

Plasmodium falciparum Associated with Severe Childhood Malaria Preferentially Expresses PfEMP1 Encoded by Group A *var* Genes

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Abstract

Parasite-encoded variant surface antigens (VSAs) like the *var* gene–encoded *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) family are responsible for antigenic variation and infected red blood cell (RBC) cytoadhesion in *P. falciparum* malaria. Parasites causing severe malaria in nonimmune patients tend to express a restricted subset of VSA (VSA_{SM}) that differs from VSA associated with uncomplicated malaria and asymptomatic infection (VSA_{UM}). We compared *var* gene transcription in unselected *P. falciparum* clone 3D7 expressing VSA_{UM} to in vitro–selected sublines expressing VSA_{SM} to identify PfEMP1 responsible for the VSA_{SM} phenotype. Expression of VSA_{SM} was accompanied by up-regulation of Group A *var* genes. The most prominently up-regulated Group A gene (*PFDD1235w/MAL7P1.1*) was translated into a protein expressed on the infected RBC surface. The proteins encoded by Group A *var* genes, such as *PFDD1235w/MAL7P1.1*, appear to be involved in the pathogenesis of severe disease and are thus attractive candidates for a vaccine against life-threatening *P. falciparum* malaria.

Key words: *var* gene • *Plasmodium falciparum* • malaria • PfEMP1 • antibody selection

Introduction

Plasmodium falciparum is the most pathogenic malaria parasite and a major cause of morbidity and mortality among children in sub-Saharan Africa. The virulence of *P. falciparum* has been linked to its expression of variant surface antigens (VSAs) that subvert acquisition of protective immunity and mediate infected RBC sequestration (for review see reference 1). Severe and life-threatening falciparum malaria is associated with parasites expressing a restricted and antigenically conserved VSA subset (VSA_{SM}; 2–4). VSA_{SM} expression appears

to confer a selective advantage on parasites in nonimmune individuals, perhaps by allowing particularly efficacious infected RBC sequestration and high growth rates (5). As VSA_{SM}-specific immunity is acquired, this advantage gradually gets smaller. Survival rates of parasites expressing less virulent and more diverse VSAs (VSA_{UM}) eventually surpass those of VSA_{SM}-expressing parasites, causing VSA_{UM}-expressing parasites to dominate infections in semi-immune individuals (5). This scenario makes it theoretically possible to protect

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Abbreviations used in this paper: CIDR, cysteine-rich interdomain region; Ct, threshold cycle; DBL, Duffy binding-like domain; DIG, digoxigenin; GST, glutathione S-transferase; PfEMP1, *Plasmodium falciparum* erythrocyte membrane protein 1; TrHBMEC, transformed human bone marrow endothelial cell; VSA, variant surface antigen.

nonimmune children against severe and complicated malaria by accelerating acquisition of VSA_{SM}-specific immunity through vaccination.

P. falciparum erythrocyte membrane protein 1 (PfEMP1) is the best characterized VSA family. PfEMP1 molecules are encoded by the *var* family comprising 40–60 highly diverse genes per haploid genome (6–8). Any single parasite expresses one PfEMP1 variant on the infected RBC surface (9, 10), but expression can switch at each reinvasion cycle (11, 12). Previous efforts to link PfEMP1 expression to particular clinical syndromes have been foiled by the extensive intergenomic and intragenomic variation of *var* genes in field isolates, simultaneous transcription of several *var* genes, and technical difficulties such as primer bias (13–15).

We have combined the availability of the entire genome sequence and the structural characteristics of the *var* genes in the *P. falciparum* clone 3D7 (16–18) with the capacity to control the VSA phenotype of this clone by in vitro antibody selection (19) or selection for adhesion to transformed human bone marrow endothelial cells (TrHBMECs; 20 and unpublished data) to investigate the relationship between VSA phenotype, *var* gene transcription, and PfEMP1 expression.

Materials and Methods

Malaria Parasites and In Vitro Selection Procedure. The *P. falciparum* clone 3D7 was cultured in 0 Rh⁺ RBCs as previously described (21). Repeated rounds of panning on DynaBeads coated by IgG from two plasma pools (SM1 and SM2) from semi-immune Ghanaian children (22) and one plasma pool (SM3) from semi-immune Tanzanian children (23) were used to select 3D7 parasites expressing VSAs that were highly recognized by IgG in these plasma pools (19).

Standard panning techniques (24) were used to select 3D7 asexual parasites for adhesiveness to TrHBMECs (20, 25). After three rounds of selection followed by cryo preservation and recovery, the ability of the selected subline and the parental culture to adhere to TrHBMEC (5,000–20,000/well) was compared. On average (six experiments), selected 3D7 bound 69 infected RBCs/100 TrHBMECs compared with 17.5 infected RBCs/100 TrHBMECs for the unselected parental parasites ($P = 0.0008$; Student's *t* test).

Flow cytometry (21) was used to verify that each of the four selected sublines expressed VSA_{SM}-type VSAs, i.e., had a plasma IgG recognition pattern resembling that of VSAs expressed by *P. falciparum* parasites isolated from children with severe malaria (2, 3). The genotypic identity of 3D7 and the selected sublines was regularly verified by PCR at the polymorphic *msp1*, *msp2*, and *glurp* loci (3).

In addition, parasites were isolated on days 8, 9, and 10 from a Dutch volunteer exposed on day 0 to mosquitoes infected by *P. falciparum* isolate NF54 (26) as part of ongoing studies of experimental infections (27). These parasites were cultured in vitro for 27 (day 8 and day 9 isolates) or 33 d (day 10 isolate) to obtain sufficient parasites for DNA/RNA analysis. Experiments involving samples of human origin received ethical clearance from the National Institute for Medical Research, Dar es Salaam, Tanzania, and the Ethical Committee of the University Medical Centre, Nijmegen, Netherlands.

DNA/RNA Extraction and cDNA Synthesis. RBCs infected by trophozoite/schizont-stage parasites (36–48 h after invasion)

from in vitro cultures were isolated by exposure to a strong magnetic field (21). In some experiments, the purified infected RBCs were cultured overnight to obtain cultures uniformly infected by ring-stage (30 h) parasites. These time points have previously been shown to be optimal for studies of *var* gene transcription (28).

Genomic DNA was isolated from infected RBCs by Nucleospin purification kits (BD Biosciences) and total RNA was prepared using Trizol (Invitrogen) as recommended by the manufacturers and treated with DNase1 (Invitrogen) for 15 min at 37°C. Absence of DNA in RNA samples was confirmed by stable base fluorescence after 40 cycles of real-time PCR with *seryl-tRNA synthetase* primers (28). Superscript II was used to reverse transcribe DNA-free RNA primed with random hexamer primers (Invitrogen) at 25°C for 10 min and 42°C for 50 min followed by 70°C for 15 min.

Quantitative Real-time PCR. Quantitative real-time PCR was performed using a Rotorgene thermal cycler system (Corbett Research) and real-time PCR-optimized and gene-specific primers for each of 59 full-length *var* genes and a pseudogene in the *P. falciparum* 3D7 genome (28 and see Table S1, available at <http://www.jem.org/cgi/content/full/jem.20040274/DC1>).

Reactions were performed in 20- μ l volumes using QuantiTect SYBR Green PCR Master Mix (QIAGEN) and 0.5 mM primers as previously described (28). Quantification was performed using Rotorgene software version 4.6. The housekeeping gene *seryl-tRNA synthetase*, which shows a uniform transcription profile in different parasite isolates and an unchanged pattern throughout the parasite life cycle, was used as an endogenous control as previously described (28), and used for calculations of fold changes in *var* gene transcription by the $\Delta\Delta$ CT method (User Bulletin no. 2; Applied Biosystems). We have previously verified the equality of the efficiency of the target (*var*) and reference (*seryl-tRNA synthetase*) amplification using the primer set on serial dilutions of genomic DNA (28).

DNA Cloning, Standard Curve, and Absolute Quantification. Genes (*PF11_0521*, *PFL0030c*, *PFL1950w*, *PFL005w*, *PF13_003*, *PFB1055c*, *PFD1235w/MAL7P1.1*, *PFE1640w*, *PF11830*, *PF08_0107*, *MAL6P1.314*, *MAL6P1.316*, *PFD0995c*, *PFA0015c*, and *seryl-tRNA synthetase*) used for absolute quantification were PCR amplified, ligated into the pCR2.1 TOPO vector, and transformed into *Escherichia coli* TOPO10 cells (TA Cloning System; Invitrogen). Plasmids were purified using QIAGEN Miniprep spin columns (QIAGEN) and the identity of inserts was verified by subsequent sequencing on an ABI Prism 310 (PerkinElmer) using the Big Dye terminator reaction mix, ABI Prism proofreading and translation software, and the *P. falciparum* 3D7 genome database (<http://www.plasmodb.org>).

Triplicate real-time measurements were made for each plasmid dilution (5×10^8 to 5×10^0 template copies) and a best fit standard curve was generated (28). The standard curves were linear across a range of seven logs of DNA concentrations with correlation coefficients between 0.9779 and 0.9969. The detection limit of the system was ≥ 20 copies (unpublished data). The coefficient of variance was calculated as $100 \times$ (standard deviation of the mean). RT-PCR was performed as described using 1 μ g total RNA in a total volume of 40 μ l, of which 0.5 μ l was subsequently used for real-time PCR. Absolute values were calculated from the standard curves.

Identification of Sequences Related to PFD1235w/MAL7P1.1. *PFD1235w/MAL7P1.1*-like *var* genes were PCR amplified from genomic DNA of Ghanaian *P. falciparum* field isolates (BM021 and BM048), cloned, and sequenced. The following primers used were: sense GTATGGCATCGTAACGCA and antisense

AGAAAGTCTTTGTATGACT. Nucleotide sequence data reported in this paper are available from GenBank/EMBL/DDBJ under accession nos. AY584238 and AY584239.

Northern Blotting. For Northern blotting, 10 µg total RNA were separated in a standard denaturing MOPS formaldehyde agarose gel and transferred to positively charged nylon membranes overnight (29, 30). RNA was cross-linked to the membrane by baking for 30 min at 120°C. Digoxigenin (DIG)-labeled RNA probes were generated using the DIG RNA labeling kit (Roche). Hybridization, washing, and detection were performed according to the manufacturer's recommendations with a hybridization temperature of 65°C in DIG Easy Hyb buffer (Roche).

Immunoblot Analysis. SDS-extracted trophozoite/schizont-stage-infected RBCs were reduced and electrophoresed in the Laemmli sodium dodecyl sulfate PAGE system (29). Immunoblots were prepared on PVDF membranes by semidry blotting using standard methods. Binding sites were blocked in Tris-buffered saline Tween (TBS-T) containing 5% skimmed milk. Blots were probed with preimmune mouse sera, antiserum was raised against a recombinant protein from the intracellular acidic segment ATS of PfEMP1 genes, and Duffy binding-like domain (DBL)5-δ of *PF1235w/MAL7P1.1* was diluted 1:100 in TBS-T. Bound antibody was detected with relevant IgG alkaline

phosphatase-conjugated antibody (DakoCytomation) and blots were developed using NBT (p-nitroblue tetrazolium phosphate; Sigma-Aldrich) and BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma-Aldrich) as substrates.

Protein Expression. The cysteine-rich interdomain region (CIDR)1-α and DBL5-δ of *PF1235w/MAL7P1.1* were subcloned into the pGEX-4T1 vector (Amersham Biosciences) by PCR using the following domain-specific oligonucleotide primers: CIDR1-α-Fw: 5'-CGGAATTCGACGCTAAAACCTG-ATAGTA-3'; CIDR1-α-Rv: 5'-ATAAGAATGCGGCCGC-ACATATATCGCCATTCAACG-3'; DBL5-δ-Fw: 5'-CGG-AATTCAGTCTCAATGCCGCATGTG-3'; and DBL5-δ-Rv: 5'-ATAAGAATGCGGCCGCCTCTACAATGTCTGCACA-3'. A conserved sequence of the intracellular acidic segment ATS was subcloned into the pGEX-4T1 vector by PCR using the following oligonucleotide primers: ATS-Fw: 5'-CGGAAT-TCAAAAACAAAATCATCAGTAG-3'; ATS-Rv: 5'-ATAAG-AATGCGGCCGCCTTGATTACCACTTAATGTG-3' (EcoRI site, underlined; NotI site, underlined italics). The proteins were expressed as fusion proteins at the carboxy terminus of glutathione S-transferase (GST) from *Schistosoma japonicum* (31) and purified by affinity chromatography on glutathione sepharose 4B (Amersham Biosciences). The DBL5-δ of *PF1235w/*

Table I. Copy Number of Selected *var* Genes and *seryl-tRNA synthetase* Transcripts in Unselected and *VSA_{UM}*-expressing 3D7 and 3D7 after Antibody Selection for *VSA_{SM}* Expression

Cluster ^a	Primer ^b	Gene	Trophozoite/schizont-stage parasites (36–48 h after invasion)				Ring-stage parasites (30 h after invasion)					
			Unselected		Antibody-selected		Unselected		Antibody-selected		Fold change	
			Copies	CV ^c	Copies	CV ^c	Copies	CV ^c	Copies	CV ^c		
A	8	<i>PF11_0521</i>	426	11	7,119	27	16.7	12,570	31	84,341	29	6.7
	20	<i>PF13_0003</i>	1,797	7	6,129	20	3.4	77,934	13	179,746	9	2.3
	35	<i>PF1235w/MAL7P1.1</i>	2,931	20	29,941	13	10.2	101,417	27	1,152,758	12	11.4
	67	<i>MAL6P1.314</i>	3,145	13	19,356	14	6.2	87,130	19	767,747	8	8.8
	97	<i>PFA0015c</i>	77,890	12	1,333	30	58.4	155,151	21	18,419	14	8.4
B/A	94	<i>MAL6P1.316</i>	13,742	15	36,637	24	2.7	247,156	29	1,057,056	28	4.3
	18	<i>PFL0005w</i>	15	21	1,106	23	73.7	928	12	13,769	23	14.8
B	21	<i>PFB1055c</i>	64	16	2,533	12	39.6	5,272	22	26,070	5	4.9
	58	<i>PFI1830c</i>	79,111	39	59,860	22	1.3	18,600	15	13,124	12	1.4
B/C	12	<i>PFL1950w</i>	226	22	524	8	2.3	13,236	19	19,053	24	1.4
C	66	<i>PF08_0107</i>	82,211	33	5,590	15	16.3	993,679	23	58,130	17	17.1
	95	<i>PF0995c</i>	23,415	19	491	12	47.7	567,205	19	9,148	19	62
<i>var2</i>	10	<i>PFL0030c</i>	907	14	12,562	19	14	12,905	30	127,631	30	9.9
<i>var1</i>	37	<i>PFE1640w</i>	12,279	21	14,239	10	1.2	8,224	17	9,111	30	1.1
		<i>seryl-tRNA synthetase</i>	609,533	16	609,533	16	1	643,271	20	643,271	20	1

^aDescribed in reference 18.

^bDescribed in reference 32 and Table S1.

^cCoefficient of variation (%) between three different quantification experiments.

MAL7P1.1 was cloned into the pBAD-TOPO vector (Invitrogen) using the following primers: DBL5- δ -Fw: 5'-CGG-AATTCAGTCTCAATGCCGCATGTG-3'; DBL5- δ Rv: 5'-TCTACAATGTCTGGCACA-3'. For production of carboxy terminally V5 epitope and histidine-tagged protein, the DBL5- δ insert was excised by EcoRI and PmeI digestion and then subcloned into the EcoRI and blunt-ended BglII sites of the *Baculovirus* transfer vector pAcGP67-A (BD Biosciences). Recombinant *Baculovirus* were generated by cotransfection of the pAcGP67-A-DBL5- δ construct gene and Bsu36I-linearized Bapkap6 *Baculovirus* DNA (BD Biosciences) into insect Sf9 cells. Recombinant DBL5- δ product was expressed by infection of insect High Five cells with recombinant *Baculovirus*. DBL5- δ protein was purified from culture supernatants on Co²⁺ metal chelate agarose columns and eluted with 25 mM Hepes-KOH, pH 7.6, 0.5 mM MgCl₂, 0.5 mM DTT, 100 mM NaCl, 10% glycerol, and 100 mM imidazole.

Generation of Antisera. All procedures complied with European or national regulations. Antibodies to ATS expressed in *E. coli*- and *Baculovirus*-expressed DBL5- δ were raised in BALB/c mice by subcutaneous injection of 5 μ g protein in complete

Freund's adjuvant followed by several boosters of ATS and DBL5- δ in incomplete Freund's adjuvant.

Immunostaining, Flow Cytometry, and Microscopy. Immunostaining and flow cytometry were performed as previously described (21) with some modifications. In brief, 2.5×10^5 MACS-purified, ethidium bromide-labeled infected RBCs were incubated for 1 h in 20 μ l murine sera or for 30 min in 5 μ l human sera depleted for anti-human RBC antibodies. For immunostaining with murine sera, infected RBCs were sequentially exposed to 100 μ l of 1:25 goat anti-mouse Ig (DakoCytomation), biotinylated anti-goat Ig (DakoCytomation), and 1:200 FITC-conjugated streptavidin (DakoCytomation) for 30 min each. For immunostaining with human sera, infected RBCs were incubated in 100 μ l of 1:25 biotinylated anti-human IgG (DakoCytomation) followed by 1:2,000 FITC-conjugated streptavidin for 30 min each. For confocal microscopy, wet mounts of immunostained parasites and a Carl Zeiss MicroImaging, Inc. scanning microscope were used. Trypsin treatment was performed as previously described (32). Cells were washed once in PBS, incubated in 10 vol of 100 μ g/ml TPCK-treated trypsin (Amersham Biosciences) in PBS for 10 min at 37°C. The reaction was

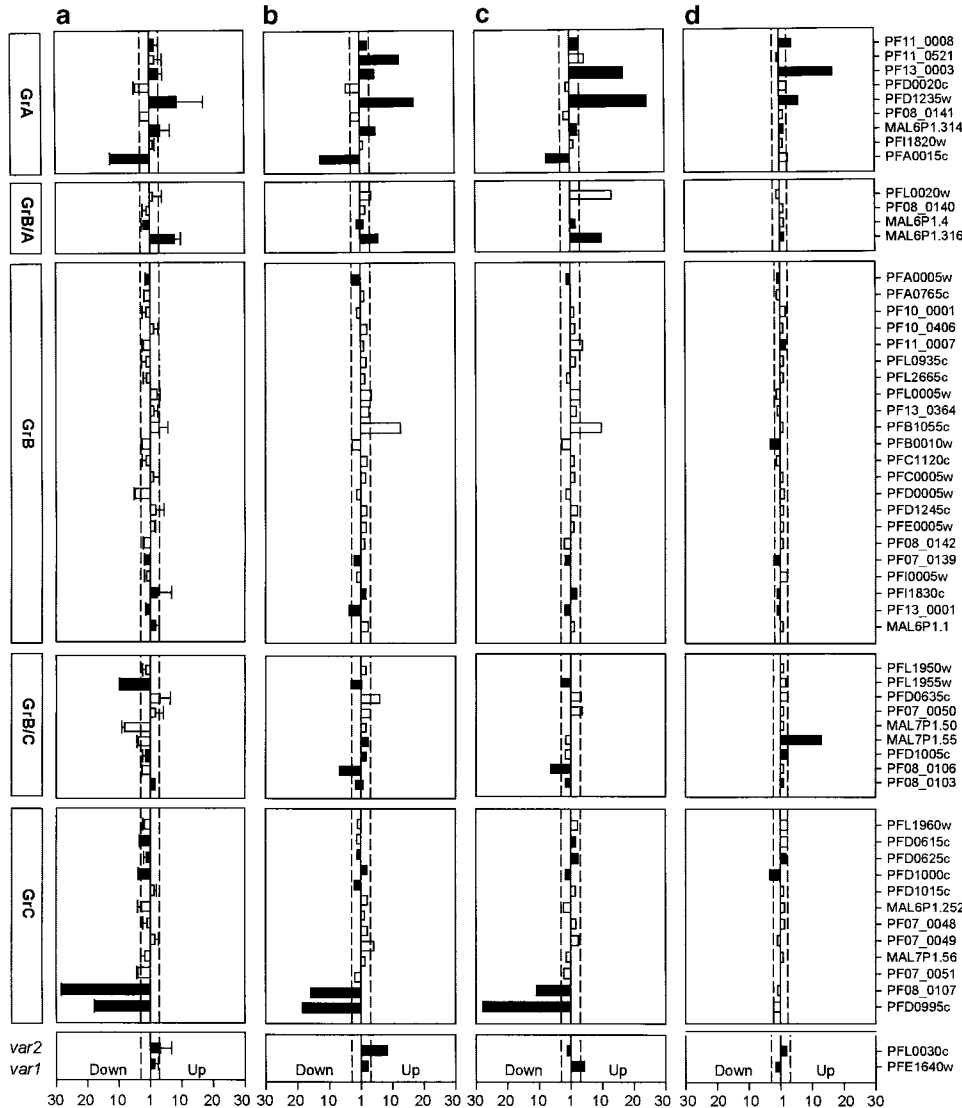


Figure 1. Fold changes in *var* gene transcription by late-stage (36–48 h after invasion) *P. falciparum* 3D7 before and after antibody selection of parasites for selection of VSA_{SM}-type antigens using DynaBeads coated with IgG from three different pools of plasma from semi-immune African children (SM1, SM2, and SM3; a–c) and for adhesion to TrHBMEC (d; see Materials and Methods for details). Transcription levels were measured using real-time PCR and primers specific for 59 *var* genes and 1 pseudogene (reference 28 and Table S1). Experiments with SM1 were repeated five times and results are shown as means \pm SD. Solid bars indicate highly transcribed *var* genes. These genes had the 15 lowest Ct values in selected and/or unselected 3D7. A threefold change in *var* gene transcription (dashed lines) was arbitrarily defined as the cut off for biologically significant changes in *var* gene transcription. Primer identity and calculation of selection-induced *var* gene transcription is described in Materials and Methods and reference 28. The grouping of *var* genes is described in reference 18.

stopped with 1 vol of 2 mg/ml soybean trypsin inhibitor (Sigma-Aldrich) in RPMI 1640/5% Albumax.

ELISA. Plasma samples from 20 children (3 to 4 and 10 to 11 yr old) and 10 adults (18 to 19 yr old) living in Mgome village in the Tanga region of Tanzania were used for ELISA analysis of antibody responses to purified recombinant GST proteins (33). Control plates were coated with GST alone. The mean ELISA unit plus 2 standard deviations obtained with sera from 13 Danish blood donors without malaria exposure was used as negative cut off value (34).

Competition ELISA. Competition ELISA was performed using recombinant CIDR1- α domains of *PFD1235w/MAL7P1.1* and *var1 (PFE1640w)* and three different plasma samples with high, medium, and low antibody reactivity to these proteins. Blocking of plasma was performed using 0.1, 1, 5, and 10 μ g/ml recombinant protein for 2 h at room temperature. The test plasma samples were diluted at 1:50 and tested as described above.

Online Supplemental Material. Primers for quantitative real-time PCR are shown in Table S1, which is available at <http://www.jem.org/cgi/content/full/jem.20040274/DC1>.

Results

Group A var Genes Are Up-regulated in 3D7 Selected for Expression of VSA_{SM}-type Antigens. Parasites associated with severe malaria express VSAs (VSA_{SM}) that are recognized at high levels by plasma IgG of most semi-immune children, whereas parasites causing uncomplicated malaria infection tend to express VSAs (VSA_{UM}) that are recognized less often and at lower levels (2, 3). Among unselected 3D7 parasites, most express VSA_{UM} with a small minority expressing VSA_{SM} (19). However, the dominating VSA phenotype changes to VSA_{SM} after selection of 3D7 using DynaBeads coated with IgG from semi-immune children (19) or adhesion to TrHBMEC. This cell line does not express scavenger receptor CD36 and thus is unlikely to bind VSA_{UM}-expressing parasites (25).

We compared *var* gene transcription in unselected and VSA_{SM}-expressing selected 3D7 using primer sets targeting

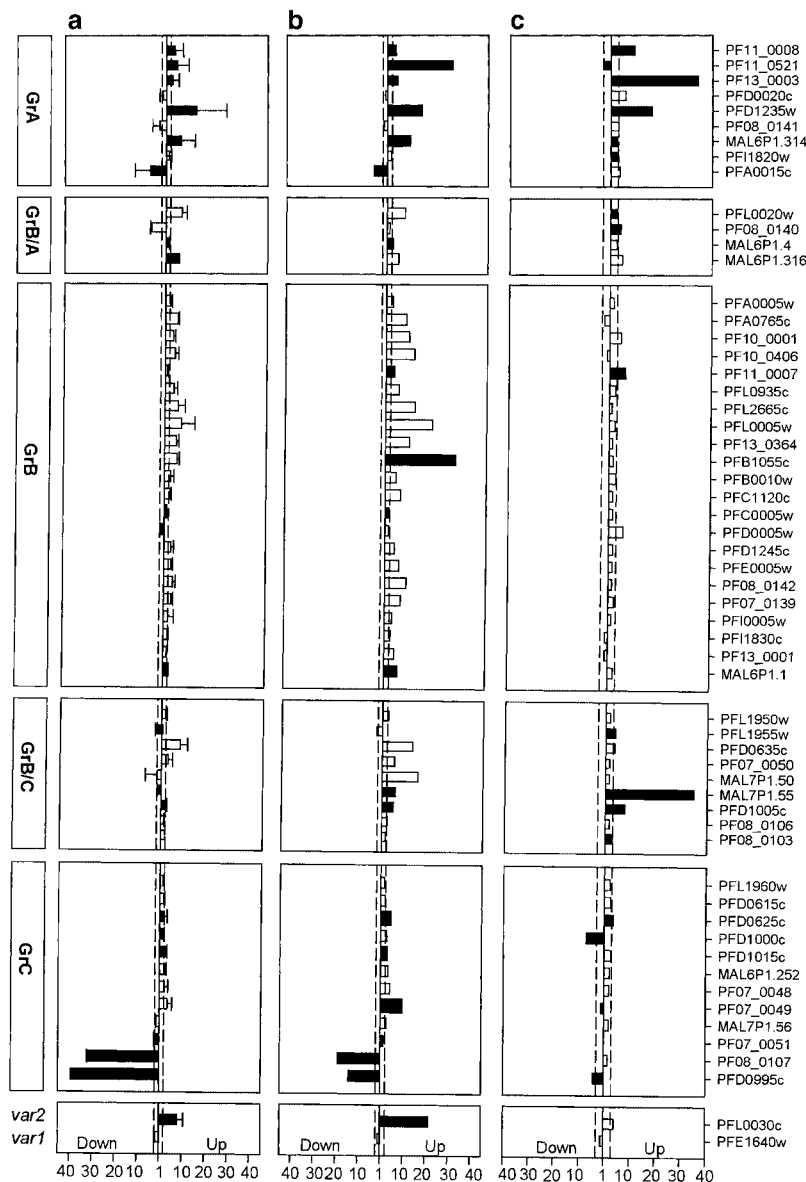


Figure 2. Changes in *var* gene transcription in synchronized ring-stage (30 h) *P. falciparum* 3D7 after antibody selection with the SM1 and SM2 pools (a and b) or selection for adhesion to TrHBMEC (c) as described in the legend for Fig. 1. A fold-change of two (dotted lines) was defined as the cut off for biological interesting changes in *var* gene transcription.

each of the 60 *var* genes in 3D7 (Table S1, which is available at <http://www.jem.org/cgi/content/full/jem.20040274/DC1>; references 16 and 28). 4 (*PF13_0003*, *PFD1235w/MAL7P1.1*, *MAL6P1.314*, and *MAL6P1.316*) of the 15 most highly transcribed *var* genes were up-regulated in late trophozoites/schizonts after antibody selection-induced change from VSA_{UM} to VSA_{SM} expression, whereas 4 other *var* genes (*PF08_0107*, *PF0995c*, *PFA0015c*, and *PFL1955w*) were down-regulated (Fig. 1). These changes were consistently observed after antibody selection of 3D7 with three different plasma pools from West (Fig. 1, a and b) and East Africa (Fig. 1 c). Comparable results were obtained after selection for binding to TrHBMEC (Fig. 1 d), although these parasites also showed high transcription of *MAL7P1.55*. Ring-stage parasites showed similar patterns, although three additional genes (*PF11_0008*, *PF11_0521*, and *PFL0030c*) were up-regulated in response to selection for VSA_{SM} expression (Fig. 2).

Most of the seven up-regulated *var* genes belong to the Group A *var* cluster and encode high mol wt PfEMP1, whereas one of the up-regulated genes belongs to the Group B/A cluster and encodes a relatively large PfEMP1 with a complex domain structure (16, 18). In contrast, two out of three of the down-regulated genes belong to the Group C cluster, encoding relatively small four-domain PfEMP1 molecules (16, 18).

To confirm the above results, we measured mRNA copy numbers of highly transcribed *var* genes that were significantly up-regulated (*PF11_0521*, *PFL0030c*, *PF13_0003*, *PFD1235w/MAL7P1.1*, *MAL6P1.314*, and *MAL6P1.316*) or down-regulated (*PF08_0107*, *PF0995c*, and *PFA0015c*). We also determined copy numbers of three Group B genes (*PFL0005w*, *PFB1055c*, and *PF11830c*), a Group B/C gene (*PFL1950w*), and *PFE1640w* (*var1*). The results (Table I)

were consistent with the threshold cycle (Ct) values and confirmed the fold change calculations (Figs. 1 and 2). Apart from two genes (*PF11830c* and *PFE1640w*), the examined *var* genes were transcribed at higher levels in ring-stage than in trophozoite/schizont-stage parasites, in agreement with previous observations (35). These results also show that selection-induced change from VSA_{UM} toward VSA_{SM} expression was accompanied by a change in *var* gene transcription pattern from Group C genes toward Group A and B/A genes. Thus, the Group C gene *PF08_0107* was both dominant and present in a much higher copy number than any of the other transcripts among unselected 3D7 (Figs. 1 and 2, and Table I), whereas two Group A genes (*PFD1235w/MAL7P1.1* and *MAL6P1.314*) and a Group B/A gene (*MAL6P1.316*) were dominant among antibody-selected 3D7 (Figs. 1 and 2, and Table I).

Northern blots of antibody-selected, ring-stage 3D7 showed strong hybridization signals corresponding to full-length transcripts of the up-regulated genes *PFD1235w/MAL7P1.1* and *MAL6P1.314* (Fig. 3, a and c). The corresponding signals were either absent or much weaker in Northern blots of unselected 3D7. By contrast, a strong signal corresponding to *PF08_0107*, which was highly transcribed but markedly down-regulated after antibody selection, could be detected in unselected, but not in antibody-selected, 3D7 (Fig. 3 b).

These results suggest that 3D7 selected for expression of VSA_{SM}-type antigens preferentially transcribes full-length Group A *var* genes.

The Up-regulated Group A var Genes Are Translated into High mol wt PfEMP1 in 3D7-expressing VSA_{SM}-type Antigens. Western blot analysis of PfEMP1 expression by unselected late trophozoite/schizont-stage 3D7 using antise-

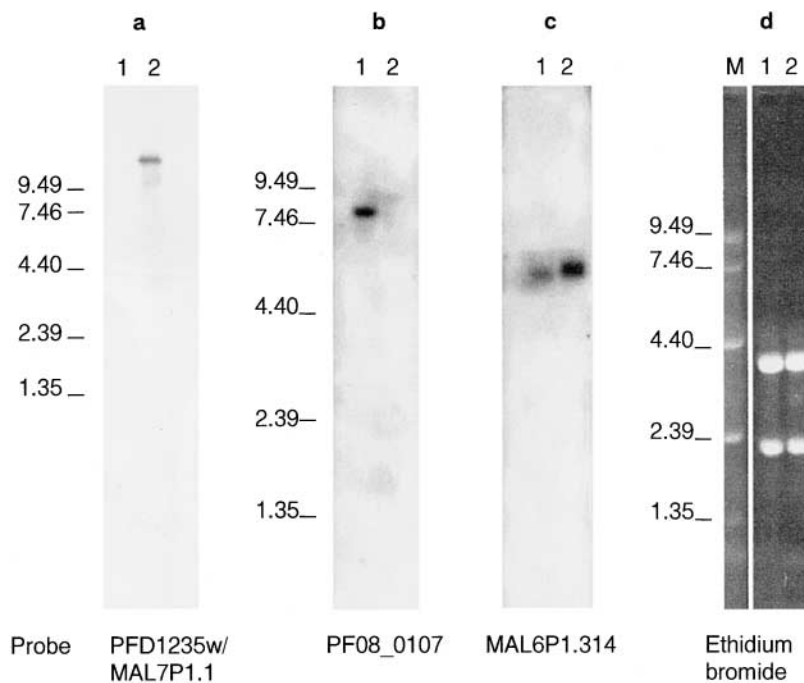


Figure 3. Relative levels of *var* transcripts in 10 μ g total RNA obtained from unselected (1) and antibody-selected 3D7 (2) ring-stage (30 h)-infected RBCs. Northern blots were probed with DIG-labeled RNA probes targeting *PFD1235w/MAL7P1.1* (a), *PF08_0107* (b), and *MAL6P1.314* (c). Transcript sizes were 12.5 (*PFD1235w/MAL7P1.1*), 8.3 (*PF08_0107*), and 5.7 kb (*MAL6P1.314*). An ethidium bromide-stained gel is shown to allow comparison of total RNA amounts (d).

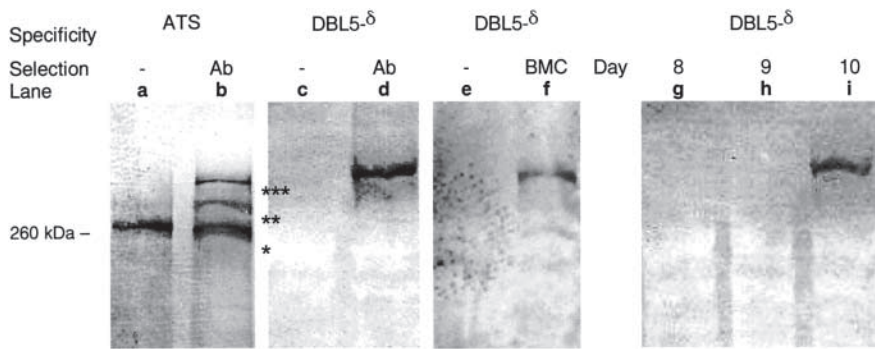
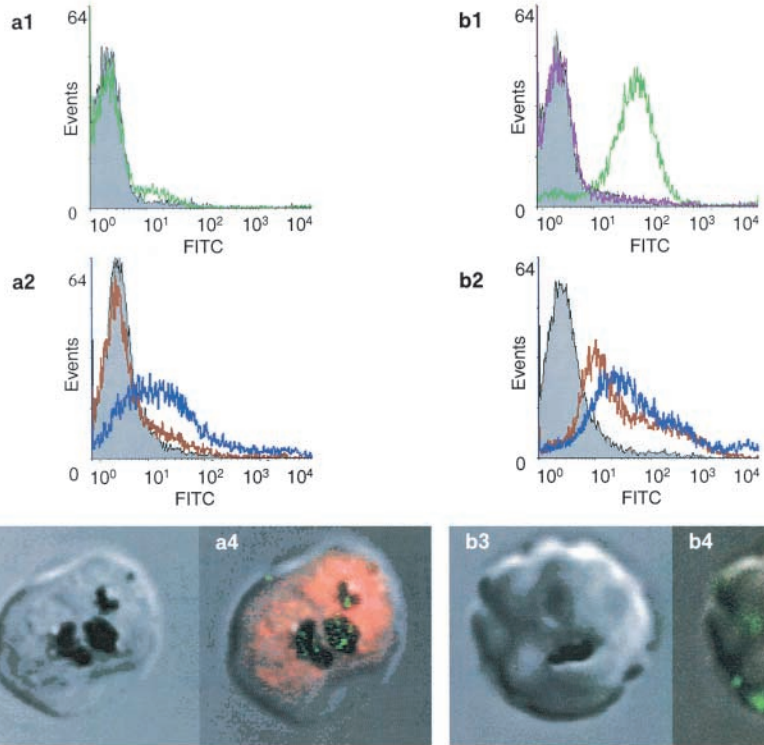


Figure 4. Expression of PfEMP1 in unselected (a, c, and e), antibody-selected (b and d), and TrHBMEC-selected (f) trophozoite/schizont stage 3D7. Samples were run in 5% SDS-PAGE gels, blotted, and probed with antiserum against a conserved part of the intracellular acidic segment ATS (a and b) or against DBL5- δ of *PF*D1235*w*/*MAL*7P1.1 (c–i). Antibody-selected parasites expressed *PF*D1235*w*/*MAL*7P1.1 (***) and a PfEMP1 with mol wt corresponding to the predicted mol wt of MAL6P1.316 (**; b). Both selected and unselected parasites expressed a PfEMP1 with mol wt corresponding to the predicted mol wt of PF08_0107 (*; a and b). TrHBMEC-selected 3D7 expressed *PF*D1235*w*/*MAL*7P1.1 (f). Western blots of SDS-extracted RBCs infected by NF54 obtained from a Dutch volunteer (reference 27) on days 8, 9, and 10 of an experimental, mosquito-induced infection (g–i) probed with the DBL5- δ antibody revealed expression of *PF*D1235*w*/*MAL*7P1.1 on day 10 (i). Surface expression of antigens in *P. falciparum* 3D7 before (a1–a4) and after (b1–b4) antibody selection. Murine plasma antiserum against recombinant DBL5- δ of *PF*D1235*w*/*MAL*7P1.1 protein reacted with the surface of most antibody-selected 3D7-infected RBCs in FACS® (b1, green line). This reaction was abolished by trypsin treatment (b1, purple line). In contrast, the antiserum only reacted with a minority of unselected 3D7 (a1, green line). Reactivity with pre-vaccination mouse serum is shown for comparison (shaded histograms). VSA-specific IgG reactivity in plasma from a semi-immune African child (a2 and b2, red line) and a clinically immune African adult (a2



and b2, blue line) confirmed the VSA_{UM} phenotype of unselected 3D7 and the VSA_{SM} phenotype of antibody-selected 3D7. Localization of the *PF*D1235*w*/*MAL*7P1.1 protein using confocal microscopy and murine plasma anti-DBL5- δ antibodies (a3, a4, b3, and b4). Ethidium bromide staining of DNA in the nuclei is red/orange and staining of *PF*D1235*w*/*MAL*7P1.1 protein using FITC-labeled antibodies is green. Pre-vaccination mouse plasma did not stain infected RBCs (not depicted).

rum against the conserved intracellular ATS *var* domain showed a single band with an estimated mol wt of 260 kD (Fig. 4 a). This indicates that most of these parasites expressed a PfEMP1 with a four-domain structure, in agreement with our absolute quantification data (Table I), showing a dominant Group C gene (*PF*08_0107) transcript encoding a PfEMP1 molecule of this size. Antibody-selected 3D7 expressed a 260 kD PfEMP1, but also expressed additional high mol wt PfEMP1 bands (Fig. 4 b). Again, the data agree with the absolute quantification results showing that the two most highly transcribed *var* genes in antibody-selected 3D7 were *MAL*6P1.316 and *PF*D1235*w*/*MAL*7P1.1, encoding proteins with predicted mol wts of ~330 and 400 kD, respectively. Furthermore, an antiserum against DBL5- δ of *PF*D1235*w*/*MAL*7P1.1 revealed a high

mol wt band in SM1-, SM2-, and SM3-selected 3D7 (Fig. 4 d and unpublished data), corresponding to the top band in the ATS-probed blot (Fig. 4 b). TrHBMEC-selected parasites showed a three-band pattern identical to that of the antibody-selected 3D7 when probed with anti-ATS serum (unpublished data) and expressed the 400-kD gene product of *PF*D1235*w*/*MAL*7P1.1 (Fig. 4 f). This indicates that *PF*D1235*w*/*MAL*7P1.1 is translated into protein. *PF*D1235*w*/*MAL*7P1.1-specific Antibodies Recognize Antigens on the Surface of VSA_{SM}-expressing 3D7. Antisera against DBL5- δ of *PF*D1235*w*/*MAL*7P1.1 were tested on infected RBCs by flow cytometry and confocal microscopy. Only a small minority of unselected 3D7 reacted with the murine DBL5- δ antibodies (Fig. 4 a1), although these infected RBCs did express VSA detectable by flow

cytometry and human plasma (Fig. 4 a2). By contrast, these antibodies recognized a large proportion of RBCs infected with antibody-selected 3D7, a reactivity that was abrogated by prior trypsin treatment of the infected RBCs (Fig. 4 b1). Confocal microscopy using the DBL5- δ antiserum showed a distinct punctate pattern on the surface of intact RBCs infected by antibody-selected 3D7 (Fig. 4 b4), but not by unselected 3D7 (Fig. 4 a4).

These results indicate that the product of *PFD1235w/MAL7P1.1* is expressed on the surface of antibody-selected 3D7 and is responsible for the *VSA_{SM}* phenotype.

VSA_{SM}-type Plasma IgG Recognition of PFD1235w/MAL7P1.1 Protein. If *PFD1235w/MAL7P1.1* mediates a *VSA_{SM}* phenotype, a high proportion of children in malaria-endemic areas would be expected to have acquired antibodies to this protein. To test this hypothesis, we mea-

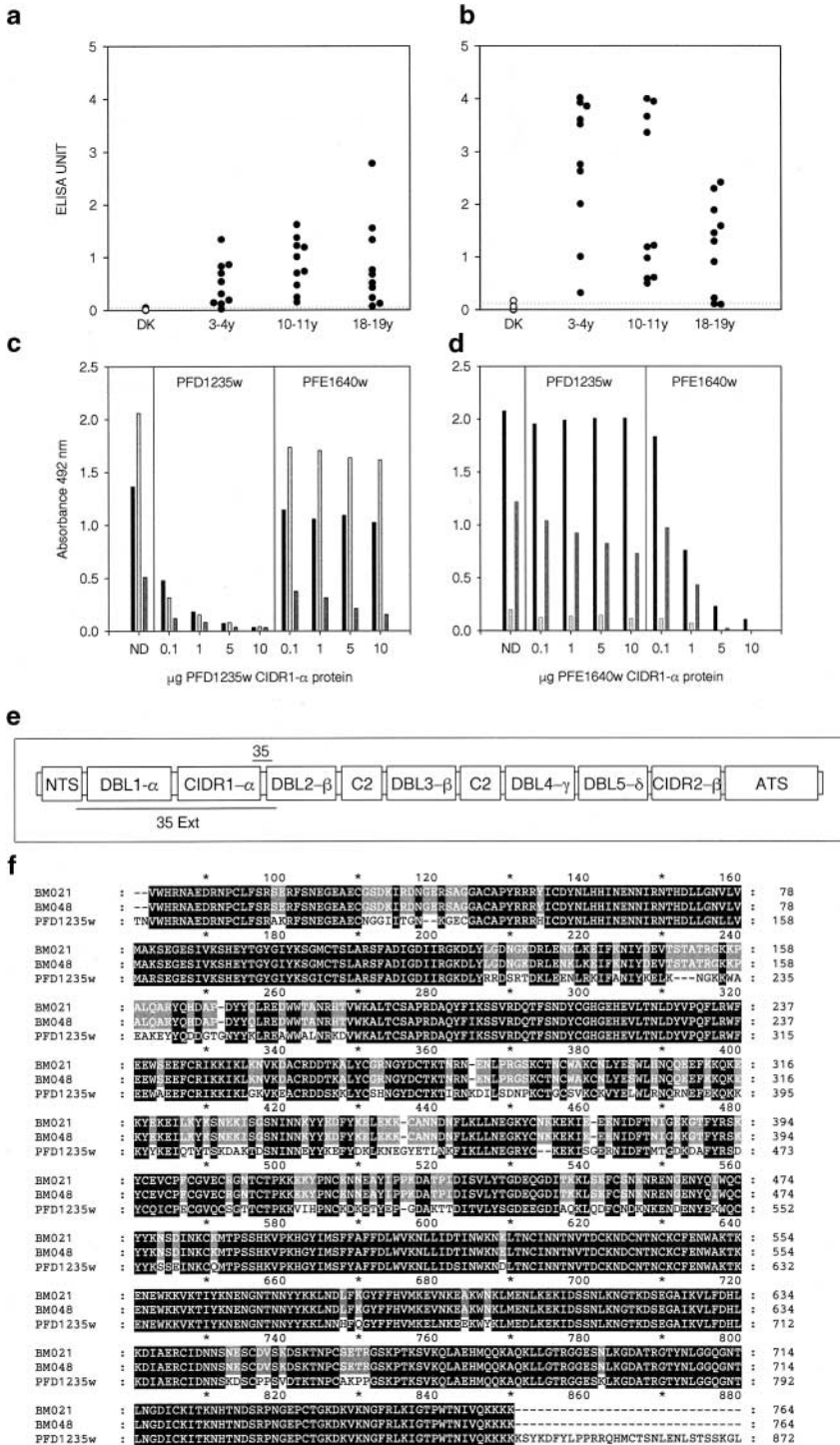


Figure 5. Plasma antibody levels to recombinant DBL5- δ (a) and CIDR1- α (b) domains of the *PFD1235w/MAL7P1.1* protein in Tanzanian children and adults, and in Danish donors without *P. falciparum* exposure (DK). For competition ELISA experiments, plates were coated with recombinant CIDR1- α domains of the proteins encoded by *PFD1235w/MAL7P1.1* (c) and PFE1640w/*var1* (d), and ELISA reactivity was measured in three different plasma samples, which had been preincubated with increasing concentrations of homologous or heterologous fusion protein as indicated in the top part of each panel. Nondepleted (ND) plasma was included for comparison. Sequence similarity between *PFD1235w/MAL7P1.1* and other genes of the *var4* family were identified in field isolates. Schematic domain structure of *PFD1235w/MAL7P1.1* (*var4*; e). The line indicates the position of the 2.3-kb fragment (35 Ext) sequenced and numbered line (35) position of the primer pair used for real-time PCR (refer to Figs. 1 and 2, and Table I). Sequence alignment of *PFD1235w/MAL7P1.1*, BM021, and BM048 (f). The sequences shown correspond to line 35 Ext in (e). These sequence data are available from GenBank/EMBL/DBJ under accession nos. AY584238 and AY584239.

sured IgG plasma levels against *PFD1235w/MAL7P1.1* by ELISA in asymptomatic individuals living under high malaria transmission intensity in Tanzania. Most children and adults had comparable levels of IgG directed against DBL5- δ (Fig. 5 a) and CIDR1- α (Fig. 5 b). We used competition ELISA with CIDR1- α domains from *PFD1235w/MAL7P1.1* and *var1* (*PFE1640w*) to test the antigen specificity of the antibody recognition. Preincubation of the plasma with homologous recombinant proteins caused a dose-dependent reduction in OD₄₉₂ values, whereas the heterologous proteins did not (Fig. 5, c and d).

These results, and the demonstration that our DBL5- δ antiserum did not cross-react with *MAL6P1.316* and *PF08_0107* in Western blotting, suggest that the antibody reactivity to recombinant *PFD1235w/MAL7P1.1* protein was the result of exposure to parasites expressing PfEMP1 resembling that encoded by *PFD1235w/MAL7P1.1*, rather than being due to a broad cross-reactivity between different CIDR or DBL domains.

A Family of PFD1235w/MAL7P1.1 var Genes. Although the exon I of many *var* genes differ markedly between different parasite isolates, conserved subfamilies such as *var1*, *var2*, and *var3* have been described (17, 28, 36, 37). Therefore, we speculated that *PFD1235w/MAL7P1.1* homologues might exist in isolates other than 3D7. Using *PFD1235w/MAL7P1.1*-specific primers, we amplified a 2,292-bp fragment from genomic DNA of two Ghanaian *P. falciparum* field isolates. These parasites carried genes showing 72% identity to *PFD1235w/MAL7P1.1* over a stretch of 764 amino acids corresponding to the 3' end of NTS into the 5' end of DBL2- β (Fig. 5, e and f). We call this new gene subfamily *var4*.

PFD1235w/MAL7P1.1 Is Up-regulated and Translated into PfEMP1 Early in an NF54-induced Malaria Infection of a Non-immune Patient. To examine the in vivo relevance of the above findings, we studied parasites rescued on days 8, 9, and 10 from a Dutch volunteer receiving a mosquito-transmitted *P. falciparum* NF54 infection on day 0. NF54 was isolated from a Dutch malaria patient (26) and is isogenic with 3D7 cloned from it (28). Western blots of day 10 parasites probed with antiserum against the DBL5- δ domain of *PFD1235w/MAL7P1.1* revealed a high mol wt band (Fig. 4 i), corresponding to that observed in antibody- and TrHBMEC-selected 3D7 (Fig. 4, d and f). This band was not detected in blots of parasites obtained on days 8 and 9 (Figs. 4, g and h). *PFD1235w/MAL7P1.1* appeared to be among the least transcribed *var* genes on day 8, ranking 55 out of 60 among day 8 ring-stage Ct values, but ranked 9 out of 60 in parasites rescued on day 10. Furthermore, the trophozoite copy numbers of *PFD1235w/MAL7P1.1* relative to *seryl-tRNA synthetase* indicated an 8.4-fold increase in *PFD1235w/MAL7P1.1* mRNA between days 8 and 10.

Taken together, these data show that *PFD1235w/MAL7P1.1* is translated into a functional protein in vivo, and indicate that this protein might be associated with fast-growing parasites in nonimmune patients.

Discussion

Asexual *P. falciparum* parasitemia can be controlled by antibodies acquired after natural exposure to the parasites (38, 39). Several studies point to parasite-encoded, clonal VSAs on the infected RBC surface as the main target of these antibodies, as acquisition of protection from *P. falciparum* malaria corresponds to a gradual accumulation of IgG with a broad range of VSA specificities (40–44). The available evidence suggests that VSA-specific immune responses steadily restrict the repertoire of VSA that are compatible with parasite survival, and drive VSA expression away from VSA_{SM} toward VSA_{UM} (2, 3, 44, 45). Therefore, VSA expression is nonrandom as it depends on the degree of immunity in the infected host. In fact, several studies have demonstrated that parasites causing severe *P. falciparum* malaria in young children with little protective immunity tend to express VSA_{SM} that are serologically distinct from VSA_{UM} expressed by most parasites causing uncomplicated malaria and subclinical infection in older, and more immune, individuals (2, 3). Importantly, VSA_{SM} appear to be serologically less diverse than VSA_{UM} (4), which is consistent with the observation that immunity to severe malaria is acquired more rapidly than to uncomplicated disease and subclinical infection (46). This suggests that it might be possible to develop disease-ameliorating vaccines that protect against mortality and severe morbidity by accelerating acquisition of immunity to VSA_{SM}-expressing parasites and forcing VSA expression away from VSA_{SM}. This study was undertaken with that goal in mind.

The best characterized VSA is PfEMP1, which mediates infected RBC adhesion to a number of host receptors and is encoded by the *var* gene family (6–8). Attempts to identify VSA_{SM}-type PfEMP1 have been hampered by the intraclonal and interclonal variability of the *var* genes, the concomitant transcription of several *var* genes during natural infections, and the problem of primer bias (13–15). However, the entire genome of the *P. falciparum* clone 3D7 is now available (16), making systematic quantitative analysis of *var* gene transcription by real-time quantitative PCR and absolute quantification possible for that clone. In vitro cultures of 3D7 generally express VSAs that are serologically similar to the VSA_{UM} expressed by parasites causing uncomplicated disease, and bind strongly to CD36. However, upon antibody selection (19) or selection for adhesion to CD36⁻ TrHBMEC, 3D7 expresses VSA_{SM}-like erythrocyte surface antigens.

We found that acquisition of the VSA_{SM}-type phenotype in 3D7 was accompanied by a shift in *var* gene transcription from dominant transcription of the Group C *var* gene *PF08_0107* to dominant transcription of the Group A genes *PFD1235w/MAL7P1.1* and *MAL6P1.314*, and the Group B/A gene *MAL6P1.316*. Northern blot data documented that the transcripts were full-length and the expressed products of several high mol wt PfEMP1 species, including *PFD1235w/MAL7P1.1*, could be detected in antibody-selected 3D7 by Western blotting. Finally, we could detect a VSA on the surface of antibody-selected

3D7-infected RBCs using a murine antiserum raised against recombinant DBL5- δ domain of the *PFD1235w/MAL7P1.1 var*. This finding directly points to the protein product of the Group A *var* gene *PFD1235w/MAL7P1.1* as a major VSA_{SM}-type antigen.

Thus, it appears that Group A and B/A genes such as *PFD1235w/MAL7P1.1*, *MAL6P1.314*, and *MAL6P1.316* encode VSA_{SM}-like proteins, and that these proteins are involved in the acquisition of the VSA_{SM} phenotype after antibody selection or selection for adhesion to TrHBMEC. The fact that almost identical results were obtained using several plasma pools from children living in East and West Africa indicate that PfEMP1 similar to those encoded by *PFD1235w/MAL7P1.1*, *MAL6P1.314*, and *MAL6P1.316* exist in *P. falciparum* parasites transmitted across Africa. This observation and the findings that Group A is structurally the most homogeneous of the *var* gene groups (17, 18) are consistent with the hypothesis that SM-type VSAs are antigenically relatively conserved (4, 19). Other characteristics of the Group A *var* genes further support their proposed relation to VSA_{SM}. Thus, most Group A *var* genes encode high mol wt PfEMP1 molecules that do not bind CD36 (18, 47). Expression of high mol wt PfEMP1 has been linked to severe malaria (48), and selection of 3D7 for expression of VSA_{SM} causes decreased adhesiveness to CD36 (19). Furthermore, Group A genes encode a characteristic head structure that includes a DBL1- α homology block lacking one to two cysteines, a feature that has been linked to severe malaria (15). The Group B/A gene *MAL6P1.316* is unusually Group A-like in that it has a 5' UTR region with low similarity to Group B genes as well as a complex domain structure and a DBL1-CIDR1 head structure that is characteristic of Group A rather than Group B *var* genes (18).

Immuno-epidemiological evidence suggests that the gradual acquisition of protective immunity steadily drives VSA expression from VSA_{SM} toward VSA_{UM}. The proposed relationship between VSA_{SM} expression and *PFD1235w/MAL7P1.1* implies that the product of this gene should be recognized at high levels and early in life. Consistent with this hypothesis, we found that Tanzanian young children and adolescents had comparable levels of IgG reacting with recombinant *PFD1235w/MAL7P1.1* DBL5- δ and CIDR1- α domains.

We have proposed that the dominance of parasites expressing VSA_{SM}-type antigens among nonimmune patients is related to their higher growth rate in such individuals and that the shift toward VSA_{UM}-type antigens occurs as this strong selective advantage of VSA_{SM}-expressing parasites gradually disappears as VSA_{SM}-specific immunity is acquired (5). In line with this hypothesis, we found that transcription of *PFD1235w/MAL7P1.1* was much higher on day 10 of infection compared with earlier time points in a Dutch non-immune volunteer infected by *P. falciparum*, indicating that a larger proportion of the parasites obtained on day 10 transcribed *PFD1235w/MAL7P1.1* than earlier in the infection.

Any malaria vaccine candidate must meet the fundamental requirement that it should be a well-defined and not too

polymorphic antigen. In general, *var* genes are believed to vary considerably between different parasite isolates, although exceptions have been recently described (17, 28, 36, 37). We found genes with a high similarity to *PFD1235w/MAL7P1.1* in parasites from patients and suggest that this new conserved *var* gene subfamily should be named *var4*.

In conclusion, we have identified 3D7 *var* genes conferring a VSA_{SM} phenotype associated with severe and life-threatening malaria. Furthermore, we have shown that one of the most highly transcribed Group A *var* genes (*PFD1235w/MAL7P1.1*) is conserved and translated into protein, which can be detected on the surface of infected erythrocytes carrying the VSA_{SM} phenotype. Finally, we have shown that recombinant domains of *PFD1235w/MAL7P1.1* are well recognized by antibodies in plasma from *P. falciparum*-exposed children, and that transcription of this Group A gene dominates among 3D7 parasites early in the infection of a nonimmune individual. Our data suggest that it is possible to develop a disease-ameliorating vaccine against severe *P. falciparum* malaria that is based on proteins encoded by Group A *var* genes such as *PFD1235w/MAL7P1.1*.

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