Jensen, ATR; Magistrado, P; Sharp, S; Joergensen, L; Lavstsen, T; Chiucciuni, A; Salanti, A; Vestergaard, LS; Lusingu, JP; Hermsen, R; Sauerwein, R; Christensen, J; Nielsen, MA; Hviid, L; Sutherland, C; Staalsoe, T; Theander, TG (2004) Plasmodium falciparum associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes. The Journal of experimental medicine, 199 (9). p. 1179. ISSN 0022-1007 DOI: https://doi.org/10.1084/jem.20040274

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DOI: 10.1084/jem.20040274

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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\textsubscript{SM}) that differs from VSA associated with uncomplicated malaria and asymptomatic infection (VSA\textsubscript{UM}). We compared var gene transcription in unselected P. falciparum clone 3D7 expressing VSA\textsubscript{UM} to in vitro–selected sublines expressing VSA\textsubscript{SM} to identify PfEMP1 responsible for the VSA\textsubscript{SM} phenotype. Expression of VSA\textsubscript{SM} was accompanied by up-regulation of Group A var genes. The most prominently up-regulated Group A gene (PFD1235w/MAL7P1.1) was translated into a protein expressed on the infected RBC surface. The proteins encoded by Group A var genes, such as PFD1235w/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\textsubscript{SM}; 2–4). VSA\textsubscript{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\textsubscript{SM}–specific immunity is acquired, this advantage gradually gets smaller. Survival rates of parasites expressing less virulent and more diverse VSAs (VSA\textsubscript{UM}) eventually surpass those of VSA\textsubscript{SM}–expressing parasites, causing VSA\textsubscript{UM}–expressing parasites to dominate infections in semi-immune individuals (5). This scenario makes it theoretically possible to protect
nonimmune children against severe and complicated malaria by accelerating acquisition of VSA-specific immunity through vaccination.

*P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is the best characterized VSA. 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 VSA s 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 *ghop 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 manufacturer 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-μl 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 ΔΔCT 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, PFEL0030c, PFEL1950u, PFEL005u, PF13_003, PFB1053s, PFD1235w/MAL7P1.1, PFE1640w, PFH1830, PF08_0107, MAL6P1.314, MAL6P1.316, PFD0995w, PFA0015c, and seryl-tRNA synthetase) used for absolute quantification were PCR amplified, ligated into the pHCR2.1 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 × 10⁶ to 5 × 10⁷ 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 *s* (standard deviation of the mean). RT-PCR was performed as described using 1 μg total RNA in a total volume of 40 μl, of which 0.5 μl 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 GTATGCGCATCTGTAAGGAA and antisense
AGAAGTCTTTGTATGACT. 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 of PFD1235w/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- and DBL5- of PFD1235w/MAL7P1.1 were subcloned into the pGEX-4T1 vector (Amersham Biosciences) by PCR using the following domain-specific oligonucleotide primers: CIDR1-Fw: 5′-CGGAATTC GACGCTAAAACTG-ATAGTA-3′; CIDR1-Rv: 5′-ATAAGAATGCGGCCGC ACATATATCGGATGCGATTCAACG-3′; DBL5-Fw: 5′-CGGAATTC GACGCTAAAACTG-ATAGTA-3′; DBL5-Rv: 5′-ATAAGAATGCGGCCGC ACATATATCGGATGCGATTCAACG-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′-CGGAATTC AAAACAAAATCATCAGTAG-3′; ATS-Rv: 5′-ATAAGAATGCGGCCGC GTTGATTACCACTTAATGTG-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 of PFD1235w/

Table I. Copy Number of Selected var Genes and seryl-tRNA synthetase Transcripts in Unselected and VSAUM-expressing 3D7 and 3D7 after Antibody Selection for VSAUM Expression

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Primera</th>
<th>Gene</th>
<th>Unselected</th>
<th>Antibody-selected</th>
<th>Fold change</th>
<th>Unselected</th>
<th>Antibody-selected</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PF11_0521</td>
<td>8</td>
<td>426</td>
<td>7,119</td>
<td>16.7</td>
<td>12,570</td>
<td>84,341</td>
<td>6.7</td>
</tr>
<tr>
<td>A</td>
<td>PF13_0003</td>
<td>20</td>
<td>1,797</td>
<td>6,129</td>
<td>3.4</td>
<td>77,934</td>
<td>179,746</td>
<td>2.3</td>
</tr>
<tr>
<td>A</td>
<td>PFD1235w/MAL7P1.1</td>
<td>35</td>
<td>2,931</td>
<td>29,941</td>
<td>10.2</td>
<td>101,417</td>
<td>1,152,758</td>
<td>11.4</td>
</tr>
<tr>
<td>B/A</td>
<td>MAL6P1.314</td>
<td>67</td>
<td>3,145</td>
<td>19,356</td>
<td>6.2</td>
<td>87,130</td>
<td>767,747</td>
<td>8.8</td>
</tr>
<tr>
<td>A</td>
<td>PFA0015c</td>
<td>97</td>
<td>77,890</td>
<td>1,333</td>
<td>58.4</td>
<td>155,151</td>
<td>18,419</td>
<td>8.4</td>
</tr>
<tr>
<td>B/A</td>
<td>MAL6P1.316</td>
<td>94</td>
<td>13,742</td>
<td>36,637</td>
<td>2.7</td>
<td>247,156</td>
<td>1,057,056</td>
<td>4.3</td>
</tr>
<tr>
<td>B</td>
<td>PFL0005w</td>
<td>18</td>
<td>15</td>
<td>1,106</td>
<td>73.7</td>
<td>928</td>
<td>13,769</td>
<td>14.8</td>
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<tr>
<td>B</td>
<td>PFB1055c</td>
<td>21</td>
<td>64</td>
<td>2,533</td>
<td>39.6</td>
<td>5,272</td>
<td>26,070</td>
<td>4.9</td>
</tr>
<tr>
<td>B</td>
<td>PFI1830c</td>
<td>58</td>
<td>79,111</td>
<td>59,860</td>
<td>1.3</td>
<td>18,600</td>
<td>13,124</td>
<td>1.4</td>
</tr>
<tr>
<td>B/C</td>
<td>PFL1950w</td>
<td>12</td>
<td>226</td>
<td>524</td>
<td>2.3</td>
<td>13,236</td>
<td>19,053</td>
<td>1.4</td>
</tr>
<tr>
<td>C</td>
<td>PF08_0107</td>
<td>66</td>
<td>82,211</td>
<td>5,590</td>
<td>16.3</td>
<td>993,679</td>
<td>58,130</td>
<td>17.1</td>
</tr>
<tr>
<td>C</td>
<td>PFD0995c</td>
<td>95</td>
<td>23,415</td>
<td>491</td>
<td>47.7</td>
<td>567,205</td>
<td>9,148</td>
<td>62.1</td>
</tr>
</tbody>
</table>

| var2    | PFL0030c | 10   | 907       | 12,562            | 14          | 12,905     | 127,631            | 9.9         |
| var1    | PFE1640w | 37   | 12,279    | 14,239            | 1.2         | 8,224      | 9,111              | 1.1         |
| seryl-tRNA synthetase | 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: DBL-5Fw: 5′-CGG-AATTCAAGTCTCAATGCCGATG-3′; DBL5-5Rv: 5′-TCTACAAATGTCTGGCACA-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 Bakpak6 Baculovirus DNA (BD Biosciences) into insect SF9 cells. Recombinant DBL5-Δ protein was purified from culture supernatants on Co_2 metal chelate agarose columns and eluted with 25 mM Hepes-KOH, pH 7.6, 0.5 mM MgCl_2, 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 μg 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 × 10^6 MACS-purified, ethidium bromide–labeled infected RBCs were incubated for 1 h in 20 μl murine sera or for 30 min in 5 μl human sera depleted for anti–human RBC antibodies. For immunostaining with murine sera, infected RBCs were sequentially exposed to 100 μl 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 μl 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 μg/ml TPCK-treated trypsin (Amersham Biosciences) in PBS for 10 min at 37°C. The reaction was stopped by adding 1/10 vol of 100 mM EDTA in PBS and centrifuging at 500 g for 10 min. The pellet was used for immunostaining.

**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 VSAAsm-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 ± 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 μg/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_M-type Antigens.** Parasites associated with severe malaria express VSA(s) (VSA_M) that are recognized at high levels by plasma IgG of most semi-immune children, whereas parasites causing uncomplicated malaria infection tend to express VSA(s) (VSA_U) that are recognized less often and at lower levels (2, 3). Among unselected 3D7 parasites, most express VSA_U with a small minority expressing VSA_M (19). However, the dominating VSA phenotype changes to VSA_M 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_U-expressing parasites (25).

We compared var gene transcription in unselected and VSA_M-expressing selected 3D7 using primer sets targeting

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**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, PFD0995c, 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).

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, PFD0995c, and PFA0015c). We also determined copy numbers of three Group B genes (PFL0005w, PFB1055c, and PFI1830c), 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 (PF11830k 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-
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 (PF08_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 MAL6P1.316 and PFD1235w/MAL7P1.1, encoding proteins with predicted mol wts of ~330 and 400 kD, respectively. Furthermore, an antiserum against DBL5-δ of PFD1235w/MAL7P1.1 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 PFD1235w/MAL7P1.1 (Fig. 4 f). This indicates that PFD1235w/MAL7P1.1 is translated into protein.

PFD1235w/MAL7P1.1-specific Antibodies Recognize Antigens on the Surface of VSA SM-expressing 3D7. Antisera against DBL5-δ of PFD1235w/MAL7P1.1 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 confocal microscopy. Pre-vaccination mouse plasma antiserum against DBL5-δ/H9254 of PFD1235w/MAL7P1.1 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 PFD1235w/MAL7P1.1 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 PFD1235w/MAL7P1.1 protein using FITC-labeled antibodies is green. Pre-vaccination mouse plasma did not stain infected RBCs (not depicted).
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\textsubscript{SM} phenotype.

**VSA\textsubscript{SM} Type Plasma IgG Recognition of PFD1235w/MAL7P1.1 Protein.** If PFD1235w/MAL7P1.1 mediates a VSA\textsubscript{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/DDBJ 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 ODₙₙ 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-Δ (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 revealed a high mol wt band (Fig. 4 i), corresponding to 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 serp1-rRNA 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_U (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_U 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_U (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_U 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 anti-body 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 indicates 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 VSA 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).

Immunophysiological 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.

The PlasmoDB database (http://www.plasmodb.org) was an essential resource for this work and we are indebted to all those who generated the PlasmoDB data. Lotte Bram, Susanne Pedersen, and Kirsten Pihl are thanked for excellent technical assistance, and Lauren Rattray for the TrHBMEC selection of 3D7.

The study received financial support from the Danish International Development Assistance (grants nos. 104.Dan.8L/306 and 91168), The Danish Council for Health Science Research (grant no. 63686), and the Commission of the European Communities (grant no. QLK2-CT-2002-01197, EUROMALVAC). A. Salanti, J.P. Lusingu, and P. Magistrado are Gates Ph.D. scholars supported by the Gates Malaria Partnership.

Submitted: 10 February 2004
Accepted: 18 March 2004

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