by *in vivo* targeted disruption of the mosquito *Anopheles gambiae* defensin gene causing the death of the mosquitoes after Gram-positive bacterial infection [8]. Members in this family have molecular weights of 3–5 kDa and three to four disulfide bridges, and share a conserved cysteine-stabilized α-helical and β-sheet (CSαβ) structural motif. These molecules represent the only one class of effector scaffold conserved across the eukaryotic kingdom [1–3]. Such a scaffold is composed of a single α-helix and one β-sheet of two strands, in which the α-helix spanning the CysXaaXaaXaaCys sequence is connected by two disulfide bridges to the carboxyl-terminal β-strand containing CysXaaCys (Xaa represents any amino acid), whereas the third disulfide bridge links the amino-terminus to the first β-strand [9]. Due to unique structural and functional features, these defensins are being recognized as ideal molecular targets for developing anti-infective drugs [10,11].

Despite significant conservation in the structural core, defensins from different origins show some structural modifications in their n-loop and carboxyl-terminal sizes as well as disulfide bridge numbers. For example, scorpine-related defensins isolated from scorpion venoms extended their amino-termini to a new antimicrobial unit [12]. Relative to ancient invertebrate-type defensins (AITDs), classical insect-type defensins (CITDs) possess a longer n-loop. Plant/insect-type defensins (PITDs) generally developed a fourth disulfide bridge and most of them display antifungal rather than antibacterial activity [2]. In bees, a duplicated defensin developed a longer carboxyl-terminus [13].

Recent computational structural analysis identified two bacteria-derived defensin-like peptides (DLPs) which could represent the ancestor of eukaryotic defensins [14]. In this work, we report the recombinant expression, *in vitro* folding, structural and functional characterization of one peptide named AdDLP from *Anaeromyxobacter dehalogenans*. We found that AdDLP efficiently inhibited the development of *Plasmodium berghei* ookinetes and killed intraerythrocytic *Plasmodium falciparum* at micromolar concentrations but was not toxic to mammalian red blood cells. These results thus provide functional evidence for myxobacterial origin of eukaryotic defensins. High-level production of pure, nontoxic anti-*Plasmodium* peptide in *Escherichia coli* makes AdDLP an inter-

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esting candidate for antimalarial drug design. At the meantime, the inhibition of *Plasmodium* ookinetes also provides possibility for the production of transgenic *Plasmodium*-resistant mosquitoes in the future.

Materials and methods

Gene synthesis and expression vector construction. The AdDLP nucleotide sequence was synthesized by Beijing BIOMED TECH (BIOMED, Beijing). Synthesized gene was ligated into pET-28a vector by BamHI and SalI sites, in which an enterokinase (EK) sequence (DDDDK) was introduced for the removal of the carrier peptide containing His-tag (Fig. 1A). The recombinant plasmid pET-28a-AdDLP was transformed into *E. coli* BL21 (DE3) plysS for protein expression.

In vitro folding of AdDLP. Expression of fusion protein was induced with 1 mM IPTG at OD_{600} of 0.25. Cells were harvested 4 h later and the pellet was suspended in resuspension buffer (100 mM Tris–HCl, 100 mM NaCl, pH 8.0). After sonication and subsequent centrifugation, the pellet was washed using isolation buffer (2 M urea and 2% Triton X-100 in the resuspension buffer). Following centrifugation, pellets were resuspended in solubilization buffer (6 M guanidinium hydrochloride, 10 mM β-mercaptoethanol and 10 mM imidazole in the resuspension buffer) for 1 h at room temperature followed by centrifugation and the supernatant was loaded to Ni–NTA resin pre-equilibrated by solubilization buffer. Refolding was initiated by a linear urea gradient from 6 to 0 M. Refolded fusion protein was eluted by elution buffer (200 mM imidazole and 3 mM β-mercaptoethanol in the resuspension buffer) and the imidazole in the eluate was completely removed by RP-HPLC (Agilent Zorbax 300SB-C18, 4.6 × 150 mm, 5 μm) using a linear gradient of 0–60% acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) in water within 40 min with a flow rate of 1 ml/min. The lyophilized fusion protein was digested in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) by EK at room temperature for 2 h.

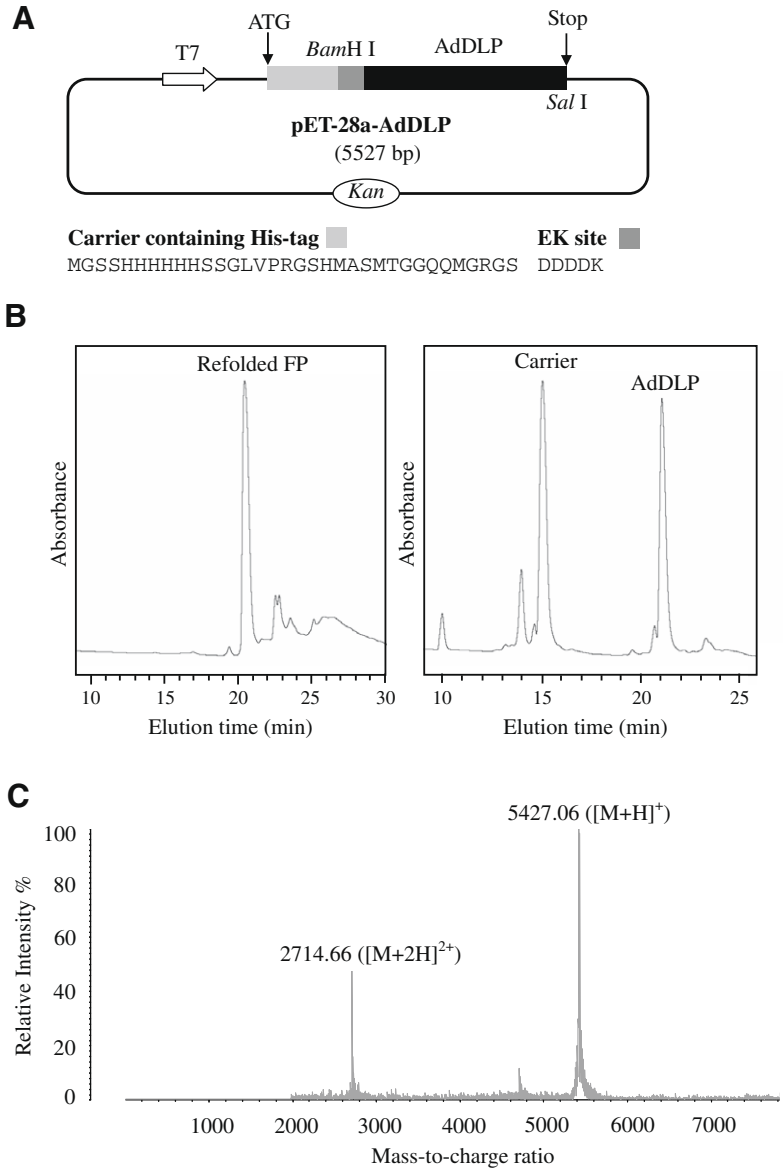

Fig. 1. Expression, purification and characterization of AdDLP. (A) Construction of pET-28a-AdDLP expression vector. The synthesized DNA sequence of AdDLP was inserted into BamHI and SalI sites of pET-28a with an EK cleavage site at the 5′ end; (B) RP-HPLC showing the refolded fusion protein (FP) and its EK-digested product. C18 column was equilibrated with 0.1% TFA and the purified proteins were eluted from the column with a linear gradient from 0% to 60% acetonitrile in 0.1% TFA within 40 min; (C) Determination of the molecular weight of recombinant AdDLP by MALDI-TOF. The spectrum has two main peaks, corresponding to the singly and doubly protonated forms of the peptide.