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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.

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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.

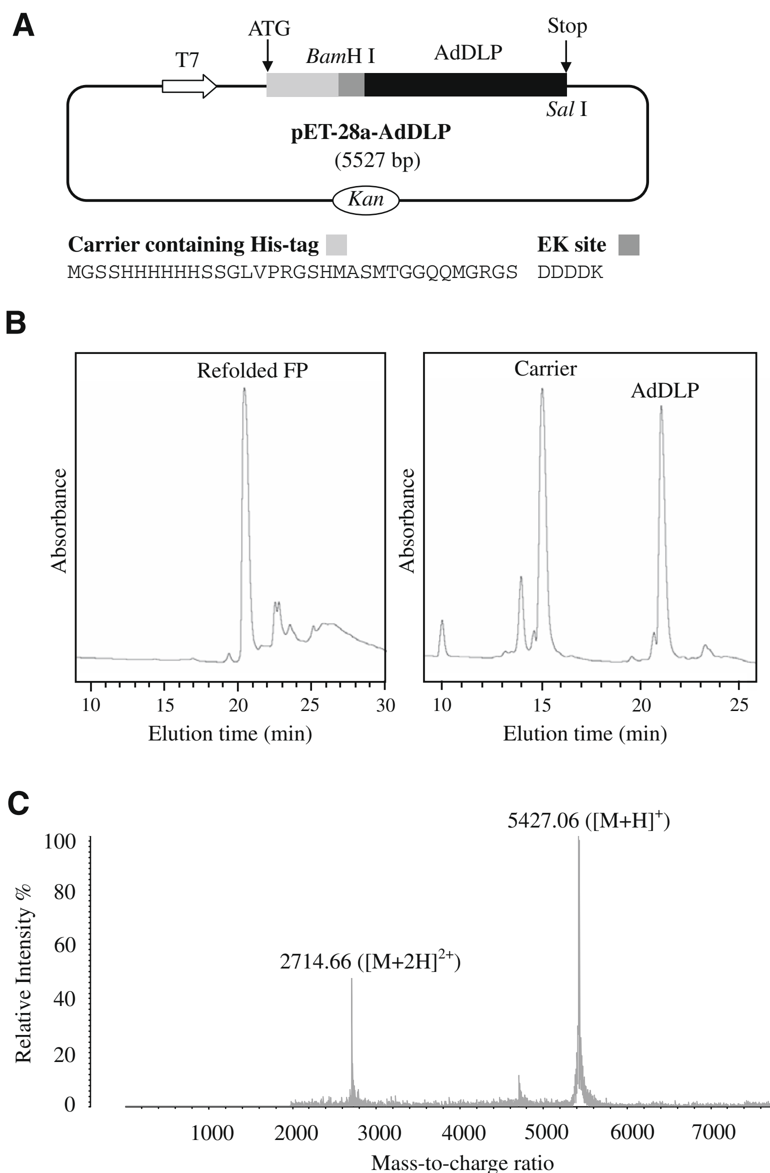

Fig. 1. Expression, purification and characterization of AdDLP. (A) Construction of pET-28a-AdDLP expression vector. The synthesized DNA sequence of AdDLP was inserted into BamHI and SalI sites of pET-28a with an EK cleavage site at the 5′ end; (B) RP-HPLC showing the refolded fusion protein (FP) and 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.

MALDI-TOF. The molecular weight of recombinant AdDLP was determined by MALDI-TOF mass spectra on a Kratos PC Axima CFR plus (Shimadzu Co., Ltd., Kyoto).

Circular dichroism spectroscopy. Circular dichroism (CD) spectra of AdDLP were recorded on a JASCO J-715 spectropolarimeter (Jasco, Tokyo, Japan) at a protein concentration of 0.3 mg/ml dissolved in water. Spectra were measured at 20 °C from 240 to 190 nm by using a quartz cell of 1.0 mm thickness. Data were collected at 0.2 nm intervals with a scan rate of 200 nm/min and CD spectra were measured by averaging three scans. Secondary structure contents in AdDLP were calculated with the DICHROWEB software (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

Antimicrobial and haemolytic assays. Antibacterial assays were carried out according to the literature [15,16]. Briefly, 50 μl of overnight bacterial culture was inoculated into 5 ml LB medium and incubated at 37 °C for 1–3 h with shaking at 200 rpm until they grew to 0.1 OD_{595}. 20 μl of AdDLP was added to 80 μl bacterial culture at a given final concentration and then absorbance at 595 nm was measured after incubation at 37 °C for 24 h; for antifungal assays [17], 20 μl of AdDLP was added to 80 μl fungal spores or yeast cells suspended in 1× MEA medium with 0.2 OD_{595} at a given final concentration. After incubation for 24 h at 26 °C, absorbance at 595 nm was measured. All experiments were repeated twice and H_2O was used as a control. Growth inhibition was evaluated by comparison of absorbances, respectively, recorded from AdDLP- and H_2O-treated microbial cultures. Microorganisms used include: (1) The Gram-positive bacterium *Micrococcus luteus.* (2) The Gram-negative bacterium *E. coli* ATCC 25922. (3) The fugus *Neurospora crassa.* (4) The yeast *Saccharomyces cerevisiae*. Haemolytic activity against fresh mouse (*Mus musculus*) red blood cells was assayed according to the standard method [15].

Antiparasitic assays. P. berghei Anka 2.34, a gametocyte producer strain, was kindly donated by R. Sinden (Imperial College, UK). Ookinete cultures were carried out as described [18]. Leucocyte-depleted infected-mouse blood was suspended 1:5 in culture medium and tested in 100 μl aliquots in flat-bottom 96-well plates. Peptide was tested at two concentrations (10 and 20 μM), and were added to triplicate wells and the numbers of ookinetes were assessed 24 h later in Giemsa-stained blood smears as described [12]; *P. falciparum* parasites were cultivated at 2% parasitemia according to standard conditions [19] or in the presence of 10 μM AdDLP. The effect of the peptide was estimated each 24 h by microscopic examination of thin blood films stained with Giemsa. Total parasitaemia of treated cultures was compared to the total parasitaemia of wild type parasites cultured in the absence of the peptide. Experiments were carried out by triplicate.

Structural prediction by I-TASSER. I-TASSER (http://zhang.bioinformatics.ku.edu/I-TASSER/), a protein structure prediction algorithm that combines the methods of threading, *ab initio* modeling and structural refinement [20], was used to construct a model structure of AdDLP. Protein structures were superimposed using MultiProt, a fully automated and high efficient technique for structural alignments of proteins. RMSD values between Cα atoms of AdDLP and related peptides were calculated at the server (http://bioinfo3d.cs.tau.ac.il/MultiProt). Structures were visualized using MOLMOL (http://hugin.ethz.ch/wuthrich/software/molmol/). Secondary structures of the AdDLP model were defined according to STRIDE [21].

Results and discussion

Production of recombinant AdDLP

It has been proposed that AdDLP represents a putative ancestor of eukaryotic CSαβ-type defensins. To provide experimental evidence to support such a hypothesis, we synthesized its coding sequence and cloned into pET-28a vector (Fig. 1A). The His-tagged fusion protein expressed as inclusion bodies was solubilized with 6 M guanidinium hydrochloride and the solubilized inclusion bodies were subsequently bound to Ni–NTA resin and subjected to on-column refolding by a linear urea gradient approach. In our experiment, about 65% of the inclusion bodies became soluble after refolding in the absence of the denaturation agent, suggesting that these fusion proteins have been correctly folded. The refolded fusion protein and its purity was characterized by RP-HPLC (Fig. 1B). The purified fusion protein was subjected to EK digestion and subsequent RP-HPLC isolation (Fig. 1B), from which we collected one major fraction, corresponding to the retention time of 21.2 min, for MALDI-TOF analysis (Fig. 1C). The experimental MW detected is 5427.06 Da, 4.15 Da less than the calculated MW (5431.21 Da) from its primary sequence, suggesting that four hydrogen atoms in the cysteines were removed to form two disulfide bridges. The final yield of the recombinant AdDLP is about 5 mg/L *E. coli* culture.

Structural features of AdDLP

The CD spectrum of AdDLP showed a typical curve of eukaryotic defensins in water, as identified by a positive maximum at 190 nm and a negative minimum at 208 nm. The spectrum quality is appropriate for calculating the secondary structure content of AdDLP because it is nearly identical with the calculated spectrum by CDSSTR method and reference data set 4 (Fig. 2A). Based on the CD data, we estimated the secondary structure content in AdDLP by DICHROWEB [22]. The results indicate that the recombinant peptide contains 25% α-helix and 32% β-sheet, compatible with some structurally known defensins. For example, for *Drosophila* antifungal drosomycin (pdb entry 1MYN), the corresponding values calculated from its NMR structure are 25% and 22.7%, respectively. The experimentally determined secondary structure content is higher than a previous model constructed by comparative modeling, where the structure of the carboxy-terminal 10 residues of AdDLP was not built due to the lack of corresponding structural information in the template [14]. To obtain a reliable full-length structure model with a compatible secondary structure content with the CD result, we applied the I-TASSER algorithm to *ab initio* model the AdDLP structure (Fig. 2B). The model built in this way exhibits an overall structural similarity to that from comparative modeling, but this new model contains 22% α-helix and 24% β-sheet, closer to the experimental data from CD. In this new model, two disulfide bridges for the formation of a typical cysteine-stabilized α-helix (CSH) motif [23] were correctly predicted. Assignment of this new model secondary structures by STRIDE identified an α-helical region that spans residues Arg^{12}-Leu^{22} and a β-sheet composed of three strands (β1: Tyr^5Arg^6; β2: Leu^{26}-Val^{30}; β3: Gly^{37}-Glu^{41}).

When VAST (vector alignment search tool) was used to search for structural neighbors in the MMDB/PDB database (http://www.ncbi.nlm.nih.gov/Structure/VAST/vastsearch.html), we detected three classes of defensins from multicellular organisms as structural homologues of AdDLP, which include: (1) ancient invertebrate-type defensins (e.g. CgDEF and Mgd-1); (2) classical insect-type defensins (e.g. PtDEF A); and (3) antifungal plant/insect-type defensins (e.g. drosomycin, Ah-AMP1, Vrd2 and Gamma 1-H (Fig. S1, provided as Supplementary material). Root mean square deviations (RMSDs) for matched Cα atoms between AdDLP and other peptides range from 1.10 to 1.59 Å, suggesting their significant structural similarity and thus providing structural evidence for their evolutionary relationship.

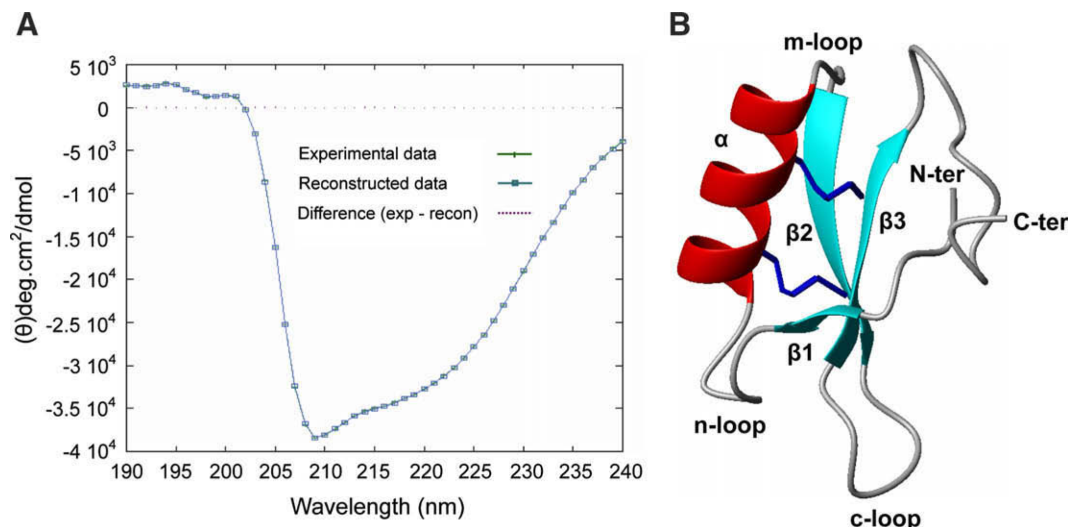

Fig. 2. Structural analysis of AdDLP by CD and computational prediction. (A) The CD spectra of recombinant AdDLP in water. Spectra were taken at a peptide concentration of 0.3 mg/ml. Green: experimental data, blue: calculated spectrum derived from the calculated output secondary structure, dotted line: difference between experimental and calculated data; (B) The *ab initio* structure of AdDLP predicted by I-TASSER (http://zhang.bioinformatics.ku.edu/I-TASSER/) which has been submitted to the Protein Model database (http://mi.caspur.it/PMDB/) under the id number of PM0075559. Blue sticks represent disulfide bridges. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Biological activity of AdDLP

We firstly evaluated antibacterial and antifungal effects of recombinant AdDLP on several representative microorganisms by liquid growth inhibition assays. The results showed that at 10 and 20 μM concentrations, this peptide exhibited no obvious antimicrobial activity (classical inhibition zone assays giving identical results even at higher peptide concentrations, data not shown). At these two concentrations, AdDLP also displayed no hemolytic effect on mouse red blood cells.

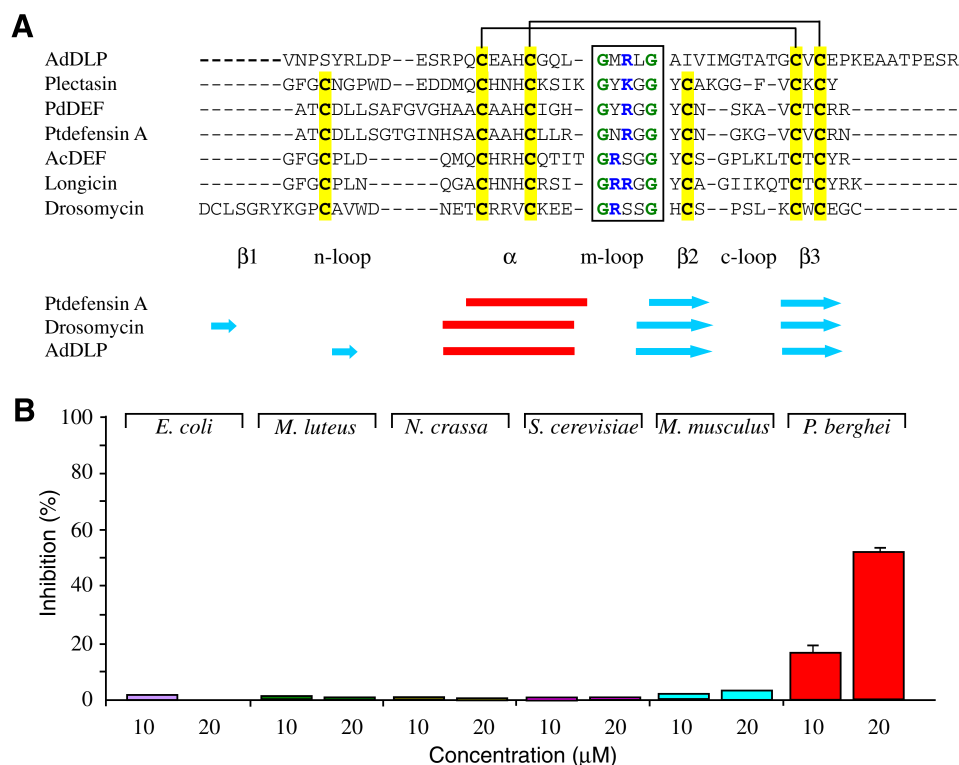

Fig. 3. Structure-guided functional discovery of AdDLP. (A) AdDLP contains a 5-residues-motif (GMRLG) in the m-loop which is similar to some previously recognized defensins with antiparasitic activity (Notice: the antiparasitic activity of plectasin is not tested even though it has such a motif). Cysteines involved in disulfide bridges are shadowed in yellow. The 5-residues-motif is boxed with conserved glycines in green and basic residues in blue. Secondary structure elements of the *ab initio* model of AdDLP, Ptdefensin A (pdb entry 1ICA) and drosomycin (pdb entry 1MYN) were defined according to STRIDE; (B) functional screening of targets of recombinant AdDLP. Only representative species for Gram-negative and Gram-positive bacteria, fungus, yeast, mammalian red blood cell and parasite were chosen for evaluation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Previous studies have shown that some insect defensins possess antiparasitic activity [4–6]. For example, Shahabuddin et al. reported that injecting insect defensins from *Phormia terranovae* and *Aeshna cyanea* negatively affected the development of *Plasmodium gallinaceum* oocysts and sporozoites [4]. Drosomycin, a *Drosophila* antifungal peptide, inhibited the development of *P. berghei* ookinetes [24]. Studies also have revealed that longicin plays a crucial role in inhibiting the transmission of *Babesia* parasites in the vector tick *Haemaphysalis longicornis* [25]. Previously, we have noticed that all these defensins have a common motif which could be related to their antiparasitic activity [24]. This highly exposed motif is located in the m-loop and its five residues contain at least one arginine and two glycines at two termini of this motif that likely facilitate the formation of flexible conformations to promote the interaction of these polypeptides with parasites. Interestingly, such a motif was also found in AdDLP which is composed of $G^{23}MRLG^{27}$ (Fig. 3A), suggesting that AdDLP could possess antiparasitic activity. Our assays showed that AdDLP efficiently inhibited the development of *P. berghei* ookinetes in the concentration range from 10 to 20 μM (Fig. 3B). At the latter concentration, the inhibitory efficiency is >50%, more potent than two well-characterized antiparasitic peptides, e.g. Shiva-3, a cecropin-like synthetic peptide [26], and gambicin, a cysteine-rich peptide of 8 kDa from *A. gambiae* [27]. Subsequently, we evaluated effects of AdDLP on the intraerythrocytic *P. falciparum*, a human malaria parasite, by exposing its trophozoite stage cultures to 10 μM AdDLP. Our results showed that this peptides significantly reduced parasite density at 48 h post-treatment in relation to controls (Fig. 4). Remarkably, no infected erythrocytes were detected after 72 h. Such a critical dependence on exposure time was also observed in RScp, a recombinant antimalarial peptide from the venom of the scorpion *Pandinus imperator* [28].

Conclusion

AdDLP is the first bacterial defensin-like peptide identified in the Gram-negative bacterium *A. dehalogenans*. Although sequence and structural feature supports its evolutionary relationship to eukaryotic defensins, experimental evidence is lacking. In this work, we successfully produced active AdDLP by recombinant techniques and analyzed its structure and function. Our study confirmed that AdDLP exhibited a typical eukaryotic defensin structure characteristic. The finding of its antiparasitic activity suggests that this feature could represent an ancient function of eukaryotic defensins. Also, the structural and functional analysis of this peptide is important for gene construction of recombinant peptides that could be used to control parasites by developing transgenic malaria-resistant mosquitoes. In particular, high-level prokaryotic production of pure anti-*Plasmodium falciparum* peptide without harming mammalian red blood cells makes AdDLP an interesting candidate for antimalarial drug design. How AdDLP kills intraerythrocytic *P. falciparum* remains an open question and awaits further investigation.

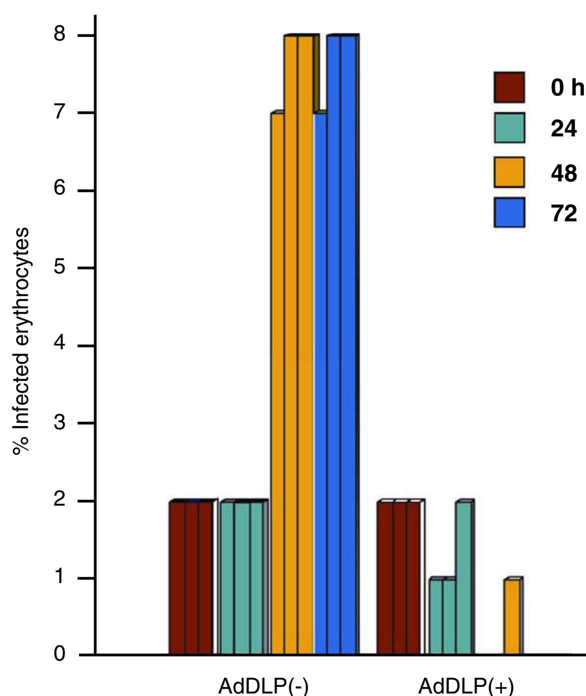

Fig. 4. Antiparasitic activity of AdDLP. Effects of AdDLP on intraerythrocytic *Plasmodium falciparum*. The peptide concentration is 10 μM and experiments were carried out by triplicate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.043.

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