ORIGINAL PAPER

In vitro selection of RNA aptamers against a conserved region of the *Plasmodium falciparum* erythrocyte membrane protein 1

Anders Barfod · Tina Persson · Johan Lindh

Abstract The *var*-gene encoding *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is known to play a major role in the pathogenicity of the *P. falciparum* parasite. The protein enables the parasite to adhere to the endothelial linings of small blood vessels (cytoadherence) as well as to non-infected erythrocytes (rosetting), thus preventing clearance from the bloodstream. The development and spread of resistance towards most anti-malarial drugs used for treatment and prevention of the most severe form of malaria truly emphasise the importance of a continuous research and development of new drugs. In this study we use Systematic Evolution of Ligands by EXponential enrichment (SELEX) methodology to isolate high-affinity ligands (aptamers). To validate the results from the SELEX in vitro selection, different aptamers have been selected against PfEMP1 in a live cell assay of *P. falciparum* strain FCR3S1.2, a highly rosetting strain. We have been able to show the rosette disrupting capacity of these SELEX-aptamers at concentrations of 33 nM and with 100% disruption at 387 nM. The described results show that RNA aptamers are promising candidates for adjunct therapy in severe malaria.

A. Barfod · T. Persson
Department of Organic Chemistry, Lund University,
22100 Lund, Sweden

J. Lindh (✉)
Department of Parasitology,
Mycology and Water & Environmental Microbiology,
Swedish Institute for Infectious Disease Control,
17182 Solna, Sweden
e-mail: johan.lindh@smi.se

Introduction

Plasmodium falciparum is the causative agent of severe malaria in humans. Millions of people worldwide are infected every year by *P. falciparum* and more than one million die, most of them small children in sub-Saharan Africa (Skeet 2005). Quinine, chloroquine and sulfadoxine/pyrimethamine (SP) is the most common malaria drugs but unfortunately parasite resistance towards all these drugs has been documented in endemic regions (White 1998). The most efficient drug used today is artemisinin and its derivates and although no clinical resistance has been demonstrated, there are indications of a developed in vitro resistance towards the drug in *P. falciparum* (Ashley and White 2005). Therefore there is a need to discover new drugs or alternative ways to fight the disease. One strategy would be to hinder the infected red blood cells from adhering to the endothelial linings of small blood vessels (cytoadherence) or to uninfected erythrocytes (rosetting), which should increase parasitic clearance from the bloodstream by the spleen (Ho and White 1999; Chen 2007). Cytoadherence and rosetting is linked to *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a protein expressed in the infected erythrocyte (Baruch et al. 1995; Chen et al. 2000). Exposed on the surface of the infected erythrocyte it binds to a number of human cell surface receptors such as heparan sulphate (HS), ICAM-1, CD36 and CSA (Chen 2007). PfEMP1 consist mainly of duffy-binding ligand domains (DBLs) and cysteine rich inter domain regions, and the number of domains and size of the protein varies depending on which of the 60 *var*-genes is expressed (Flick and Chen 2004). The parasite expresses a single *var*-gene giving rise to one specific PfEMP1 protein. By switching to another *var*-gene and hence another PfEMP1, antigenic variation of the infected erythrocyte

surface is achieved which facilitates parasite avoidance of host immune system. Structural conservation is expected due to the adhesive function of this protein irrespective of the isoform expressed. Rosetting, the virulence associated phenotype, is mediated by the N-terminal duffy-binding-like domain (DBL1α) which has a degree of sequence conservation relative to other PfEMP1 domains and the one domain that is present in almost all the different PfEMP1s (Normark et al. 2007). This makes DBL1α an attractive candidate for the development of a novel drug against severe malaria or as a vaccine candidate; however, antibody recognition of the structural semi-conserved epitopes in DBL1α have shown limited cross-reactivity between different *P. falciparum* strains (Ahuja et al. 2006). Due to the smaller size of aptamers compared to antibodies they have a potential to reach more buried conserved regions of the protein, and bind its target with high affinity. In some reported cases even with higher specificity than antibodies (Kusser 2000; Stoltenburg et al. 2007). Based on these characteristics we designed a Systematic Evolution of Ligands by EXponential enrichment (SELEX) protocol (Ellington and Szostak 1990; Tuerk and Gold 1990) which could be designed to allow the selection of serum-stable RNA aptamers to bind with specificity to the structurally conserved parts of DBL1α. A similar strategy has been reported for other pathogenic parasites where aptamers have successfully been selected against surface proteins (Ulrich et al. 2002; Lorger et al. 2003). The SELEX method is based on an iterative process of in vitro selection cycles where the initial DNA/RNA library of 10^{14}–10^{15} different molecules is reduced to a smaller pool of different molecules that have affinity towards the target in question. We also investigated whether specific affinity binding aptamers were able to bind to PfEMP1 on the surface of live parasites and whether they had the capacity to disrupt rosettes. We demonstrate a set of aptamers that can inhibit the formation of rosettes.

Material and methods

Culturing of P. falciparum Blood stage parasites of *P. falciparum* strain FCR3S1.2 was cultivated according to standard methods with 10% AB^+Rh^+ serum added to buffered medium (Flick et al. 2004)

Protein expression in Escherichia coli Recombinant $DBL1\alpha^{His}$ from FCR3S1.2 was expressed as follows, SG13009 (pREP4) cells from Qiagen harbouring plasmids pQE-TriSystem His·Strep 2 ($DBL1\alpha^{his}$), or pQE-60 ($DBL1\alpha^{his}$) (Moll et al. 2007) were grown in LB-medium containing ampicillin (100 μg/ml) and kanamycin (30 μg/ml) at either 22°C or 37°C. At OD_{600}=0.8 cells were induced

with 0.1 mM IPTG for 3 h; 1 l cell suspension was harvested. Pellet was resuspended in 25 ml lysis buffer (50 mM NaH_2PO_4/NaOH pH7.4, 300 mM NaCl, 1 mM PMSF, 0.05% Triton x-100, 10 mM imidazole). Cells were incubated with Lysozyme (Sigma) on ice for 30 min, and sonicated. Cell debris was removed by centrifugation (4°C, 30 min, 18,000×g). Supernatant was treated with DNAse (Fermentas) and protein was purified according to manufacturer (Qiagen). Elution was dialysed in phosphate-buffered saline (PBS) overnight at 4°C in dialysis tube with 8,000 molecular weight cut-off (Gentaur). Protein purity was estimated by running samples on 10% SDS-PAGE and protein concentration was determined by Bradford assay (Bradford 1976). Alternatively, His-tagged DBL1α was purified on FPLC. *E. coli* extracted soluble fraction was loaded onto nickel column on FPLC (Amersham Biosciences) with a flow speed of 0.5 ml/min. Bound protein was washed with 5–70 mM imidazole gradient (60 ml at 1 ml/min). Protein was eluted with 400 mM imidazole and dialysed and analysed as previously described. A fusion protein of glutathione *S*-transferase (GST) and DBL1α was expressed from a previously cloned pGEX-4 T1 vector in *E. coli* (Flick et al. 2004).

In vitro selection—SELEX The DNA library was generated using oligo B (5′-CGACTGCAGAGCTTGCTACG (N)$_{50}$ GGTACCGAGCTCGAATTCCC-3′) and oligo A (5′-GCGTAATACGACTCACTATAGGGAATT CGAGCTCGGTACC-3′), sequence for T7 promoter underlined. Oligonucleotides were synthesised and purchased from IBA, Germany. Oligo B contains a central sequence (N)$_{50}$ of 50 randomised nucleotides flanked by constant regions. Double-stranded DNA Library was created by annealing 3 μM of Oligo A and Oligo B (95°C for 5 min and cool for 15 min at 25°C), subsequently adding Klenow fragment (Fermentas) in the supplied buffer at 37°C for 2 h, purified by Microcon Ym-30 column (Millipore) and eluted in 30 μl RNAse-free water. 2′F-modified library was created by T7 RNA transcription of 40 μg template using T7 R&DNA™ Polymerase (Epicentre) in supplied transcription buffer adding DTT (Epicentre) to 10 mM and ATP, GTP, 2′F-dCTP, 2′F-dUTP (Epicentre) to 1.25 mM. RNA was labelled by adding 0.37 MBq [α-^{32}P]-ATP (Amersham Biosciences) in 20-μl reaction volume. Reaction ran at 37°C for 5 h and DNA/RNA was precipitated using 0.2 M NaOAc (pH=5.2), 70% EtOH. Sample ran on 10% 8 M UREA PAGE and RNA was exercised and extracted from the gel with 1 M NaOAc (pH=4.7) overnight at 4°C. After centrifugation with glass wool RNA was precipitated with 70% EtOH and glycogen to 0.05%. The first selection cycle was performed with 30 μg (1 nmol) radioactive labelled 2′ F-RNA and a minimum of 60 μg (1.3 nmol) purified DBL1α. Subsequent cycles were performed with 300 pmol

RNA and from 0.45 to 1.2 nmol His-tagged DBL1α bound to Ni-NTA agarose (Qiagen) in all cycles. A pre-selection on 100 μl Ni-NTA agarose was performed in every cycle before incubation with DBL1α to avoid enrichment of matrix binders. Incubation of RNA with DBL1α was performed at 37°C with gentle agitation for 60 min in starting cycles and decreased to 15 min in later cycles. Beads were washed with 2×500 μl PBSM (PBS, 1 mM MgCl$_2$) and RNA/protein eluted with 7 M UREA, 400 mM imidazole, 50 mM NaH$_2$PO$_4$ (pH=7.4). The resulting mixture was subjected to phenol/chloroform extraction for eluting RNA aptamers from DBL1α. RNA was precipitated as previously described and ssDNA was generated by adding primer B (5′-CGACTGCAGAGCTTGCTACG-3′) in excess and 20 U of M-MuLV-RT (Fermentas) in supplied buffer with 1 mM dNTP. 2′F-RNA was hydrolyzed in 0.1 M NaOH at 37°C for 30 min. ssDNA was purified using Ym-30 Microcon column. Oligo A was added in a 1:1 ratio to the produced ssDNA. After annealing the two oligonucleotides, dsDNA was created by Klenow fill out. The dsDNA was amplified by polymerase chain reaction (PCR) using Taq DNA polymerase (Fermentas) with a maximum of 14 cycles using primer A (5′-GCGTAATACGACTCACTATAG-3′) and primer B. PCR product was pooled and purified and used as template for next SELEX cycle. The RNA recovery in each cycle was determined by measuring the radioactivity of all collected fractions during the experiment by diluting samples in 3 mL BetaMax ES™ Liquid Scintillation Fluid and recorded in a Beckman LS 3801 All DNA/RNA concentrations were determined with NanoDrop™ 1000 (Thermo Scientific).

Cloning and sequencing of RNA aptamers PCR amplified dsDNA from SELEX pool 8 was cloned into TOPO vector pCR®4 or pCR®2.1 (Invitrogen) and transformed into *E. coli* strain Top10 (Invitrogen). Colonies were isolated and insert was confirmed by Colony PCR with M13 primers. Positive clones were sequenced by AGOWA (Germany). Sequences were aligned and screened for conserved motifs using the MEME system motif discovery search version 3.5.4 (Grundy et al. 1997).

Secondary RNA structure prediction Selected RNA molecules were subjected to secondary structure prediction using the Mfold software. The programme was run with defaults settings listed on the updated homepage from the Institute for Theoretical Chemistry at Vienna University. http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi

Transcription of individual aptamers and their binding to DBL1α^{His} Individual *E. coli* clones were grown in 5 ml LB-amp (100 μg/ml) at 37°C overnight. Plasmid was isolated using mini-prep kit (Sigma). The template for T7 transcription was generated using primer A and primer B for PCR. PCR product was purified by centrifugation on MICROCON® Ym-30 (Millipore). T7 transcription of 500 ng dsDNA template was performed in 20 μl with 40 U T7 R&DNA™ polymerase, 1.25 mM 2′F-dUTP, 2′F-dCTP (Epicentre) and ATP, GTP (Fermentas) and 10 mM DTT, 0.37 Mbq [α-^{32}P]-ATP (Amersham Bioscience) at 37°C for 4 h. Template was digested using 1 U of DNAse at 37°C for 15 min. RNA was purified using MICROCON® ym-30. The RNA was mixed with PBSM and denatured at 95° for 3 min and stored at room temperature 15 min. Fifty-nanomolar aptamers across different motifs were tested for binding on 500 nM DBL1α^{His} bound to ~30 μl Ni-NTA agarose beads. Five hundred micrograms yeast RNA was added and RNA was incubated with protein in PBSM for 45 min followed by four washes with 1 ml PBS and 0.05% Triton x-100. Bound radioactive RNA was eluted with 7 M UREA, 400 mM imidazole, 50 mM NaH$_2$PO$_4$ (pH=7.4) and quantified with scintillator as previously described.

5′ end-labelling of aptamers and their binding to GST-DBL1α Purified RNA aptamer, 0.5–1 μg, was dephosphorylated with 1 U of shrimp alkaline phosphatase and labelled with [γ-^{32}P]-ATP (Perkin Elmer) using T4 polynucleotide kinase according to manufacturer's instructions. Forty nanograms labelled RNA (1.2 nM) and 1.2 μg non-labelled RNA (35.6 nM) was added to 20 μl pre-coated glutathione beads (~5–8 μg GST-DBL1α) in 1.1 ml PBS with 0.1% Triton x-100 and 300 μg yeast RNA in Eppendorf tube. Suspension incubated for 45 min with rotation at room temperature. Beads washed three times with 1 ml PBS with 0.1% Triton x-100. Bound radioactive RNA was eluted with 50 mM Tris pH=8.0, 10 mM glutathione and quantified as previously described. The experiments were performed either twice or three times with different protein and RNA batches. When standard deviation is shown a two-tailed Student's *t* test is performed and significance is indicated in the figure.

Incubation of 5′ end-labelled aptamers with live culture of FCR3S1.2 Three hundred nanograms (~10 nM) of 5′-^{32}P-labelled RNA was incubated with 1 ml FCR3S1.2 culture (5% parasitemia, 5% hematocrit) for 45 min at 37°C with slow rotation. Cells were spun at 200×g and washed four times with 1 ml PBS. Cell pellet was resuspended in 100 μl PBS and mixed with 3 ml scintillation fluid, vortexed, left to settle and radioactivity was measured with scintillator. The experiment was performed in triplicates on three different occasions with different cell batches.

Rosetting disruption assay 2′F-RNA samples for disruption assay were prepared as previous experiments. *P. falciparum* strain FCR3S1.2 was cultured as previously described

(Vogt et al. 2006). The cell culture was mixed with 2′F-RNA samples to a finale volume of 100 µl in concentrations from 33 nM to 1 µM. Unselected 2′F-RNA generated with T7 transcription from unselected pool 0 was used as a negative control. A reference culture with the addition of the same volume of PBSM without 2′F-RNA was analysed during the time course of the experiment to observe if there was any change in the rosette rate during the experiment. Parasites were incubated 1 h at room temperature with gentle agitation. Culture was mixed with RPMI/acridine-orange and analysed using UV-microscope for rosetting status. Rosetting parasites were defined as infected erythrocytes with two or more attached uninfected erythrocytes. A minimum of 400 parasites were counted for each sample. Upon auto-agglutination each individual parasite in a cluster was counted as a rosetting parasite. Rosetting rate was calculated as (rosetting parasites/(rosetting parasites+ non-rosetting parasites)×100). In the given experiment the rosetting rate was correlated to the pool 0 control. The experiments were performed at least three times for 4 and 8 µg/mL and twice at the other concentrations. Each experiment was done on different occasions using different 2′F-RNA batches.

Results

Expression of DBL1α in E. coli The entire sequence of DBL1α from FCR3S1.2 was optimised for the codon-usage of *E. coli* to facilitate and increase the expression in the bacteria (Flick et al. 2004). By His-tag high-affinity purification soluble DBL1α^{His} was purified. Also a fusion protein of glutathione *S*-transferase and DBL1α was expressed in *E. coli* and purified with glutathione agarose beads. Verification of the two recombinant proteins was confirmed with immunoblots using DBL1α-specific antibodies (data not shown). A previous study has confirmed that the recombinant protein expressed in *E. coli* binds to heparin in both free and bound form (Flick et al. 2004).

Selection of DBL1α-specific RNA aptamers For the in vitro selection a combinatorial library of 5×10^{14} unique RNA sequences (Ellington and Szostak 1990; Tuerk and Gold 1990) was used. The strategy was to enrich for serum-stable aptamers (Pieken et al. 1991) that would have affinity towards the recombinant DBL1α domain from the high-rosetting strain FCR3S1.2 (Chen et al. 2004). Eight rounds of selection and amplification on DBL1α^{His} pre-bound to nickel beads were performed. The course of the experiment was monitored by determining the percentage of bound RNA in every SELEX cycle (Fig. 1). Increasing recovery of RNA was observed through the selection rounds and
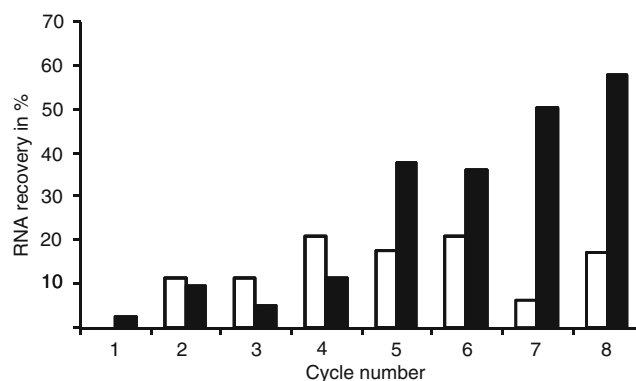

Fig. 1 SELEX selection on recombinant DBL1α^{His}. A 2′-F substituted RNA library of 10^{14} sequences was incubated with 0.45–1.3 µM recombinant DBL1α^{His} bound to Ni-NTA beads. 2′F-RNA was internally labelled with ^{32}P-[αATP] and the percentage of bound 2′F-RNA recovery was quantified using scintillation count of obtained fractions and listed for each of the eight selection rounds. *White bars* represent pre-selection on the 100 µl Ni-NTA matrix. *Black bars* are selection on Ni-NTA with bound DBL1α^{His}

maximum binding was achieved in cycle 8 with approximately 58% RNA recovery. The amplified DNA pool from cycle 8 was cloned into the TOPO® TA vector, and the nucleotide sequences of the randomised region of 85 individual clones were determined by sequencing (S 1).

Grouping of sequenced clones using MEME and testing of individual aptamers on recombinant DBL1α^{His} Eighty-five clones from the in vitro selection on recombinant DBL1 α^{His} were sequenced and analysed for conserved motifs using the MEME software (Grundy et al. 1997). The MEME software was set to discover a sequence of six to seven conserved nucleotides in the dataset within the randomised region from the 85 sequenced clones. Twenty of the most common motifs are listed (S 2). In order to characterise the different motifs, individual aptamers from each different group were tested for binding on DBL1α^{His}. The radioactive labelled aptamers were analysed and listed according to their capacity to associate to the recombinant protein (Fig. 2). The unselected pool 0 together with aptamer c07 was found to have the lowest retention towards DBL1α^{His} and b02, d12 and e05 the highest. These aptamers were further tested in three individual binding experiments (Fig. 2). Also the aptamers b02, d12 and e05 were detected in nine different motifs from the MEME data and were chosen for further investigation (Table 1). Theoretical structures of b02, d12 and e05 were achieved using the Mfold software (Zuker 2003) and the structures with the lowest free energies are depicted (Fig. 3). These predicted structures indicate that the three aptamers have different structures. Two isolated motifs in b02 are located within two suggested adjacent stem/loop regions (Fig. 3, b02). The motif AUCAA is suggested to be located in an

Fig. 2 Testing individual aptamers on recombinant DBL1α^{His}. Radioactive labelled aptamers containing different motifs were tested for binding on DBL1α^{His} bound to Ni-NTA agarose beads. Non-selected RNA (Pool 0) is adjusted to the value 1 and the recovery of different aptamers is related to this value. Clones are listed with increasing recovery from *left* to *right*. Clones b02, d12 and e05 are tested in triplicates. *Error bars* present SDs
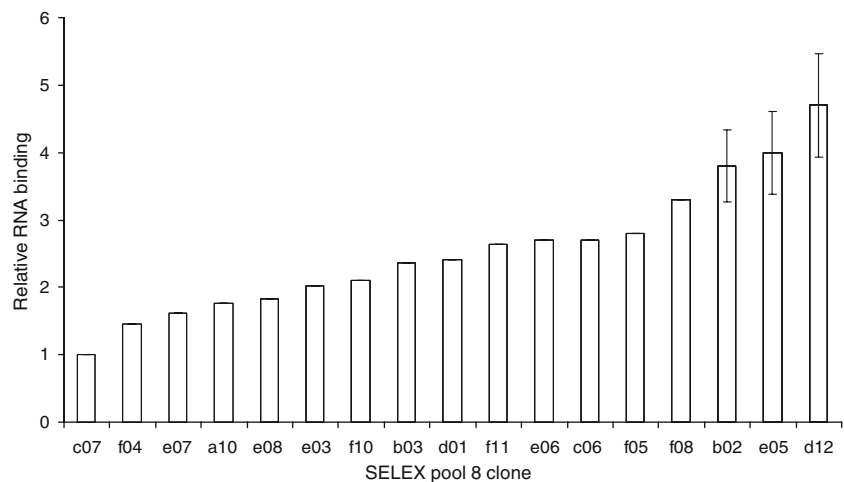
open loop structure found in b02, d12 and e05 (Fig. 3, b02, d12 and e05). Three other motifs are found in e05. The motif at position 21 is a part of a stem/loop. Two motifs at 55 and 69 are parts of a long transcending stem within the RNA molecule (Fig. 3, e05).

Aptamer binding to GST-DBL1α To confirm that the interaction of the aptamers with the protein was specific an additional experiment was designed. Non-labelled RNA was to compete with radioactive 5′-labelled RNA for binding to a GST-DBL1α fusion protein. The recovery of labelled RNA decreases 40% with the addition of 30-fold molar excess of non-labelled RNA (Fig. 4). Furthermore, the decrease is significant ($P \leq 0.05$). The two negative controls in the experiment, unselected RNA (pool 0) and e02, gave 85–90% lower recovery when compared to b02, d12, e05 ($P \leq 0.001$).

To further confirm that the observed decrease is specific, radioactive labelled d12 was incubated in the presence of

30-fold molar excess of e02 and pool 0 (Fig. 5). No decrease was observed with the addition of RNA from these two pools. In the experiment the observed decrease upon competing labelled d12 with excess non-labelled d12 and the binding of d12 compared to e02 and pool 0 were highly significant. This proposes that the decrease observed is due to interaction between selected aptamers and its target GST-DBL1α.

Aptamer binding to the surface of infected erythrocytes As the selected aptamers were binding to the recombinant DBL1α domain the next step was to verify if they could be associated to the surface of the infected erythrocyte. Two of these aptamers was compared to unselected RNA from pool 0 and the non-binding aptamer e02. Equal molar amounts of RNA were end-labelled with ^{32}P and incubated with 5% parasitemia culture of FCR3S1.2. The results demonstrate that the selected RNA is retained fourfold higher than RNA from unselected pool or the non-binding aptamer e02

Table 1 Summary of groups identified using the MEME software containing b02, d12 and e05

	St.	P value		Sites		No.
b02	48	1.68e-04	CGCGT	**TTTTTCA**	AACAC	10
b02	35	3.37e-04	CACCA	**GCAATCA**	ACGCG	12
d12	60	2.86e-05	GCATC	**GAAGGTT**	GGCA	12
d12	53	2.80e-04	TTATC	**TTGCAT**	CGAAG	10
d12	24	8.58e-04	AAC	**AATACGA**	CTACA	10
d12[a]	38	1.03e-03	ACACC	**ATCAAA**	AGTAT	10
e05[a]	41	1.03e-03	AATGA	**ATCAAA**	AACAA	10
e05	21	3.07e-04		**GTGACCA**	CGCGG	10
e05	69	7.96e-04	TTCGA	**CTACGC**	TAATT	10
e05	55	2.76e-05	AACAA	**CCAGGGC**	CGTTC	10

All groups contain 10 or more different aptamers. The start column (St.) indicates where in the aptamer, from the 5'-end, the conserved site start. The P value is an estimate of the expected number of motifs with the given log-likelihood ratio (or higher), and with the same width and number of occurrences, that one would find in a similarly sized set of random sequences. No. indicates the number of aptamers within the group

[a] Site found in both d12 and e05
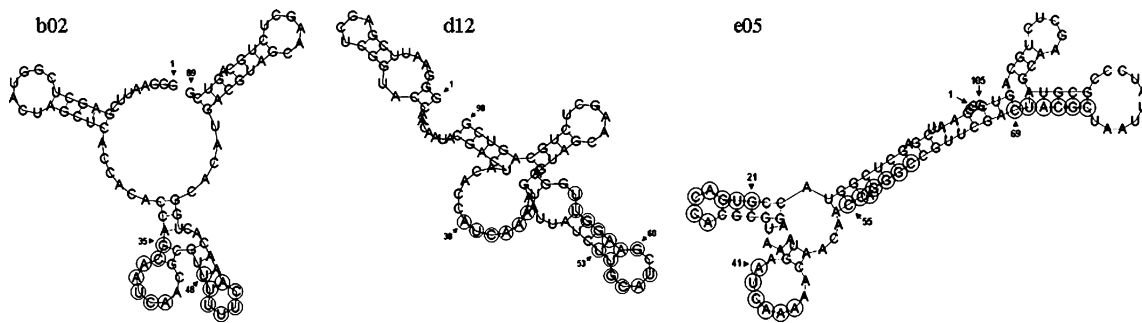
Fig. 3 Mfold prediction on RNA structure from clones b02, d12 and e05. Structures represent predictions with low free energy. Nine motifs isolated from the MEME search (S 2) are included. Location of the motif within the aptamer is shown with numbers listed from the 5′ end

(Fig. 6). The difference between these two groups is highly significant ($P \leq 0.001$).

Effects of RNA aptamers on rosetting in FCR3S1.2 in vitro Since two of the selected aptamers showed significantly higher association to the infected erythrocytes than unselected RNA, the next step was to analyse whether this interaction could affect the rosetting status of a blood culture of the highly rosetting and multi-adhesive clone FCR3S1.2. The rosetting and cytoadherence of FCR3S1.2 is sensitive to heparin and heparan sulphate and rosettes can be disrupted with an addition of 100 μg/ml heparin (~7 μM) in a 5% hematocrit culture with 5% parasitemia (Vogt et al. 2006). In a similar fashion we investigated whether the SELEX-selected RNA aptamers could disrupt the interaction between infected erythrocyte and non-infected erythrocytes. The aptamers were tested in blood cultures at a concentration of 1 μg/ml (33 nM) to 12 μg/ml (387 nM), and with unselected RNA from pool 0 as a negative control. A significant ($P \leq 0.001$) decrease in rosette rate to 35% in relation to the control was observed at 8 μg/ml (258 nM) and a total disruption was seen at 12 μg/ml for the clones b02, d12 and e05 (Fig. 7a). Even though minimal effect on rosette state was observed at a concentration of 33 nM, there was a clear visible effect when observing the

culture in the microscope as giant rosettes were decreased to smaller rosettes. An example of rosette disruption can be seen in the fluorescence image (Fig. 7b). The large rosettes which are present in the sample treated with unselected RNA (Fig. 7 (B1)) are almost completely disrupted in the aptamer treated sample (Fig. 7 (B2)). The infected erythrocytes are not adhering to other infected or non-infected erythrocytes. RNA from pool 0 did not disrupt rosettes in the concentration range 1–12 μg/ml, but a small degree of disruption (5–10% lower than untreated cells) was observed at an unselected RNA concentration of 31 μg/ml (1 μM).

Discussion

It is generally accepted that severe malaria is most likely caused by sequestration of the infected erythrocytes, yet there is no specific anti-sequestration drug available today. PfEMP1 is a key molecule used by *P. falciparum* to interact with the human host in multiple ways (Flick and Chen 2004). The adhesive function of this protein makes it possible for the parasite to adhere to endothelial lining and uninfected erythrocytes. Disrupting PfEMP1/receptor inter-

Fig. 4 Binding of ^{32}P labelled aptamers on GST-DBL1α. Radioactive labelled aptamer b02*, d12*, e02* and e05* and unselected RNA pool 0* were incubated with GST-DBL1α bound to glutathione beads. Labelled RNA from binding aptamers was competed with 30-fold excess non-labelled corresponding RNA (b02, d12, e05). Bound RNA* was quantified with scintillator. Unit on *y* axis= Bound RNA (mol). *Error bars* present SDs. *$P \leq 0.05$ and **$P \leq 0.001$, from a two-tailed Student's *t* test
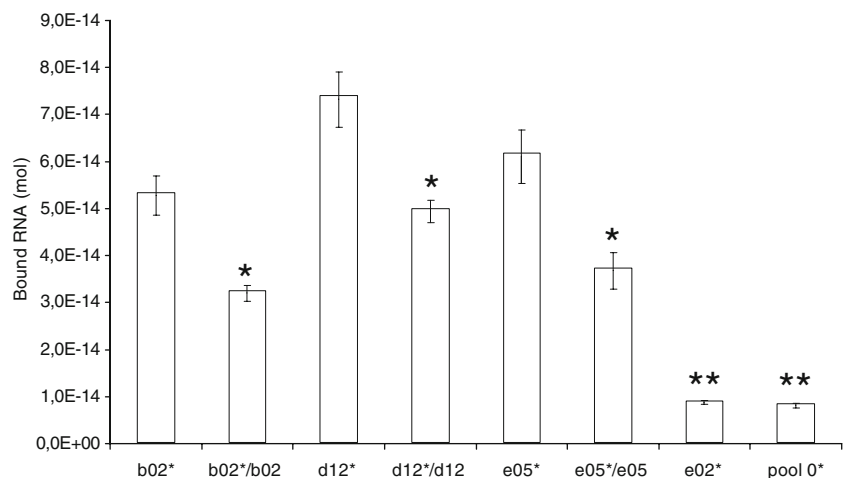
Fig. 5 Selective competition of ^{32}P labelled d12 aptamer on GST-DBL1α. Labelled aptamer d12*, e02* and unselected RNA pool 0* incubated with GST-DBL1α bound on glutathione beads. Thirty-fold non-labelled RNA from d12, e02, and pool 0 was added. Bound labelled RNA was quantified with scintillator. Units on y axis=Bound RNA (mol). *Error bars* present SDs. **$P\leq0.001$, from a two-tailed Student's t test
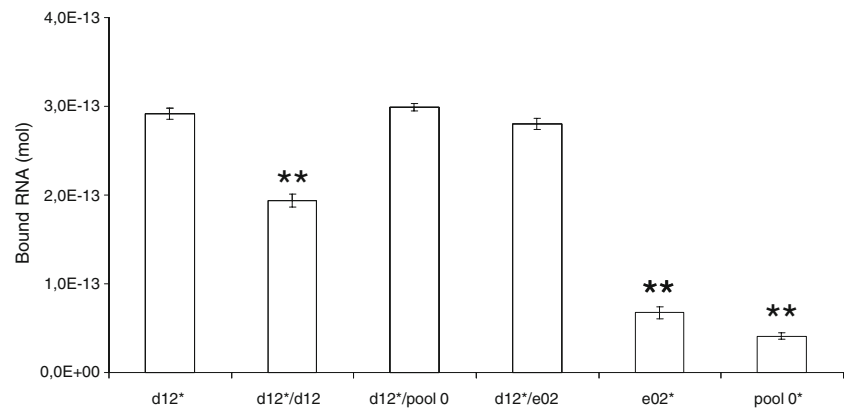
actions using aptamers might be advantageous due to their smaller size. This might give them greater potential to reach more buried structures which antibodies are not able to access due to steric hindrance. In addition, the RNA aptamers are easy to chemically modify and elicit little or no immunogenicity in therapeutic applications (Richardson et al. 1999; Pendergrast et al. 2005). Furthermore, previous work done with 2′F-modified RNA in serum demonstrate the half-life of the RNA to be approximately 15 h (Lorger et al. 2003). By SELEX technique aptamers were raised against the semi-conserved DBL1α region of PfEMP1. In this project we focused on generating RNA aptamers and testing their binding capacity to recombinant DBL1α as well as examining their capacity of rosette disruption. The first step in a SELEX screening is to isolate the target for selection. More complex and expensive systems such as a cell-free-based system and baculovirus expression system did not give better yields than our choice; *E. coli*. In all systems protein solubility was limiting expression yields.

Through eight selection rounds on recombinant DBL1α^{His} a major increase in specific RNA recovery was observed. To identify conserved motifs among the selected RNA aptamers, 85 clones were sequenced and analysed. The outcome of the MEME search revealed 20 conserved groups found within the 85 clones. The large variation among the

sequenced clones could be a result of the adherent nature of DBL1α due to its overall positive charge giving rise to low affinity interaction with RNA molecules. It is common to increase selection pressure in later cycles by lowering the protein concentration in relation to RNA concentration. Due to the limited stability of the protein this was not performed. Instead, shorter incubation times were chosen.

The initial screening of single clones on DBL1α^{His} showed that selected RNA aptamers bind with higher affinity than unselected RNA. The aptamers b02, d12 and e05 gave up to fivefold higher recovery after incubation with recombinant protein compared to unselected RNA (Fig. 2). Later experiments with GST-DBL1α proved that the binding was significant and specific since recovery of the labelled aptamer could be decreased by adding an excess of non-labelled RNA from b02, d12 and e05. No decrease was observed by the addition of non-binding aptamer e02 or pool 0, non-selected RNA. There is also a similarity between the dose and response of these aptamers (Fig. 7). This could be due to that they share the same AUCAA motif found within loop regions in all three aptamers (Fig. 3). In general the recovery rates were low in relation to the estimated protein concentrations used in the experiments. This could be due to the limited stability and aggregation of the recombinant DBL1α. All work on the
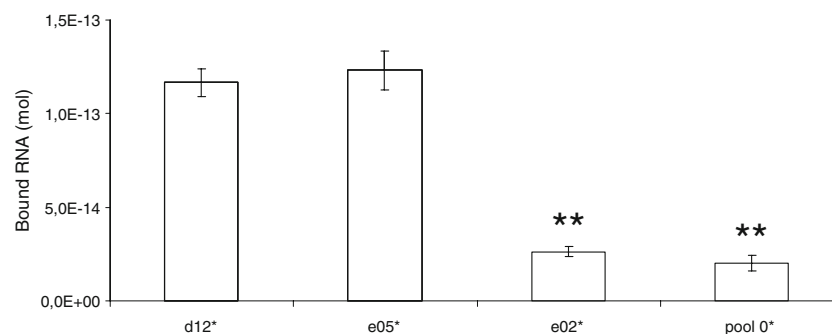
Fig. 6 RNA association to the surface of FCR3S1.2 infected erythrocytes. RNA, 9.8 pmol (10 nM), from clone d12, e05, e02 and pool 0 was incubated with 1 ml blood culture of FCR3S1.2. Cell pellet was washed with PBS. Bound labelled RNA from resuspended cell pellet was measured with scintillator. Units on y axis=Bound RNA in mol. *Error bars* present SDs, **$P\leq0.001$, from a two-tailed Student's t test

Fig. 7 Effect of 2′F-RNA of selected SELEX clones on the rosette state of FCR3S1.2. **a** One hundred microlitres aliquots of rosetting parasite cultures (FCR3S1.2) were treated with 2′F-RNA at different concentrations (1, 2, 4, 8, 10 and 12 μg/ml). The rosetting rate was counted after 1-h incubation and compared with 2′F-RNA from non-selected pool 0. *Closed triangle* pool 0, *open triangle* e05, *open square* b02, *closed square* d12. *Error bars* present SDs. **b** Rosette disruption of FCR3S1.2 visualised by fluorescence microscopy of cells stained with acridine-orange with 400 times magnification. Image *B1* is control cells, which have been incubated with non-selected 2′F-RNA from pool 0 for 1 h. Image *B2* are cells treated 1 h with 10 μg/ml 2′F-RNA from SELEX clone d12 (**b**)
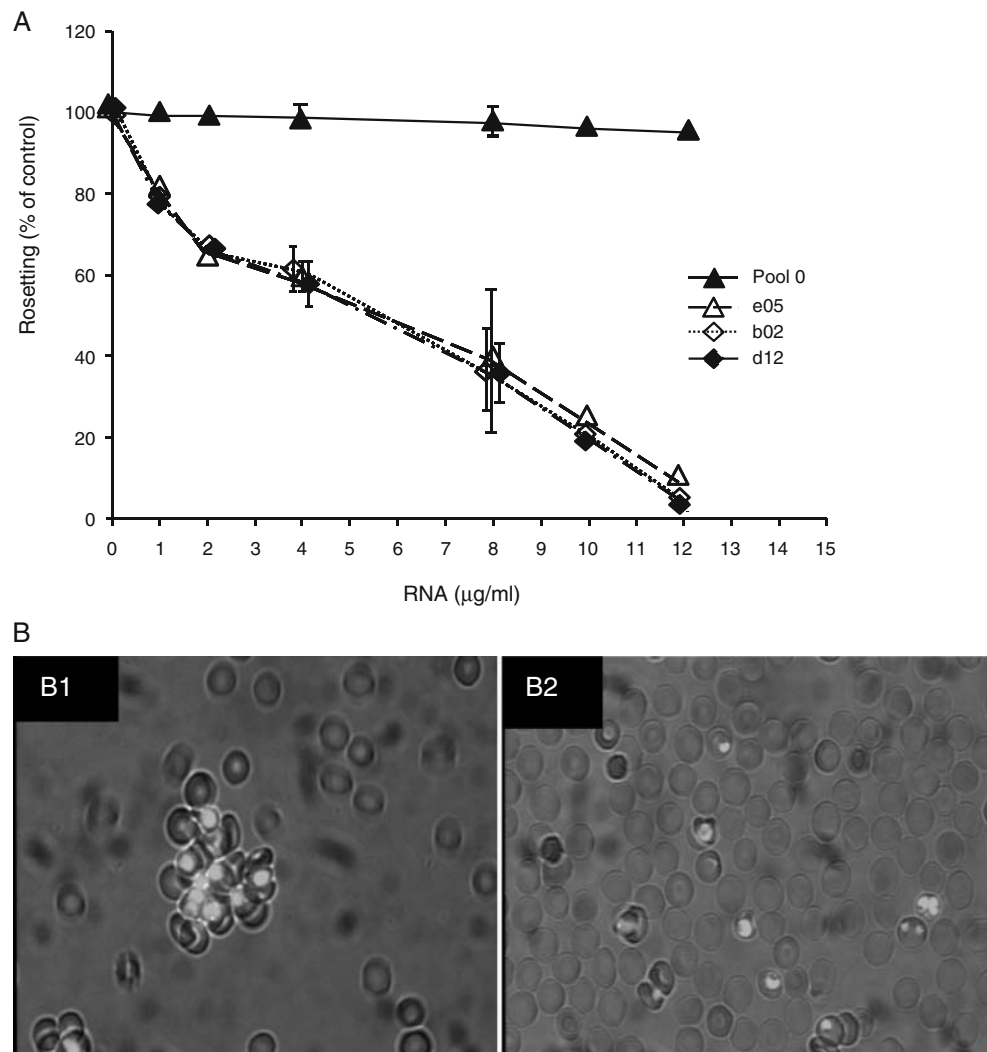
recombinant protein has no value, if aptamers do not bind to the domain in its native environment (PfEMP1 on the surface of the infected erythrocytes). The first approach to verify biological relevance were to ^{32}P-label the selected aptamers and observe if they associate to the infected erythrocyte to a higher degree than unselected RNA. Selected RNA is retained significantly more on infected erythrocytes than unselected RNA (Fig. 6). Binding levels were generally low. We believe the limiting factor to be the number of DBL1α binding sites in 1 ml parasite culture. Even though no precise estimate of the number of PfEMP1 molecules per cell has been published, the number is expected to be relatively low since detection by mass spectrometry has failed (Ducret et al. 1998; Fried et al. 2004; Wu and Craig 2006).

Rosetting in FCR3S1.2 is likely to be due to the interaction between DBL1α and heparan sulphate on the non-infected erythrocyte since FCR3S1.2 is sensitive to heparin (Chen et al. 2000). Rosetting strains which are less

sensitive to heparin might exploit other erythrocyte receptors such as blood group A antigen (Barragan et al. 2000). If the aptamers have a higher affinity towards DBL1α than heparan sulphate, they might be able to compete with the binding and thereby exclude and disrupt the HS/DBL1α interaction, visualised by disrupted rosettes. This was tested for the three SELEX selected clones b02, d12 and e05. They were all able to disrupt rosettes in an in vitro culture of FCR3S1.2 (Fig. 7a). A concentration as low as 33 nM had an effect on the rosette status of the culture visualised as a major decrease in size of the rosettes. Unselected RNA did not have any effect at these concentrations. The selected RNA has the ability to disrupt rosettes at 15 times lower concentration than heparin in a similar experiment (Vogt et al. 2006). Besides FCR3S1.2, the strain Dd2 was tested for rosette disruption with similar results (data not shown). The structures of the three aptamers were predicted using the software for RNA folding. These given structures of the aptamers are only theoretical and secondary characteriza-

tion of the RNA aptamers must be performed to verify the secondary structure of the given RNA.

To summarise, the results show that DBL1α-specific RNA aptamers have the potential to locate the protein on the surface of infected erythrocytes. More importantly, the aptamers can putatively be used as a novel anti-rosetting drug. The next step will be to test the aptamers for rosette disruption and sequestration of parasites in an animal model to see if the aptamers are active in vivo (Moll et al. 2007). Since the use of aptamers as putative therapeutic drugs is an expanding field, knowledge on how to increase stability and activity of aptamers in vivo is being generated as well as decreasing the production cost. This will help in facilitating future work in applying aptamers in vivo.

References

Ahuja S, Pettersson F, Moll K, Jonsson C, Wahlgren M, Chen Q (2006) Induction of cross-reactive immune responses to NTS-DBL-1alpha/x of PfEMP1 and in vivo protection on challenge with Plasmodium falciparum. Vaccine 24:6140–6154

Ashley EA, White NJ (2005) Artemisinin-based combinations. Curr Opin Infect Dis 18:531–536

Barragan A, Kremsner PG, Wahlgren M, Carlson J (2000) Blood group A antigen is a coreceptor in Plasmodium falciparum rosetting. Infect Immun 68:2971–2975

Baruch DI, Pasloske BL, Singh HB, Bi X, Ma XC, Feldman M, Taraschi TF, Howard RJ (1995) Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. Cell 82(1):77–87

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Chen Q (2007) The naturally acquired immunity in severe malaria and its implication for a PfEMP-1 based vaccine. Microbes Infect 9:777–783

Chen Q, Heddini A, Barragan A, Fernandez V, Pearce SF, Wahlgren M (2000) The semiconserved head structure of Plasmodium falciparum erythrocyte membrane protein 1 mediates binding to multiple independent host receptors. J Exp Med 192:1–10

Chen Q, Pettersson F, Vogt AM, Schmidt B, Ahuja S, Liljestrom P, Wahlgren M (2004) Immunization with PfEMP1-DBL1alpha generates antibodies that disrupt rosettes and protect against the sequestration of Plasmodium falciparum-infected erythrocytes. Vaccine 22:2701–2712

Ducret A, Van Oostveen I, Eng JK, Yates JR 3rd, Aebersold R (1998) High throughput protein characterization by automated reverse-phase chromatography/electrospray tandem mass spectrometry. Protein Sci 7:706–719

Ellington AD, Szostak JW (1990) In vitro selection of RNA molecules that bind specific ligands. Nature 346:818–822

Flick K, Chen Q (2004) var genes, PfEMP1 and the human host. Mol Biochem Parasitol 134:3–9

Flick K, Ahuja S, Chene A, Bejarano MT, Chen Q (2004) Optimized expression of Plasmodium falciparum erythrocyte membrane protein 1 domains in Escherichia coli. Malar J 3:50

Fried M, Wendler JP, Mutabingwa TK, Duffy PE (2004) Mass spectrometric analysis of Plasmodium falciparum erythrocyte membrane protein-1 variants expressed by placental malaria parasites. Proteomics 4:1086–1093

Grundy WN, Bailey TL, Elkan CP, Baker ME (1997) Meta-MEME: motif-based hidden Markov models of protein families. Comput Appl Biosci 13:397–406

Ho M, White NJ (1999) Molecular mechanisms of cytoadherence in malaria. Am J Physiol 276:C1231–1242

Kusser W (2000) Chemically modified nucleic acid aptamers for in vitro selections: evolving evolution. J Biotechnol 74: 27–38

Lorger M, Engstler M, Homann M, Goringer HU (2003) Targeting the variable surface of African trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. Eukaryot Cell 2:84–94

Moll K, Pettersson F, Vogt AM, Jonsson C, Rasti N, Ahuja S, Spangberg M, Mercereau-Puijalon O, Arnot DE, Wahlgren M, Chen Q (2007) Generation of cross-protective antibodies against Plasmodium falciparum sequestration by immunization with an erythrocyte membrane protein 1-duffy binding-like 1 alpha domain. Infect Immun 75:211–219

Normark J, Nilsson D, Ribacke U, Winter G, Moll K, Wheelock CE, Bayarugaba J, Kironde F, Egwang TG, Chen Q, Andersson B, Wahlgren M (2007) PfEMP1-DBL1alpha amino acid motifs in severe disease states of Plasmodium falciparum malaria. Proc Natl Acad Sci U S A 104:15835–15840

Pendergrast PS, Marsh HN, Grate D, Healy JM, Stanton M (2005) Nucleic acid aptamers for target validation and therapeutic applications. J Biomol Tech 16:224–234

Pieken WA, Olsen DB, Benseler F, Aurup H, Eckstein F (1991) Kinetic characterization of ribonuclease-resistant 2'-modified hammerhead ribozymes. Science 253:314–317

Richardson FC, Tennant BC, Meyer DJ, Richardson KA, Mann PC, McGinty GR, Wolf JL, Zack PM, Bendele RA (1999) An evaluation of the toxicities of 2'-fluorouridine and 2'-fluorocytidine-HCl in F344 rats and woodchucks (Marmota monax). Toxicol Pathol 27:607–617

Skeet J (2005) WHO global report on malaria indicates progress on prevention. Nurs Times 101:42

Stoltenburg R, Reinemann C, Strehlitz B (2007) SELEX–a (r) evolutionary method to generate high-affinity nucleic acid ligands. Biomol Eng 24:381–403

Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505–510

Ulrich H, Magdesian MH, Alves MJ, Colli W (2002) In vitro selection of RNA aptamers that bind to cell adhesion receptors of Trypanosoma cruzi and inhibit cell invasion. J Biol Chem 277:20756–20762

White NJ (1998) Drug resistance in malaria. Br Med Bull 54:703–715

Vogt AM, Pettersson F, Moll K, Jonsson C, Normark J, Ribacke U, Egwang TG, Ekre HP, Spillmann D, Chen Q, Wahlgren M (2006) Release of sequestered malaria parasites upon injection of a glycosaminoglycan. PLoS Pathog 2:e100

Wu Y, Craig A (2006) Comparative proteomic analysis of metabolically labelled proteins from Plasmodium falciparum isolates with different adhesion properties. Malar J 5:67

Zuker M (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 31:3406–3415