Isolation and characterization of Psalmopeotoxin I and II: two novel antimalarial peptides from the venom of the tarantula *Psalmopoeus cambridgei*

Soo-Jin Choi[a], Romain Parent[b,1], Carole Guillaume[c], Christiane Deregnaucourt[c], Christiane Delarbre[d], David M. Ojcius[d], Jean-Jacques Montagne[a], Marie-Louise Célérier[e], Aude Phelipot[f], Mohamed Amiche[f], Jordi Molgo[g], Jean-Michel Camadro[a,*], Catherine Guette[b,2]

[a]*Laboratoire d'Ingénierie des Protéines et Contrôle Métabolique, Dept. de Biologie des Génomes, Institut Jacques Monod, UMR 7592, CNRS – Universités Paris 6 & 7, 2 place Jussieu, 75251 Paris Cedex 05, France*
[b]*Laboratoire de Chimie des Substances Naturelles, Muséum National d'Histoire Naturelle, 63 rue Buffon, 75231 Paris Cedex 05, France*
[c]*USM 0504, Biologie Fonctionnelle des Protozoaires, Muséum National d'Histoire Naturelle, 61 rue Buffon, 75231 Paris Cedex 05, France*
[d]*Laboratoire d'Immunologie Cellulaire, Institut Jacques Monod, UMR 7592, CNRS – Universités Paris 6 & 7, 2 place Jussieu, 75251 Paris Cedex 05, France*
[e]*Laboratoire d'Ecologie, UMR 7625, CNRS – Université Pierre et Marie Curie – Paris 6, Bât. A – case 237, 7 Quai Saint-Bernard, 75252 Paris Cedex 05, France*
[f]*Laboratoire de Bioactivation des Peptides, Institut Jacques Monod, UMR 7592, CNRS – Universités Paris 6 & 7, 2 place Jussieu, 75251 Paris Cedex 05, France*
[g]*Laboratoire de Neurobiologie Cellulaire et Moléculaire, UPR 9040, CNRS, 1 avenue de laTerrasse, 91198 Gif-sur-Yvette Cedex, France*

Received 26 May 2004; revised 24 June 2004; accepted 9 July 2004

Available online 21 July 2004

Edited by Felix Wieland

Abstract Two novel peptides that inhibit the intra-erythrocyte stage of *Plasmodium falciparum* in vitro were identified in the venom of the Trinidad chevron tarantula, *Psalmopoeus cambridgei*. Psalmopeotoxin I (PcFK1) is a 33-residue peptide and Psalmopeotoxin II (PcFK2) has 28-amino acid residues; both have three disulfide bridges and belong to the Inhibitor Cystine Knot superfamily. The cDNAs encoding both peptides were cloned, and nucleotide sequence analysis showed that the peptides are synthesized with typical signal peptides and pro-sequences that are cleaved at a basic doublet before secretion of the mature peptides. The IC_{50} of PcFK1 for inhibiting *P. falciparum* growth was 1.59 ± 1.15 μM and that of PcFK2 was 1.15 ± 0.95 μM. PcFK1 was adsorbed strongly to uninfected erythrocytes, but PcFK2 was not. Neither peptide has significant hemolytic activity at 10 μM. Electrophysiological recordings in isolated frog and mouse neuromuscular preparations revealed that the peptides (at up to 9.3 μM) do not affect neuromuscular transmission or quantal transmitter release. PcFK1 and PcFK2 do not affect the growth or viability of human epithelial cells, nor do they have any antifungal or antibacterial activity at 20 μM. Thus, PcFK1 and PcFK2 seem to interact specifically with infected erythrocytes. They could therefore be promising tools for antimalaria research and be the basis for the rational development of antimalarial drugs.

Keywords: Tarantula venom; ICK peptide; Malaria; *Plasmodium falciparum*

1. Introduction

Animal venoms are valuable sources of novel pharmacological tools whose specific actions are useful for characterizing their receptors. Hundreds of toxins from snakes, scorpions, spiders and marine invertebrates with a range of pharmacological activities have all been characterized [1–3].

Spider venoms contain a wide spectrum of biologically active substances, which selectively target a variety of vital physiological functions in both insects and mammals (for review, see [4,5]). The spider toxins are "short" polypeptides with molecular mass of 3–8 kDa and a structure that is held together by several disulfide bonds. There are two main groups of these peptides, the neurotoxins that target neurone receptors, neurone ions channels or presynaptic proteins involved in neurotransmitter release, and the non-neurotoxic peptides, such as necrotic peptides and antimicrobial peptides (for a review, see [6]). Recent studies have characterized the venoms of the genuses *Brachypelma*, *Pterinochilus* and *Theraphosa* [7–10]. Several peptides that inhibit atrial fibrillation [11], block the Kv2 and Kv4 subfamilies of voltage-dependent potassium channels [12,13], or multiple sodium channels [14], and proton-gated sodium channels [15] have been recently isolated. Although spider toxins bind to their receptors with high affinity, specificity, and selectivity, little is known about them and little work has been done to develop these toxins for therapeutic use.

Malaria is one of the most serious and widespread parasitic diseases in the world. There are an estimated 300–500 million clinical cases each year, and 1–1.5 million people die each year due to malaria, mainly young children (for a review, see [16]). The causative agent of malaria, an intracellular protozoan of the genus *Plasmodium*, spends much of its life cycle within the host erythrocyte. *Plasmodium falciparum* is the most virulent of the four species of *Plasmodium* that infect humans. The

* Corresponding author. Fax: +33-1-4427-5716.
E-mail address: camadro@ijm.jussieu.fr (J.-M. Camadro).

[1] Present address: INSERM U271, 151 cours Albert Thomas, 69424 Lyon Cedex 03, France.
[2] Present address: USM 0505, Ecosystèmes et Interactions toxiques, Muséum National d'Histoire Naturelle, 12 rue Buffon, 75231 Paris Cedex 05, France.

0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.07.019

increasing resistance of the parasite to classical antimalarial drugs means that new chemotherapeutic approaches are urgently needed [17]. Classical strategies are based on modifying known antimalarial drugs, while others that seek new biological targets require a better understanding of the *Plasmodium* biology, life cycle and interaction with its host [18]. Various plant and animal substances have emerged recently as interesting tools for exploring new potential antimalarial targets [19–22].

As part of a general screening for antimalarial drugs, we investigated the potential of toxins from *Psalmopoeus cambridgei*, the Trinidad chevron tarantula. This report describes two novel peptides (PcFK1 and PcFK2, *P*salmopoeus *c*ambridgei *F*alciparum killer) that possess great antiplasmodial activity against the intra-erythrocyte stage of *P. falciparum* in vitro, but do not lyse erythrocytes or nucleated mammalian cells and do not inhibit neuromuscular function, despite their structural similarity to known neurotoxins.

2. Materials and methods

2.1. Venom and toxin purification

Psalmopoeus cambridgei (*Araneae theraphosidae*) venom was obtained by electrical stimulation of venom glands. The crude venom was centrifuged ($5000 \times g$, 4 °C, 20 min), filtered through 0.45-μm microfilters (SJHVL04NS, 4-mm diameter, Millipore) and stored at −20 °C. It was then diluted twofold in 10% acetic acid and fractionated by reverse-phase high pressure liquid chromatography (RP-HPLC) on a C18 column (Supelcosil, 5 μm, 4.6×250 mm, Supelco) eluted with a linear 0–60% gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) for 70 min (flow rate: 0.75 ml/min). Fractions (0.75 ml) were collected and freeze-dried. The peptides were dissolved in RPMI 1640 (Life Technologies, Inc.) and screened for antiplasmodial activity. The peptides in active fractions were further purified by RP-HPLC on the same column with a gradient of 35–65% of solvent B (A, 0.1% TFA in water; B, 60% acetonitrile and 0.1% TFA in water). A total of 90 μl of venom was purified as six 15 μl batches. All solvents used were of HPLC grade. Chromatography was done on a Waters IonSep HPLC system coupled to a model 994 diode array detector and elution was monitored at 220 nm.

2.2. Amino acid sequence analysis

Sequences were determined on a gas phase automatic protein sequencer (Applied Biosystems 476A gas phase peptide sequanator). Phenylthiohydantoin-derivatives were detected with an on-line Applied Biosystems 120A analyzer. Data collection and analysis were performed with an Applied Biosystems 900A module calibrated with 32.5 pmol of phenylthiohydantoin-derivative standards.

Homologies of the peptide sequences were determined using the "search for short nearly exact matches" tool of the BLAST server at NCBI (http://www.ncbi.nlm.nih.gov) and non-redundant databases. The "Blast 2 Sequences" tool (http://embnet.cifn.unam.mx/blast/wblast2.html) was used to search for homologies between Psalmopeotoxins and specific published sequences when necessary. Percentages of similarity and multiple alignments were computed using the ClustalW software and phylogenetic trees were constructed using the TreeView program.

2.3. Mass spectrometry analysis

The mass spectra of positive ions were recorded in reflectron mode with a single stage reflectron matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager DE-PRO; PerSeptive Biosystems, Inc., Framingham, MA) equipped with a delayed extraction device. The delayed extraction time was set at 100 ns. A saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA was used as matrix for all MALDI experiments. Spectra were calibrated using close external standard peptide mixtures.

2.4. Cloning

Total RNAs were extracted from *P. cambridgei* venom glands frozen in liquid nitrogen, powdered and homogenized, as described by Chomczynski and Sacchi [23]. Poly(A)$^+$ RNAs were purified using the Invitrogen Micro-FastTrack kit, and a cDNA library was constructed [24], using a standard procedure with a *Not*I adaptor in the cDNA synthesis step and a *Sal*I adaptor for cloning into the pSPORT1 vector (Life Technologies, Inc.). Recombinant plasmids were prepared by transformation of JM109 competent *Escherichia coli* cells. The quality of the cDNA library was estimated by determining the size of the inserts in a random sample of 20 plasmids linearized by digestion with *Mlu*I.

A combination of 3′-RACE (Rapid Amplification of cDNA End) and nested-PCR steps was used to screen the cDNA library for sequences encoding PcFK. The reaction was performed using an antisense universal primer (primer UP: 5′-TAAAACGACGGCCAGT-3′) and a sense degenerated primer corresponding to the amino acids HDNCVYV of PcFK1 (primer PSA-I: 5′-CAYGAYAAYTGYG-TKTAYGT-3′) and the amino acids PAGKTCV of PcFK2 (primer PSA-II: 5′-CCKGCKGGRAARACKTGYGT-3′) under the following conditions: 94 °C for 240 s, followed by 30 cycles of 94 °C for 40 s, 56 °C for 30 s, and 72 °C for 60 s. An aliquot of the first round of PCR was used as template for nested-PCR, using an antisense universal primer and a sense degenerated primer corresponding to the amino acids YVPAQNPC of PcFK1 (primer Nest-PSA-I: 5′-TAY-GTKCCKGCKCARAAYCCKTGY-3′) and the amino acids TCVRGPMR of PcFK2 (primer 2: 5′-TGYGTTKMGKGGKCCK-ATGMG-3′) under the following conditions: 94 °C for 240 s, followed by 25 cycles of 94 °C for 40 s, 52 °C for 30 s, and 72 °C for 60 s. At the end of the last cycle, the sample was incubated at 72 °C for a further 5 min and separated by electrophoresis in 1.2% agarose gels. DNA fragments were excised from the gel and purified using the Qiaquick gel extraction kit (Qiagen). The PCR product was cloned in the pGEMT-Easy vector system (Promega). Nucleotide sequences were analyzed by the dideoxy chain termination technique in a double-stranded pGEMT-Easy vector using the BigDye fluorescent labeling kit and an ABI 377 automatic sequencer. We used an antisense T7 primer (primer T7: 5′-TAATACGACTCACTATAGGGA-3′) and a 3′ sense gene-specific primer deducted from the 5′ cDNA of PcFK1 (primer 3: 5′-TTTCTGTGCTTTTGTTGCT-3′) or PcFK2 (primer 4: 5′-CTCA-TTAACTTCGTTTCATCAGGTAC-3′) to determine the sequences of the 5′-end of the Psalmopeotoxins. The following thermal cycle profile was used for the RACE-PCRs: 94 °C for 240 s, 25 cycles of 94 °C for 40 s, annealing at 54 °C for 30 s and 72 °C for 60 s, and a final extension step of 72 °C for 5 min. PCR products were purified with the Qiaquick kit (Qiagen), cloned in pGEMT-Easy vector (Promega), and sequenced as above.

2.5. Structural modeling

We prepared a structural model of PcFK1 and PcFK2 using a combination of dedicated bioinformatic tools (TITO [25], Fugue [26], and 3D-PSSM [27]) on the meta-server at the CBS, Montpellier, France [28] (http://bioserv.cbs.cnrs.fr/).

2.6. Effect of PcFK1 and PcFK2 on parasite viability

The Columbian strain FcB1 of *P. falciparum* was used in all experiments. The parasites were cultured [29] in human red blood cells in complete medium and a 3% CO_2, 6% O_2, and 91% N_2 atmosphere. The complete medium was RPMI 1640 (Life Technologies, Inc.) containing 25 mM HEPES, 11 mM glucose, 27.5 mM $NaHCO_3$, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 7% (v/v) autologous heat-inactivated human serum. The in vitro life cycle of the FcB1 strain was 48 h under our culture conditions.

Increasing concentrations of the peptides in complete medium were distributed (100 μl/well) in a 96-microwell plate for screening antiplasmodial peptides and the dose–response assay. An asynchronous culture of *P. falciparum* at 1–1.5% parasitemia and 4% hematocrit in complete medium was added to each well (100 μl/well), and the cells were allowed to grow in a candle jar system for 24 h. [^{3}H]hypoxanthine was added (0.5 μCi/well) and incubation continued for a further 24 h. The red blood cells were broken by freeze-thawing and harvested on filters. The dried filters were submerged in a liquid scintillation mixture (OptiScint Hisafe) and counted in a 1450 Microbeta counter (Wallac, Perkin–Elmer). Inhibition of growth was calculated from the radioactivity of the parasites (incorporated into nucleic acids). The IC_{50} was determined according to Desjardins et al. [30].

Healthy erythrocytes were incubated for 48 h with the minimum concentration of PcFK1 and PcFK2 that caused 100% parasite inhibition to distinguish between non-specific hemolysis due to each toxin and their antiplasmodial effects. Hemolytic activity was correlated to hemoglobin leakage into the suspension and monitored by measuring the absorbance of the supernatant at 540 nm. One hundred percent hemoglobin release was determined on a lysate prepared by repeated freeze-thawing of the corresponding batch of red blood cells.

Toxins were suspended (at 20 μM) in 200 μl complete medium with 2×10^7 RBCs and incubated for 90 min at 37 °C to determine whether PcFK1 and PcFK2 bound to uninfected erythrocytes. Uninfected erythrocytes were then removed by centrifugation and the culture supernatant was tested for its capacity to inhibit the growth of *P. falciparum* in dose–response assays.

2.7. Analysis of PcFK1 and PcFK2 cytotoxicity

The bactericidal action of PcFK1 and PcFK2 was assayed using wild type strains of *E. coli* (Gram(−), MG1655) and *Bacillus subtilis* (Gram(+), Mahrburg 168) plated on solid rich media (LB 2% agar). PcFK1 and PcFK2 (0.1–20 μM) were applied to the plates as 10 μl dots to allow ring inhibition of the bacterial growth. The plates were examined after incubation for 48 h at 37 °C.

The fungicidal action of PcFK1 and PcFK2 was evaluated using wild type strains of *Saccharomyces cerevisiae* (BY4741) and *Candida albicans* (SC5314) plated on solid rich media (YPD 2% agar). PcFK1 and PcFK2 (0.1–20 μM) were applied to the plates as 10 μl dots to allow ring inhibition of the yeast growth. The plates were examined after incubation for 48 h at 30 °C.

The effects of PcFK1 and PcFK2 on mammalian cells were tested using HeLa 229 epithelial cells (obtained from the American Type Culture Collection, Manassas, VA). The HeLa cells were cultivated in Dulbecco's modified minimal essential medium (DMEM) with 2 mM Glutamax-1 (Life Technologies, Inc.), 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin. Confluent HeLa cells (6×10^4 cells/well) were incubated with the indicated concentrations of PcFK1 and PcFK2 in DMEM for 48 h. Cell death was measured by cytofluorimetry using double-staining of cells with propidium iodide (PI) and Annexin-V-FITC (Roche Diagnostic, Mannheim, Germany) as previously described [31]. Cells treated with 1 μM staurosporine were used as positive controls. Each experiment was repeated at least three times on separate days.

2.8. Electrophysiological recordings on isolated neuromuscular preparations

Cutaneous pectoris nerve–muscle preparations were removed from double-pithed male frogs (*Rana esculenta*) weighing 20–25 g. Isolated nerve–muscle preparations were suffused with standard frog physiological saline (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 5 mM HEPES, pH 7.25). In some experiments, excitation–contraction was uncoupled by treating preparations with 2 M formamide for 21 min [32,33]. Left and right hemidiaphragm muscles with their associated phrenic nerves were isolated from Swiss Webster mice (20–25 g) killed by dislocation of the cervical vertebrae followed by immediate exsanguination. Each hemidiaphragm was mounted in a Rhodorsil-lined organ bath (2 ml volume) superfused with physiological saline (154 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES buffer, and 11 mM glucose). The solution gassed with pure O_2 had a pH value of 7.4. Membrane potentials and synaptic potentials were recorded with intracellular microelectrodes filled with 3 M KCl (8–12 MΩ resistance) at room temperature (22–24 °C) using an axoclamp 2B system (Axon Instruments, Union City, CA) and conventional techniques [33]. The motor nerve of the isolated neuromuscular preparation was stimulated via a suction microelectrode, and recordings were made continuously before and throughout application of the peptides tested.

3. Results

3.1. Isolation, purification and characterization of Psalmopeotoxin I and Psalmopeotoxin II

Analysis of the fractions obtained by RP-HPLC of the crude *P. cambridgei* venom by mass spectrometry (manuscript in preparation) revealed the presence of at least 150 individual peptides with masses of 1000–6000 Da. Many of them contained multiple disulfide bonds, as determined by comparing native and reduced peptides.

Screening for antiplasmodial activity revealed the presence of two active fractions (Fig. 1A). The active peptides in these fractions were purified to homogeneity by chromatography on reverse-phase columns with an appropriate elution gradient (Fig. 1B and D). The purity of each peptide was assessed by MALDI-TOF (Fig. 1C and E), ESI-Qq-TOF mass analysis, amino-acid composition and Edman sequential N-terminal analysis. The two active peptides were named Psalmopeotoxin I (PcFK1) and Psalmopeotoxin II (PcFK2).

PcFK1 contained 33 amino acid residues and PcFK2 contained 28. They both had six cysteines forming three disulfide bridges. Their full sequences were established by N-terminal Edman degradation of the reduced alkylated toxins (Fig. 2A). The calculated molecular mass (3616.69 Da) of PcFK1 was consistent with the measured molecular mass (3615.60 Da, Fig. 1C), but the 1 Da difference mass suggested an amidated carboxylic acid at the C-terminus. The calculated molecular mass (2948.34 Da) of PcFK2 was consistent with the measured molecular mass (2948.30 Da, Fig. 1E), suggesting a free carboxylic acid at the C-terminus.

The amino acid sequence of PcFK1 showed only 35% identity with PcFK2, but the two peptides had identical disulfide bridge patterns (Fig. 2A). The conserved residues included two prolines (Pro13 and Pro17) that could play critical roles in the structure of the peptides. A database search for similar amino acid sequences revealed that PcFK1 is 48% identical to tachystatin A, a 44 residue antimicrobial peptide found in the hemocytes of the horseshoe crab (*Tachypleus tridentatus*) [34]. PcFK2 is very similar (60% identity) to a peptide found in the venom of an American tarantula (*Eurypelma californicum*) [35]. The *Eurypelma* sequence missed one cysteine residue, perhaps due to an error in sequencing. No specific activity has been reported for the *Eurypelma* toxin. PcFK2 is also very similar (41% identity) to the omega-conotoxin SVIA of *Conus striatus* venom, which binds to and blocks voltage-sensitive calcium channels on presynaptic membranes [36] (Fig. 2B and C).

3.2. Comparative analysis of PcFK1 and PcFK2 cDNAs

Several thousand *E. coli* colonies were obtained by transformation with the cDNA library constructed from 0.4 μg of poly(A)$^+$ RNA obtained from seven venom glands. A random sampling of 20 individual colonies allowed the characterization of all inserts with different sizes, ranging from 100 bp to more than 800 bp. The library was therefore representative of the sequences encoding short peptide toxins. We then characterized the cDNAs encoding PcFK1 and PcFK2 obtained from the cDNA library by a combination of 3′-RACE, nested-PCR and 5′-RACE. The nucleotide sequence of PcFK1 was quite unlike that encoding PcFK2. The cDNAs were 459 and 392 bp long with a 69/80 bp 5′-UTR, the coding sequence, and a 129/114 bp 3′-UTR. A putative polyadenylation signal (AATAAA) was found 17 nt upstream of the poly(A) tail of PcFK1 and 14 nt upstream of the poly(A) tail of PcFK2 cDNA. The precursors of PcFK1 and PcFK2 deduced from their nucleotide sequences contained a putative signal peptide (21 residues in PcFK1 and 19 residues in PcFK2), with a basic residue in their N-termini and a hydrophobic central core. Each also had a propeptide (30 residues in PcFK1 and 19 residues in PcFK2)

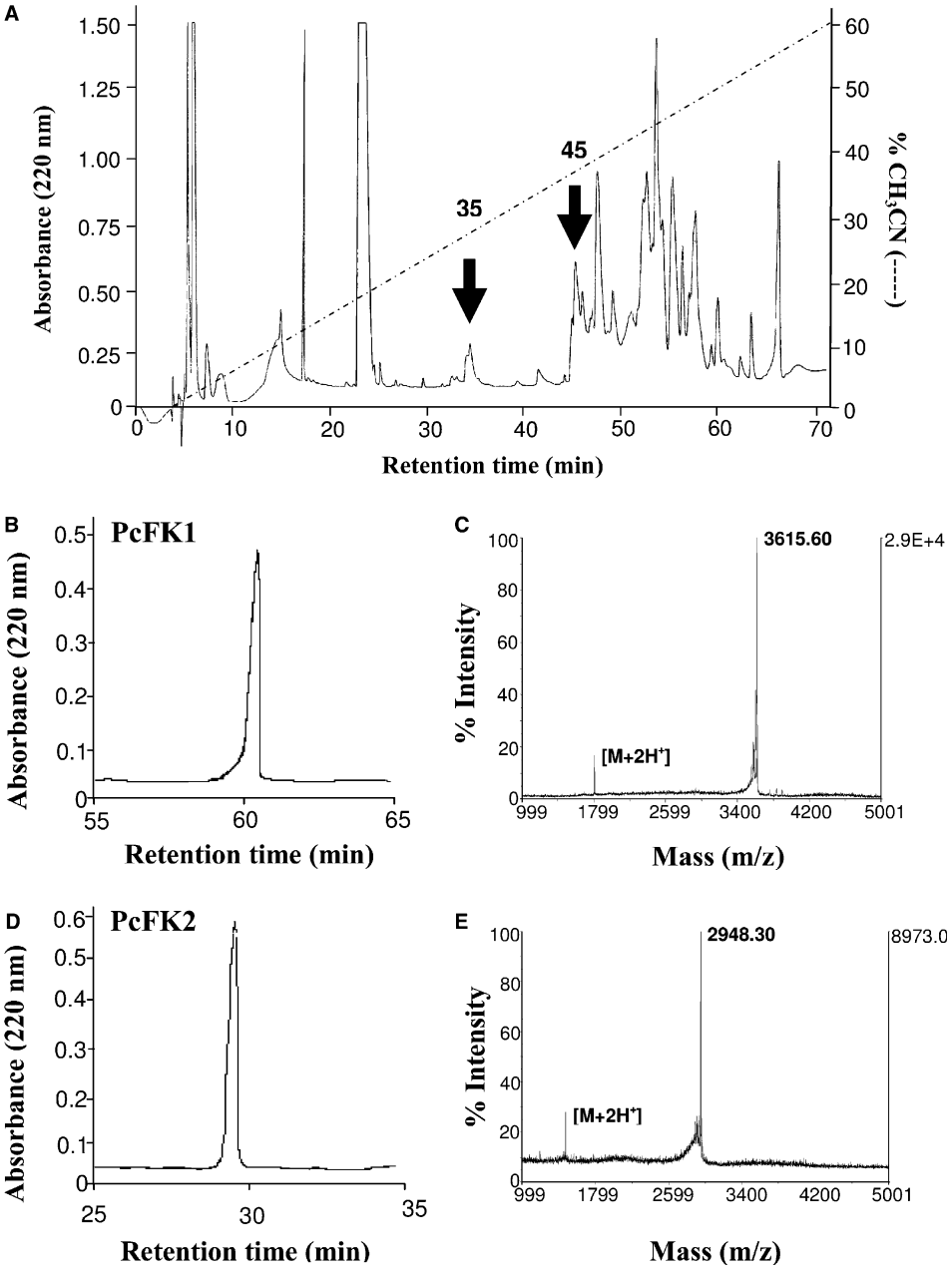

Fig. 1. Purification and characterization of PcFK1 and PcFK2. (A) RP-HPLC separation of crude *P. cambridgei* venom with a linear 0–60% gradient of acetonitrile in 0.1% aqueous TFA (70 min at 0.75 ml/min). Arrows indicate the fractions containing PcFK2 [35] and PcFK1 [45]. (B)–(E) RP-HPLC and MALDI-TOF analysis of purified PcFK1 (B, C) and PcFK2 (D, E); RP-HPLC using a 35–65% gradient of 60% acetonitrile (80 min at 0.75 ml/min) $[M + 2H^+]$ corresponds to doubly charged ions.

that was rich in acidic residues and ended with a basic doublet (Lys-Arg in the PcFK1 precursor, Arg-Arg in the PcFK2 precursor). The third part was the sequence of the mature toxin (Fig. 3A and B).

3.3. Structural modeling

The structural models of PcFK1 and PcFK2, generated using a combination of dedicated bioinformatic tools, were used to compute the most likely structure by homology modeling of both peptides with the same entry of the PDB database 1NIX, as a support. 1NIX reports the 2D ^{1}H NMR solution structure of hainantoxin-I (HnTx-I), a peptide from the venom of *Ornithoctonus (Selenocosmia) hainana*, a chinese bird spider [37]. The structures differed mainly in the conformation of the loop formed by residues 9–15 (Fig. 4). The two-residue insertion between C_{IV} and C_V had little effect on the overall structure of the peptides.

3.4. Antiplasmodial activity of PcFK1 and PcFK2

Both PcFK1 and PcFK2 inhibited the development of *P. falciparum* in erythrocytes in vitro, with IC_{50} of 1.59 ± 1.15 and 1.15 ± 0.95 μM, respectively. Although the IC_{50} values of PcFK1 and PcFK2 could be influenced by experimental parameters, such as the source of serum and red blood cells, and/

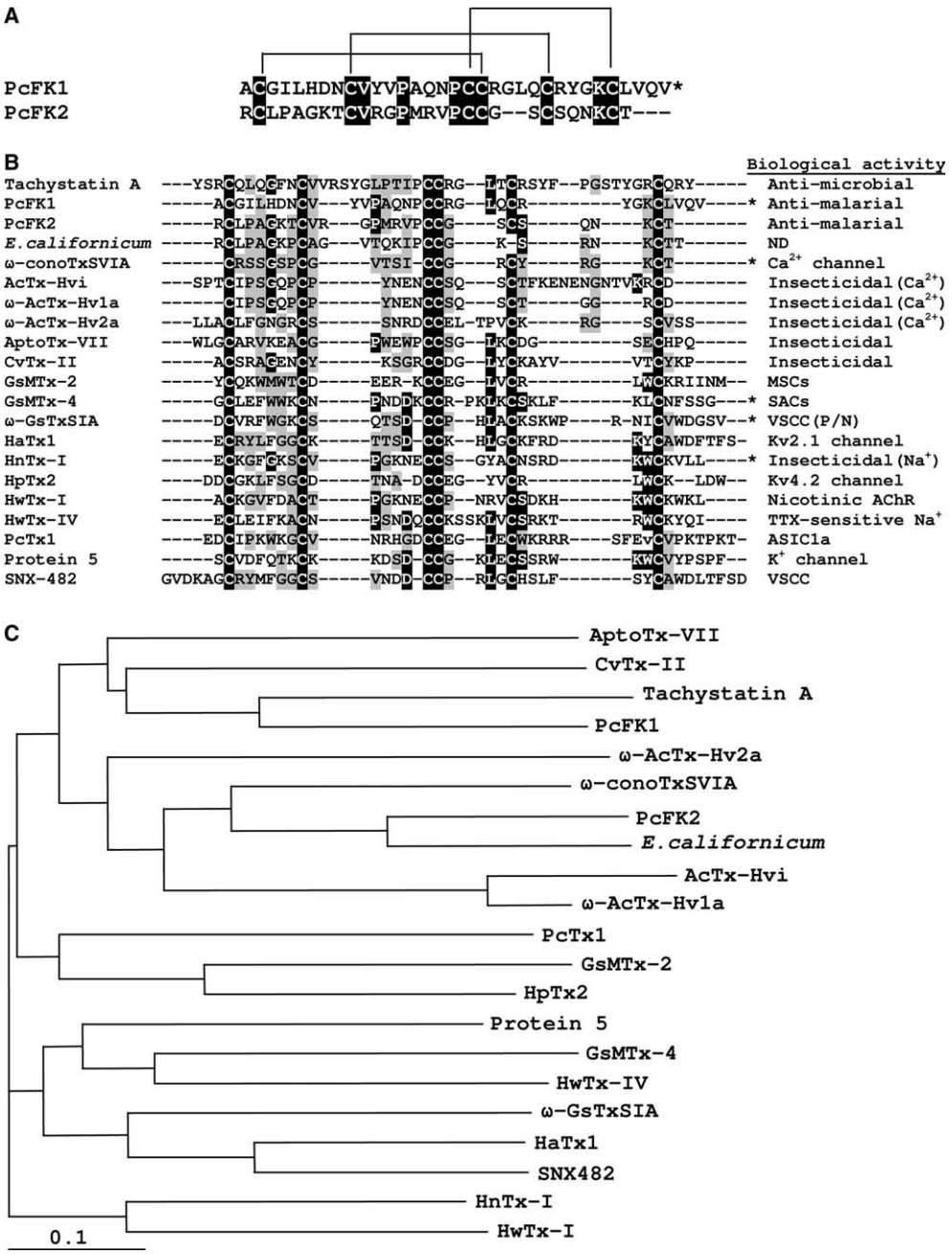

Fig. 2. Sequence alignment of PcFK1 and PcFK2. (A) Sequence alignment of PcFK1 and PcFK2 (*, C-terminal amidation). (B) Multiple alignment with structural family of inhibitor cystine knot peptides, on the six conserved half-cystine residues; conserved residues are in black boxes; homologous regions are gray. Sequences from NCBI (http://www.ncbi.nlm.nih.gov): tachystatin A; 1CIXA, *E. californicum*; S07826, ω-conoTxSVIA; AAB23763, AcTx-Hvi; 1AXH, ω-AcTx-Hv1a; P56207, ω-AcTx-Hv2a; P82852, AptoTxVII; B44007, CvTx-II; P82601, GsMTx-2; 1LUPA, GsMTx-4; A59371, ω-GsTxSIA; AAB28031, HaTx1; P56852, HnTx-I; P83591, HpTx2; P58426, HwTx-I; A37479, HwTx-IV; P83303, PcTx1; 1LMM, Protein 5; AAB32862, SNX482; P56854. All peptides except tachystatin A and ω-conoTxSVIA are from spider venoms. (C) Phylogenetic tree of cystine knot peptides based on a ClustalW multiple alignment.

or how long the erythrocytes had been stored before infection, they were always in the low micromolar range. Dermaseptin B2, a 45-residue antimicrobial peptide from frog skin, was used as control in dose–response assays to assess the toxic specificity of the peptides. At the highest concentration tested (20 μM), dermaseptin B2 had little (25%) or no effect on the viability of *P. falciparum* (not shown). We determined whether toxicity was due to the unspecific lysis of erythrocytes by measuring the capacity of PcFK1 and PcFK2 to hemolyse uninfected RBCs. Neither PcFK1 nor PcFK2 had any hemolytic activity at 10 μM, the highest concentration tested, with each peptide lysing <1.5% of the erythrocytes.

3.5. Depletion of PcFK1, but not PcFK2, by normal RBCs

We examined the possibility that the *Plasmodium*-specific toxicity of PcFK1 and PcFK2 resulted from their non-specific

```
A  CTGACATTTGACGACGACAACCCGAAGTTTCTTTCCTTCACGTCGTAGGTACCGTTCATCTGAACAAAT ATG
                                                                        M
   ATG AGA GTT TTG ATC GTA ACT GCT GTG TTT ACT TTC TTT CTG GTT CTT ACA TCT TCG
    M   R   V   L   I   V   T   A   V   F   T   F   F   L   V   L   T   S   S
   GGA CAT GAT GAG GAC AAT GAG CAA AGA AAT ATT TTG GAA GGT ATG TTT TTG GAC AGG
    G   H   D   E   D   N   E   Q   R   N   I   L   E   G   M   F   L   D   R
   GCC ATT GAA ACA CCG AAA GGA TTA GAA GAG AAA AGG GCC TGT GGC ATA TTG CAT GAC
    A   I   E   T   P   K   G   L   E   E   K   R   A   C   G   I   L   H   D
   AAC TGT GTC TAC GTC CCA GCA CAA AAC CCG TGT TGC AGG GGA CTC CAG TGC AGA TAT
    N   C   V   Y   V   P   A   Q   N   P   C   C   R   G   L   Q   C   R   Y
   GGA AAA TGC CTT GTA CAA GTT GGT CGC TAG CAACAAAAGCACAGAAAATGCTCTAGACGGAATGA
    G   K   C   L   V   Q   V   G   R   *
   TGATGCACAGCACTTTGTTAACAGGGCCTGTATTTTTGTCTTTGTAGAAATCATTATCAAGCTACTCAATTAATA
   AATATGACTTGATATCAAT-poly(A)

B  TTTCACGTTTCACAGTATCAACATAGCTTATTTTCATTTTTCGTAAAAAGCAACAGAATTTGTTTCACTTGAA
   GCAAGAA ATG GGA TTC AAA CTC GTG CTT TTT ATT GCT GTT CTA ACT CTG GTT GGC TCC
             M   G   F   K   L   V   L   F   I   A   V   L   T   L   V   G   S
   AGC AAT GCA GAA ATT AGT GCA AAA ATG GAC AGC CGA GAC TCG CCA ATG ATT CAG GAG
    S   N   A   E   I   S   A   K   M   D   S   R   D   S   P   M   I   Q   E
   CGG AGA TGC TTA CCA GCA GGC AAG ACT TGT GTG AGG GGA CCT ATG AGA GTA CCG TGC
    R   R   C   L   P   A   G   K   T   C   V   R   G   P   M   R   V   P   C
   TGT GGA TCA TGC TCA CAG AAT AAA TGT ACC TGA TGAAACGAAGTTAATGAGAATTGATTTAACT
    C   G   S   C   S   Q   N   K   C   T   *
   AAAGAAGAATGAAACAAAAGCACATATTAAAAAATGAAAATATTTCTTCGAGAGGTCTGCGTCAATAAAATTACT
   CTTAAATG-poly(A)
```

Fig. 3. Nucleotide sequences of cDNAs encoding precursors of PcFK1 (A) and PcFK2 (B) from *P. cambridgei*. The putative signal peptide is underlined with dots and the propeptide is underlined. Gray boxes indicate a basic doublet. The nucleotide sequences of the matured PcFK1 and PcFK2 are underlined in bold. A potential polyadenylation signal AATAAA is underlined twice.

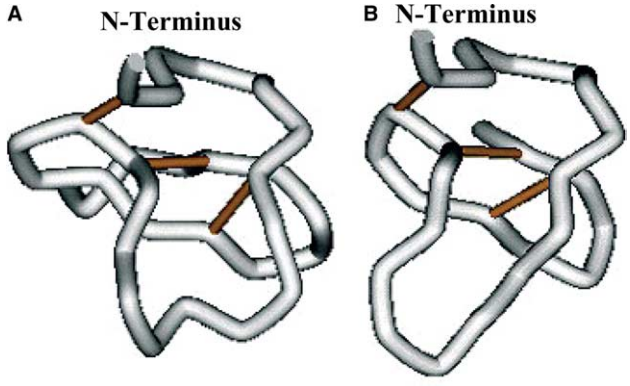

Fig. 4. The three-dimensional structure model of PcFK1 (A) and PcFK2 (B) based on the NMR structure of HnTx-I.

adsorption onto erythrocyte. Each peptide was incubated for 90 min with uninfected erythrocytes, and the culture supernatant was then tested for its capacity to inhibit parasite growth. Each experiment was repeated twice with similar qualitative results. The results from a representative experiment are shown in Fig. 5. The adsorptions of PcFK1 and PcFK2 differed considerably. The dose–response curve of *P. falciparum* growth inhibition by PcFK1 was shifted to a higher apparent IC_{50} that reflected a loss of peptide after incubation with normal RBCs (the actual concentration of the peptide is lower than the theoretical maximum concentration reported on the *X*-axis of the plot in Fig. 5A). If the IC_{50} was not altered by its incubation with the RBC, then more than 85% of PcFK1 remained bound to the normal RBCs ($IC_{50}^{app} = 0.74$ μM before incubation with RBC and 6.37 μM after incubation) (Fig. 5A). The IC_{50} of PcFK2 did not change significantly (0.28 μM before incubation with RBC and 0.46 μM afterwards, Fig. 5B). The IC_{50} of PcFK1 was also slightly higher after incubation in RPMI containing 8% serum but no erythrocytes (Fig. 5A), indicating that part of the toxin is probably lost through electrostatic and/or hydrophobic interactions with plastic wells or components of the medium. Thus PcFK2, unlike PcFK1, binds selectively to parasitized erythrocytes. Conversely, the toxic, active concentration of PcFK1 determined in previous dose–response assays could be an overestimate.

3.6. Effect of PcFK1 and PcFK2 on bacteria, yeast and mammalian cells

The specificities of PcFK1 and PcFK2 for *P. falciparum* were tested by measuring their effects on bacteria, yeast and mammalian cells. The growth of neither Gram(+) nor Gram(−) bacteria, nor that of yeast grown on solid media in antibiogram-like disk assays was inhibited by toxin concentrations up to 20 μM (data not shown). Neither were PcFK1 or PcFK2 toxic for HeLa cells in culture at concentrations up to 50 μM (PcFK1; 20 μM for PcFK2), as measured by FACS analysis of PI and annexin-V-FITC labeled cells (data not shown).

3.7. Effect of PcFK1 and PcFK2 on neuromuscular transmission

We tested the effects of the peptides on isolated frog and mouse neuromuscular preparations to determine whether PcFK1 and PcFK2 altered voltage-dependent ion channels. Neither PcFK1 nor PcFK2 (0.1–9.3 μM) had any significant effect on various electrophysiological parameters related to neuromuscular transmission (nerve conduction through intramuscular axons, latency of endplate potentials, and threshold potential for action potential generation in muscle fibers). Thus the peptides, in the concentration range used, have no effect on nerve and muscle excitability and conduction. PcFK1 and PcFK2 also had no effect on spontaneous or

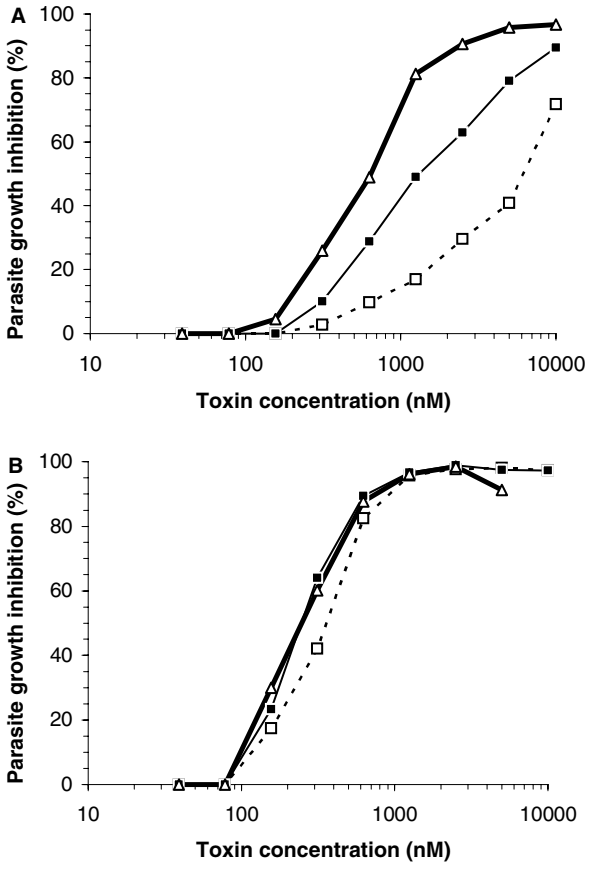

Fig. 5. Dose–response curves for PcFK1 and PcFK2 acting on *P. falciparum* viability. PcFK1 (20 μM) (A) and PcFK2 (20 μM) (B) in RPMI plus 8% serum were incubated with 2×10^7 erythrocytes (□) or without erythrocytes (■) for 90 min at 37 °C in a 96-well microplate. The supernatants were then assayed for their ability to inhibit the development of the FcB1 strain of *P. falciparum* in erythrocytes. Decreasing concentrations of the supernatants in RPMI plus serum were incubated with a 1.5% parasitemia and 2% hematocrit asynchronous culture of the parasite in a 96-well plate for 24 h. 0.5 μCi [^{3}H]hypoxanthine was then added to each well and incubation continued for a further 24 h. The cells were harvested on filters and broken by freeze-thawing. [^{3}H]hypoxanthine incorporation was measured in a 1450 Microbeta counter (Wallac). 100% incorporation was measured in the absence of peptide. Dose–response control without incubation (△).

evoked transmitter release (recorded as miniature endplate potentials and endplate potentials), indicating that synaptic transmission is unaffected by the peptides. In contrast to PcFK1 and PcFK2, in the presence of the inorganic ion Cd^{2+}, a non-specific voltage-dependent calcium channel blocker, nerve-evoked transmitter release was unable to trigger muscle action potentials (data not shown). Similarly, PcFK1 and PcFK2 had no effect on mouse hemidiaphragm preparations (data not shown).

4. Discussion

Our search for novel antimalarial peptides led us to investigate the toxins in tarantula venom. We have isolated and characterized two novel peptides, PcFK1 and PcFK2, that inhibit the growth of *P. falciparum* in erythrocytes. PcFK1 and PcFK2 are both cystine knot-containing peptides, and their molecular scaffolds are probably similar to those of conotoxins and spider toxins. The general structures of the precursors of PcFK1 and PcFK2, deduced from their nucleotide sequence, are very similar to those of the sequences encoding many secreted peptide toxins from spiders, scorpions and other invertebrates (for a review, see [4]). Both are composed of a putative signal peptide (21 and 19 residues) with a basic residue within their N-termini and a hydrophobic central core; a propeptide rich in acidic residues ending with a basic doublet (Lys-Arg in the PcFK1 precursor, Arg-Arg in the PcFK2 precursor) that is usually the target of a specific processing endopeptidase; and the sequence of the mature toxin. Although this peptidase usually cleaves the propeptide at the C-terminus of the basic doublet (as found in the PcFK1 sequence), it cleaves the precursor of PcFK2 within the doublet. Native PcFK1 has 33 residues, whereas the mature peptide deduced from the nucleotide sequence has two extra residues, Gly-Arg, in the C-terminus of PcFK1. This feature is found in many precursors of amidated peptides [38,39]. The Arg residue is probably removed in the processing step by a basic residue-specific carboxypeptidase, after which the remaining Gly-extended peptide is converted into a des-Gly peptide amine by α-amidation [40].

PcFK1 is very much like tachystatin A, a 44 residue antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*) [34]. This peptide belongs to the ω-agatoxin IVA structural family of P-type calcium channel antagonists [41]. Our antiplasmodial dose–response assays with ω-agatoxin IVA showed no parasite killing at concentrations as high as 51 μM (unpublished results). However, the antimicrobial activity of tachystatin A is not due to its effect on ionic channels, but due to its binding to chitin [34]. PcFK1 lacks two essential features of tachystatin A – a conserved phenylalanine residue between Cys_I and Cys_{II} involved in the binding of tachystatin A to chitin and two large loops between strands 1 and 3 – making it unlikely that the two peptides act via similar mechanisms.

Like tachystatin A, PcFK1 and PcFK2 do not inhibit voltage-dependent ion channels, as determined in our electrophysiological studies on isolated frog and mouse neuromuscular preparations. However, although the activity of tachystatin A was ascribed to its chitin-binding properties, neither PcFK1 nor PcFK2 inhibit the growth of yeast – neither *S. cerevisiae* nor *C. albicans*. The peptides also have no effect on the growth of bacteria such as *B. subtilis* or *E. coli*. This raises the question of how PcFK1 and PcFK2 act on *P. falciparum*. The best characterized antiplasmodial peptide is dermaseptin S4, a 28-residue peptide from the skin of a frog (*Phyllomedusa* genus) [42]. Dermaseptin S4 (IC_{50} of 0.3–2.2 μM) efficiently inhibits the growth of *Plasmodium*, but it deforms the infected red blood cells due to its high hemolytic activity [21]. PcFK1 and PcFK2 cause no swelling or shrinking of the red blood cells. This characteristic of PcFK1 and PcFK2 is very interesting considering that the most potent antiplasmodial dermaseptin S4 derivatives (IC_{50} of 0.2–7.7 μM) still have great hemolytic activity [19,20]. Other defensin-like peptides seem to act via hemolysis [43,44], and derivatives of gramicidin A inhibit *Plasmodium* growth by stimulating pore-formation, leading to the leakage of intracellular ions through the erythrocyte membrane [45,46]. Thus, the next step towards a better understanding of how PcFK1 and PcFK2 act will be to identify the targets of these peptides in the infected red blood cells. Our initial structural models of the two peptides

will help address this question. All ICK peptides usually have similar overall folding and disulfide bond patterns, but their specificities and selectivities rely on subtle variations in the charge, hydrophobicity and/or electrostatic potential distributions. We used bioinformatic search engines to look for sequence and structure features that contain all these parameters [28]. Inputting the PcFK1 and PcFK2 primary sequences led to the selection of the support structure of HnTx-I, a sodium channel inhibitor [37], suggesting that the peptides share several structural features although their sequences are quite dissimilar. This does not demonstrate that PcFK1 and PcFK2 have the same molecular target. Indeed, our data show at least one clear difference in the behavior of the peptides towards erythrocytes. PcFK2 interacts selectively with infected RBCs, while PcFK1 also binds to healthy erythrocytes. This may be because they act differently, as PcFK1 is more hydrophobic than PcFK2. Its adsorption to RBCs may thus be based on hydrophobic interactions with the lipid bilayer of the erythrocyte membrane. Nonetheless, PcFK1 is not hemolytic, so the interactions do not alter the permeability of the membrane. Another possibility is that PcFK1 may interact with a specific receptor on the RBC membrane, which could be an ion channel whose function is not critical for the normal structure or function of the RBC. Such specific ion channels might play a critical role in malaria-infected erythrocytes, because of the importance of the balance between translocation via transport pumps and leakage of Na^+ and K^+ [47]. The specific interaction of PcFK2 with infected erythrocytes raises several questions. PcFK2 may target the same receptor as PcFK1 but react with a different site that is unmasked in the infected RBC. PcFK2 and PcFK1 might also have different targets. PcFK2 may bind to a parasite protein targeted to the RBC membrane or a protein that is already present on healthy RBC, but is cryptic or inactive until activated by infection [48]. It is also possible that PcFK1 and PcFK2 enter the infected erythrocytes, affect the parasite membrane and interfere with parasite growth. However they act, the unique biochemical features of PcFK1 and PcFK2, their ability to inhibit the growth of *Plasmodium*, and their lack of hemolytic, cytotoxic or neurotoxic activity in mammalian systems make these peptides promising tools for malaria research and fascinating structures of peptidomimetics in the preparation of antiparasitic agents. Their activities on other strains of *P. falciparum* and other *Plasmodium* species will now be tested.

Acknowledgements: We thank Laurent Masson, François Teyssié and Jean-Michel Verdez for providing the juveniles of the spider, and Marie-Claude Gendron for her expertise in FACS analysis. The English text was edited by Dr. Owen Parkes. This work was supported in part by grants from the Centre National de la Recherche Scientifique (GDR 1077 Parasitology and IFR 63), the Ministère de l'Education Nationale, de la Recherche et de la Technologie (Programme de Recherches Fondamentales en Microbiologie, Maladies Infectieuses et Parasitaires, VIH-PAL, PAL+) and the Université Paris 7 – Denis-Diderot. S.-J.C. is the recipient of a grant-in-aid from the French Government (French Embassy in Republic of Korea). We thank the Region Ile-de-France (SESAME) for funding part of the MALDI-TOF system.

References

[1] Pal, S.K., Gomes, A. and Dasgupta, S.C. (2002) Indian J. Exp. Biol. 40, 1353–1358.
[2] Goudet, C., Chi, C.W. and Tytgat, J. (2002) Toxicon 40, 1239–1258.
[3] Craig, A.G., Bandyopadhyay, P. and Olivera, B.M. (1999) Eur. J. Biochem. 264, 271–275.
[4] Escoubas, P., Diochot, S. and Corzo, G. (2000) Biochimie 82, 893–907.
[5] Escoubas, P. and Rash, L. (2004) Toxicon 43, 555–574.
[6] Rash, L.D. and Hodgson, W.C. (2002) Toxicon 40, 225–254.
[7] Escoubas, P., Celerier, M.L. and Nakajima, T. (1997) Rapid Commun. Mass Spectrom. 11, 1891–1899.
[8] Escoubas, P., Chamot-Rooke, J., Stocklin, R., Whiteley, B.J., Corzo, G., Genet, R. and Nakajima, T. (1999) Rapid Commun. Mass Spectrom. 13, 1861–1868.
[9] Escoubas, P., Corzo, G., Whiteley, B.J., Celerier, M.L. and Nakajima, T. (2002) Rapid Commun. Mass Spectrom. 16, 403–413.
[10] Legros, C., Célérier, M., Henry, M. and Guette, C. (2004) Rapid Commun. Mass Spectrom. 18, 1024–1032.
[11] Bode, F., Sachs, F. and Franz, M.R. (2001) Nature 409, 35–36.
[12] Escoubas, P., Diochot, S., Celerier, M.L., Nakajima, T. and Lazdunski, M. (2002) Mol. Pharmacol. 62, 48–57.
[13] Ebbinghaus, J., Legros, C., Noltinga, A., Guette, C., Célérier, M., Pongs, O. and Bahring, R. (2004) Toxicon 43, 923–932.
[14] Middleton, R.E., Warren, V.A., Kraus, R.L., Hwang, J.C., Liu, C.J., Dai, G., Brochu, R.M., Kohler, M.G., Gao, Y.D., Garsky, V.M., Bogusky, M.J., Mehl, J.T., Cohen, C.J. and Smith, M.M. (2002) Biochemistry 41, 14734–14747.
[15] Escoubas, P., De Weille, J.R., Lecoq, A., Diochot, S., Waldmann, R., Champigny, G., Moinier, D., Menez, A. and Lazdunski, M. (2000) J. Biol. Chem. 275, 25116–25121.
[16] Snow, R.W., Trape, J.F. and Marsh, K. (2001) Trends Parasitol. 17, 593–597.
[17] Price, R.N. and Nosten, F. (2001) Drug Resist. Updat. 4, 187–196.
[18] Bozdech, Z., Llinas, M., Pulliam, B.L., Wong, E.D., Zhu, J. and De Risi, J.L. (2003) PLoS Biol. 1, E5.
[19] Krugliak, M., Feder, R., Zolotarev, V.Y., Gaidukov, L., Dagan, A., Ginsburg, H. and Mor, A. (2000) Antimicrob. Agents Chemother. 44, 2442–2451.
[20] Dagan, A., Efron, L., Gaidukov, L., Mor, A. and Ginsburg, H. (2002) Antimicrob. Agents Chemother. 46, 1059–1066.
[21] Efron, L., Dagan, A., Gaidukov, L., Ginsburg, H. and Mor, A. (2002) J. Biol. Chem. 277, 24067–24072.
[22] Conde, R., Zamudio, F.Z., Rodriguez, M.H. and Possani, L.D. (2000) FEBS Lett. 471, 165–168.
[23] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[24] Seon, A.A., Pierre, T.N., Redeker, V., Lacombe, C., Delfour, A., Nicolas, P. and Amiche, M. (2000) J. Biol. Chem. 275, 5934–5940.
[25] Labesse, G. and Mornon, J. (1998) Bioinformatics 14, 206–211.
[26] Shi, J., Blundell, T.L. and Mizuguchi, K. (2001) J. Mol. Biol. 310, 243–257.
[27] Kelley, L.A., MacCallum, R.M. and Sternberg, M.J. (2000) J. Mol. Biol. 299, 499–520.
[28] Douguet, D. and Labesse, G. (2001) Bioinformatics 17, 752–753.
[29] Deregnaucourt, C. and Schrevel, J. (2000) J. Biol. Chem. 275, 39973–39980.
[30] Desjardins, R.E., Canfield, C.J., Haynes, J.D. and Chulay, J.D. (1979) Antimicrob. Agents Chemother. 16, 710–718.
[31] Perfettini, J.-L., Gissot, M., Souque, P. and Ojcius, D.M. (2002) Methods Enzymol. 358, 334–344.
[32] Favreau, P., Gilles, N., Lamthanh, H., Bournaud, R., Shimahara, T., Bouet, F., Laboute, P., Letourneux, Y., Menez, A., Molgo, J. and Le Gall, F. (2001) Biochemistry 40, 14567–14575.
[33] Le Gall, F., Favreau, P., Benoit, E., Mattei, C., Bouet, F., Menou, J.L., Menez, A., Letourneux, Y. and Molgo, J. (1999) Eur. J. Neurosci. 11, 3134–3142.
[34] Osaki, T., Omotezako, M., Nagayama, R., Hirata, M., Iwanaga, S., Kasahara, J., Hattori, J., Ito, I., Sugiyama, H. and Kawabata, S. (1999) J. Biol. Chem. 274, 26172–26178.
[35] Savel-Niemann, A. (1989) Biol. Chem. Hoppe Seyler 370, 485–498.
[36] Ramilo, C.A., Zafaralla, G.C., Nadasdi, L., Hammerland, L.G., Yoshikami, D., Gray, W.R., Kristipati, R., Ramachandran, J., Miljanich, G. and Olivera, B.M., et al. (1992) Biochemistry 31, 9919–9926.
[37] Li, D., Xiao, Y., Hu, W., Xie, J., Bosmans, F., Tytgat, J. and Liang, S. (2003) FEBS Lett. 555, 616–622.

[38] Romi-Lebrun, R., Lebrun, B., Martin-Eauclaire, M.F., Ishiguro, M., Escoubas, P., Wu, F.Q., Hisada, M., Pongs, O. and Nakajima, T. (1997) Biochemistry 36, 13473–13482.
[39] Johnson, J.H., Bloomquist, J.R., Krapcho, K.J., Kral Jr., R.M., Trovato, R., Eppler, K.G., Morgan, T.K. and DelMar, E.G. (1998) Arch. Insect Biochem. Physiol. 38, 19–31.
[40] Kapuscinski, M., Green, M., Sinha, S.N., Shepherd, J.J. and Shulkes, A. (1993) Clin. Endocrinol. (Oxford) 39, 51–58.
[41] Fujitani, N., Kawabata, S., Osaki, T., Kumaki, Y., Demura, M., Nitta, K. and Kawano, K. (2002) J. Biol. Chem. 277, 23651–23657.
[42] Ghosh, J.K., Shaool, D., Guillaud, P., Ciceron, L., Mazier, D., Kustanovich, I., Shai, Y. and Mor, A. (1997) J. Biol. Chem. 272, 31609–31616.
[43] Shin, S.Y., Lee, M.K., Kim, K.L. and Hahm, K.S. (1997) J. Pept. Res. 50, 279–285.
[44] Shin, S.Y., Kang, J.H. and Hahm, K.S. (1999) J. Pept. Res. 53, 82–90.
[45] Gumila, C., Ancelin, M.L., Delort, A.M., Jeminet, G. and Vial, H.J. (1997) Antimicrob. Agents Chemother. 41, 523–529.
[46] Otten-Kuipers, M.A., Coppens-Burkunk, G.W., Kronenburg, N.A., Vis Mde, A., Roelofsen, B. and Op den Kamp, J.A. (1997) Parasitol. Res. 83, 185–192.
[47] Staines, H.M., Ellory, J.C. and Kirk, K. (2001) Am. J. Physiol. Cell Physiol. 280, C1576–C1587.
[48] Desai, S.A., Bezrukov, S.M. and Zimmerberg, J. (2000) Nature 406, 1001–1005.