Whole-Transcriptome Analysis of *Plasmodium falciparum* Field Isolates: Identification of New Pathogenicity Factors

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**Background.** Severe malaria and one of its most important pathogenic processes, cerebral malaria, involves the sequestration of parasitized red blood cells (pRBCs) in brain postcapillary veinules. Although the pathogenic mechanisms underlying malaria remain poorly characterized, it has been established that adhesion of pRBCs to endothelial cells (ECs) can result in cell apoptosis, which in turn may lead to disruption of the blood-brain barrier. The nature of the parasite molecules involved in the pathogenesis of severe malaria remains elusive.

**Methods.** Whole-transcriptome profiling of nonapoptogenic versus apoptogenic parasite field isolates obtained from Gabonese children was performed with pan-genomic *Plasmodium falciparum* DNA microarrays; radiolabeled instead of fluorescent cDNAs were used to improve the sensitivity of signal detection.

**Results.** Our methods allowed the identification of 59 genes putatively associated with the induction of EC apoptosis. Silencing of *Plasmodium* gene expression with specific double-stranded RNA was performed on 8 selected genes; 5 of these, named “*Plasmodium* apoptosis–linked pathogenicity factors” (PALPFs), were found to be linked to parasite apoptogenicity. Of these genes, 2 might act via parasite cytoadherence.

**Conclusion.** This is the first attempt to identify genes involved in parasite pathogenic mechanisms against human ECs. The finding of PALPFs illuminates perspectives for novel therapeutic strategies against cerebral complications of malaria.

Severe malaria and one of its most important pathogenic processes, cerebral malaria (CM), are responsible for a high proportion of malaria-related child mortality. In humans, previous pathology studies have shown a definitive association between sequestration of parasitized red blood cells (pRBCs) within the brain and CM [1–5], implying that pRBC adhesion to cerebral endothelial cells (ECs) may be one factor underlying the dysfunction of the blood-brain barrier (BBB). Recently, using ex vivo cocultures of human lung ECs (HLECs) and *Plasmodium falciparum*, we demonstrated that pRBC adhesion induces apoptosis of human ECs [6]. Others have shown a direct cytotoxic effect of pRBCs on brain ECs [7], leading to a decrease in the integrity of BBB monolayers [8]. Therefore, we and others have hypothesized that, in areas of the brain where pRBCs are bound in large numbers, pRBC-mediated microvessel apoptosis might contribute to the amplification of the complex pathological

Received 20 March 2007; accepted 30 May 2007; electronically published 25 October 2007.

Potential conflicts of interest: none reported.

Financial support: Microarray analysis was performed using the Pitié-Salpêtrière genomic core facility (P3S), which is supported by Institut National de la Santé et de la Recherche Médicale U511, Région Île-de-France, Fondation de Recherche HMR-Aventis, and Université Paris 6. The Centre International de Recherches Médicales de Franceville is funded by Total Gabon, the Gabonese Government, and the French Ministry of Foreign Affairs. *Plasmodium* pan-genomic microarrays were financed by grants from the Délégation Générale pour l’Armement (DGA-vers/n°22120/DSP/SREAF), the Programme PAL/H11001/Fond National pour la Science, the Institut Pasteur, and the Programme Génopole. A.S. was supported by MENRT, the Fondation de la Recherche Médicale, and the Fondation des Treilles.

The Journal of Infectious Diseases 2007; 196:1603–12

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0022-1899/2007/19611-0006$15.00

DOI: 10.1086/522012


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process involved in the functional alteration of the BBB [9, 10].

The aim of the present study was to investigate, using field isolates, parasite factors involved in EC apoptosis. Toward this goal, we used approaches involving the analysis of whole transcripts by DNA microarrays. Field isolates have rarely been explored because of the scarcity of parasite RNA. To overcome this difficulty, various methods have been developed, such as RNA amplification coupled with Affymetrix GeneChip arrays [11, 12]. An alternate approach to increase the sensitivity of signal detection without amplification bias has been to use radiolabeled cDNA [13, 14]. Thus, we performed a whole-transcriptome profiling of apoptogenic and nonapoptogenic plasmodial field isolates obtained from Gabonese children.

**PATIENTS, MATERIALS, AND METHODS**

**Patients.** Children with acute malaria were selected for participation by a medical doctor, and informed consent was obtained from their parents or tutors. Ethical clearance was granted by the Gabonese Ministry of Health. All procedures were performed in accordance with the Helsinki Declaration.

**Parasites.** pRBC samples were cultivated to reach schizont stage after white blood cell removal by use of a Ficoll gradient, as described elsewhere [15]. Schizonts were then enriched by use of plasmagel [16] and adjusted to 5% hematocrit for all further assays. Parasitemia obtained before incubation with HLECs is shown in table I. Parasitemia was used for functional characterization after ex vivo maturation and plasmagel enrichment.

**Culture of HLECs.** HLECs were isolated and cultivated as described elsewhere [17].

**Cytoadherence assay.** pRBCs were incubated with HLECs following protocols described elsewhere [18].

**Apoptosis assay.** Cell apoptosis was determined in HLECs incubated for 4 h with pRBCs and compared with that in HLECs incubated with unparasitized red blood cells (RBCs), according to a protocol developed for strain 3D7 [6]. Apoptogenicity was estimated by the specific enrichment of nucleosomes released into the cytoplasm of HLECs, using the following ratio: optical density of HLECs incubated with pRBCs/optical density of HLECs incubated with RBCs.

**Microarray protocol.** Cultures of field isolates were incubated with HLECs for 4 h and washed to remove noncytoadherent pRBCs. Cytoadherent pRBCs and HLECs were processed together for RNA extraction. To minimize the difference between the *Plasmodium* isolates and to obtain sufficient quantity of total RNA, transcripts from either the apoptogenic or the nonapoptogenic isolates were pooled equimolarly. The microarray protocol was performed with 2 μg of *Plasmodium* total RNA substantially contaminated with human RNA, following a protocol optimized from Gisso et al. [13]. Briefly, an RNA mix containing total RNA, random primers, and poly-dT was first denatured at 65°C for 5 min, after which the temperature was ramped to 22°C (0.1°C/s) and the mix was kept at room temperature. The reaction mix, which included bovine serum albumin (50 ng/μl) and 3 μCi/μL [3H]-dATP (Amersham), was preincubated at 42°C and added to the RNA mix. SuperScript II (Invitrogen) was added for reverse transcription at the initiation and after 40 min of incubation at 42°C. Radiolabeled cDNAs were hybridized to a DNA microarray containing 12,037 individual 70mer oligonucleotides (representing most of the *P. falciparum* open reading frames [19]) in the presence of 20 μg of human total RNA and 2 μg of human genomic DNA. Because it was impossible to normalize the gene expression levels with another labeled cDNA, we performed 8 technical replicates for each mixed RNA sample and normalized the data according to the median value of the total intensities of all spots. Acquisition of the arrays was done as described elsewhere [20], and statistical discrimination of genes expressed differentially between the apoptogenic and the nonapoptogenic field isolates was performed using SAM software (version 2.0; http://www-stat.stanford.edu/~tibs/SAM/) [21], with a false-discovery rate <1%. Bioinformatics tools such as PlasmoDB [22, 23], BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), SMART (http://smart.embl-heidelberg.de/), SignalP (version 3.0) [24], and TMAP (http://humpback.bii.a-star.edu.sg/cgi-bin/emboss/emboss.pl?_action=input&_app=tmap) were used for further gene annotation.

**Multiplex quantitative polymerase chain reaction (qPCR).** Multiplex TaqMan PCRs were performed using the MX4000 multiplex qPCR system (Stratagene), with the primers and probes shown in (table 2). None of these cross-reacted with human DNA (data not shown). PCRs were performed in quadruplicate, and additional reactions were performed without reverse transcriptase (RT) to verify the absence of DNA contami-

### Table 1. Characteristics of ex vivo isolates used for microarray analysis and comparison with the apoptogenic strain 3D7.

<table>
<thead>
<tr>
<th>Isolate characteristic</th>
<th>Nonapoptogenic samples</th>
<th>Apoptogenic samples</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NA1</td>
<td>NA2</td>
</tr>
<tr>
<td>Cytoadherence, pRBCs/mm²</td>
<td>998</td>
<td>592</td>
</tr>
<tr>
<td>HLEC intracytoplasmic nucleosome enrichment</td>
<td>1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Parasitemia, %a</td>
<td>3</td>
<td>30</td>
</tr>
</tbody>
</table>

**NOTE.** HLEC, human lung endothelial cell; pRBCs, parasitized red blood cells.

a Parasitemia was used for functional characterization after ex vivo maturation and plasmagel enrichment.
performed twice, with 3 technical replicates.

brane. [32P]-UTP-radiolabeled RNA probes were synthesized per lane were size-fractionated and blotted on a nylon mem-

hematocrit with trophozoite or schizont 3D7 cultures were adjusted to 1%–2% rate dsRNA, using a T7 transcription system.

quences (table 2). Purified PCR products were then used to gen-

gene. Genomic DNA and primers containing T7 promoter se-

method [25].

RNA 18S cDNA level, and relative gene expression was expressed

to obtain mature-stage parasites. No statistical association was

unpublished data). After coculture of 45 field isolates with

Apoptogenic characterization of 8 P. falciparum isolates and comparison to 3D7 strain. The pRBC samples obtained from

nation. Gene expression data were normalized to the parasite RNA 18S cDNA level, and relative gene expression was expressed

via the log2 of the ratio expression, determined using the 2-ΔΔCt method [25].

dsRNA preparation. DNA fragments (350–550 bp) from

each selected gene were amplified by PCR, using 3D7 P. falcipa-

rum genomic DNA and primers containing T7 promoter se-

quences (table 2). Purified PCR products were then used to gen-

drRNA, using a T7 transcription system.

Apoptosis inhibition assay. Synchronized young-

trophozoite or schizont 3D7 cultures were adjusted to 1%–2%

hematocrit with ~30% pRBCs and incubated for 3 and 16 h,

respectively, with dsRNA (100 µg/mL) in triplicate until they

reached the mature schizont stage. pRBCs, dsRNA-treated

pRBCs, RBCs, and irrelevant dsRNA–treated pRBCs were sub-

jected to apoptosis assays in triplicate.

Parasite growth assay. [3H]-hypoxanthine uptake was

evaluated as described elsewhere [26], using parasite cultures

treated with 100 µg/mL dsRNA or sterile water. Assays were

performed twice, with 3 technical replicates.

Northern blot analysis. Twenty micrograms of total RNA per lane were size-fractionated and blotted on a nylon mem-

brane. [32P]-UTP-radiolabeled RNA probes were synthesized using PCR products and amplified using primers containing a

T7 promoter (table 2). Signal intensities were quantified using a Fujifilm FLA-2000 phosphorimager, with subsequent image

analysis provided by AIDA software. Signal intensities were nor-

malized to the corresponding signal of P. falciparum GAPDH gene.

Statistical analysis. Data obtained after functional assays were analyzed for statistical significance using 1-way analysis of

variance followed by the Tukey multiple-comparisons test.

RESULTS

Apoptogenic characterization of 8 P. falciparum isolates and comparison to 3D7 strain. The pRBC samples obtained from

infected children almost exclusively contained early-stage para-
sites and were cultivated during the first cycle of parasite growth to obtain mature-stage parasites. No statistical association was

observed between parasitemia and either cytoadherence or para-

unpublished data). After coculture of 45 field isolates with HLECs, only 8 samples provided sufficient yields of total RNA for microarray hybridization. The apoptogenic properties of

these 8 field isolates were quantified by the specific enrichment of nucleosomes released into the cytoplasm of HLECs incubated

with pRBCs and were compared with those of HLECs and RBCs only (table 1). Three isolates with ratios of intracytoplasmic nu-

cleosome enrichment <2 compared with RBC controls were considered to be nonapoptogenic (isolates NA1 to NA3). In con-

trast, 5 isolates with a nucleosome enrichment >3 were consid-

ered to be apoptogenic (isolates A1 to A5). The apoptosis levels induced by the apoptogenic 3D7 laboratory strain [6] was also
determined using a schizont culture presenting 35% parasitemia and 1%–2% hematocrit. Under these conditions, HLEC nucle-

osome release was significantly increased (~3.3-fold), com-

pared with that for HLECs incubated with RBCs.

Transcriptome profiling of P. falciparum field isolates. By

whole-transcriptome analysis, 59 genes were identified as being

up-regulated (>2-fold magnitude) in pooled apoptogenic ver-

sus nonapoptogenic isolates, whereas expression of 27 other
genes was reduced (<2-fold magnitude) (table 3). Among the 59

up-regulated genes, 39 encode proteins with 1 or several pre-
dicted transmembrane domains with, for some of them, signal

sequences or PEXEL motifs [31]. After additional bioinformat-

ics analyses, it was possible to annotate and classify 41 genes in 11 families according to the putative functions of their encoded proteins (table 3). These included genes encoding proteins in-

olved in parasite pathogenicity, metabolic processes, nucleic

metabolism, the ubiquitin-signaling pathway, proteolytic and

signalization pathways, transportation, translocation machin-

ery, the cytoskeleton, stress response, and protein synthesis.

To validate the accuracy of the microarray data, the expres-
sion levels of some selected up-regulated genes within the pooled

apoptogenic versus nonapoptogenic isolates were analyzed by

TaMan RT-qPCR (table 4). Only 25 genes (10 without any pre-
dictable function and 15 corresponding to annotated proteins

representative of the aforementioned families) of 59 could be

analyzed, because of the scarce RNA quantities obtained from

Plasmodium field strains. Gene expression was higher in the

pooled apoptogenic than in the pooled nonapoptogenic group,

thus validating the microarray data (table 4).

In addition, RT-qPCR was performed for the same 25 selected

genes on every isolate (i.e., the 5 apoptogenic and the 3 non-
apoptogenic isolates described in table 1). Transcript expression

levels were normalized to the mean expression levels in non-
apoptogenic isolates (table 3). Comparison of individual expres-
sion profiles revealed that, except for isolate A2, the expression

level of 20 of the 25 genes (figure 1) was increased in apoptogenic

Table 2. Primers used for TaqMan reverse-

Table 2. Primers used for TaqMan reverse-

transcriptase quantitative polymerase chain re-

action (RT-qPCR) experiments and long dsRNA

preparation.

Table 3. Microarray results coupled to Taq-
Man reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) analysis.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Microarray, A/NA</th>
<th>RT-qPCR A/NA ± SD</th>
<th>3D7/NA ± SD</th>
<th>PlasmoDB description</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothetical transmembrane proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFI0130ca/b</td>
<td>6.2</td>
<td>8.7 ± 0.7</td>
<td>35.6 ± 6.4</td>
<td>Intergenic sequence</td>
<td>Contains a signal peptide, a PEXEL/VTS motif, and a PHISTb domains [27]</td>
</tr>
<tr>
<td>PFD0875ca/b</td>
<td>3.0</td>
<td>4.2 ± 0.8</td>
<td>8.4 ± 0.8</td>
<td>Hypothetical protein</td>
<td>Shows slight similarity with SAPS/SIT4 phosphatase-associated protein family</td>
</tr>
<tr>
<td>PFO7_0008ab</td>
<td>2.5</td>
<td>2.7 ± 0.5</td>
<td>3.1 ± 0.9</td>
<td>Hypothetical protein</td>
<td>Identified in pRBC membrane by mass spectroscopy [28]</td>
</tr>
<tr>
<td>PFI1610ca</td>
<td>10.1</td>
<td>2.6 ± 0.5</td>
<td>1.7 ± 0.1</td>
<td>Hypothetical protein</td>
<td>Contains a SGC domain identified by Pfam</td>
</tr>
<tr>
<td>PFI0_0226a</td>
<td>7.9</td>
<td>3.8 ± 0.2</td>
<td>6.7 ± 1.9</td>
<td>Hypothetical protein, conserved</td>
<td></td>
</tr>
<tr>
<td>PFI0440wa</td>
<td>5.4</td>
<td>22.4 ± 3.7</td>
<td>4.6 ± 0.5</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>PFO7_0032ab</td>
<td>4.9</td>
<td>4.2 ± 0.2</td>
<td>20.2 ± 2.1</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>MAL13P1.62a</td>
<td>8.3</td>
<td>6.2 ± 0.4</td>
<td>4.9 ± 0.8</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>PF13_0032ab</td>
<td>2.3</td>
<td>2.0 ± 0.3</td>
<td>2.4 ± 0.3</td>
<td>Hypothetical protein</td>
<td>Identified in infected red blood cell membrane by mass spectroscopy [28]</td>
</tr>
<tr>
<td>Metabolic process</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFD0670ca</td>
<td>2.4</td>
<td>2.5 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>Hypothetical protein</td>
<td>Contains a lysine decarboxylase domain</td>
</tr>
<tr>
<td>PFD0670ca</td>
<td>2.4</td>
<td>2.5 ± 0.2</td>
<td>4.1 ± 0.4</td>
<td>Hypothetical protein</td>
<td>Contains a lysine decarboxylase domain identified in pRBC membrane by mass spectroscopy [28]</td>
</tr>
<tr>
<td>Nucleic metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFI0_0369a</td>
<td>12.1</td>
<td>5.5 ± 0.5</td>
<td>6.2 ± 1.9</td>
<td>Helicase, putative</td>
<td></td>
</tr>
<tr>
<td>MAL13P1.134a</td>
<td>18.8</td>
<td>7.7 ± 1.2</td>
<td>1.2 ± 0.4</td>
<td>Helicase, putative</td>
<td></td>
</tr>
<tr>
<td>PF13_0080a</td>
<td>3.6</td>
<td>2.2 ± 0.1</td>
<td>4.1 ± 1.1</td>
<td>Hypothetical protein</td>
<td>Identified as telomerase reverse transcriptase [29]</td>
</tr>
<tr>
<td>Ubiquitin-signaling pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF10_0081</td>
<td>17.3</td>
<td>6.6 ± 1.3</td>
<td>6.2 ± 1.9</td>
<td>26S proteasome regulatory subunit 4, putative</td>
<td></td>
</tr>
<tr>
<td>PF10_0330a</td>
<td>15.6</td>
<td>3.4 ± 0.3</td>
<td>2.4 ± 0.7</td>
<td>Ubiquitin-conjugating enzyme, putative</td>
<td></td>
</tr>
<tr>
<td>PFB0260wa</td>
<td>4.5</td>
<td>4.2 ± 0.7</td>
<td>2.1 ± 0.1</td>
<td>Proteasome 26S regulatory subunit, putative</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>PFB0345ca</td>
<td>2.7</td>
<td>4.3 ± 0.1</td>
<td>1.4 ± 0.2</td>
<td>Cysteine protease, putative</td>
<td></td>
</tr>
<tr>
<td>Signalization pathway</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFL2365wa</td>
<td>3.4</td>
<td>4.7 ± 0.3</td>
<td>13.7 ± 0.5</td>
<td>Hypothetical protein</td>
<td>Contains a protein phosphatase 2C domain identified by Pfam</td>
</tr>
<tr>
<td>PF11_0239a</td>
<td>3.1</td>
<td>9 ± 1.3</td>
<td>2.5 ± 0.1</td>
<td>Hypothetical protein</td>
<td>Contains both a Ser/Thr protein kinases catalytic domain and a Ca2+-binding protein domain</td>
</tr>
<tr>
<td>PF11_0139a</td>
<td>2.6</td>
<td>3.7 ± 0.5</td>
<td>5.9 ± 0.4</td>
<td>Protein tyrosine phosphatase, putative</td>
<td>Contains a dual-specificity (Ser/Thr and Tyr) phosphatases domain</td>
</tr>
<tr>
<td>Transporter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAL13P1.206ab</td>
<td>4.2</td>
<td>3.6 ± 0.7</td>
<td>3.9 ± 0.9</td>
<td>Phosphate transporter, putative</td>
<td></td>
</tr>
<tr>
<td>Translocation machinery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PF13_0118ab</td>
<td>11.4</td>
<td>4.4 ± 0.7</td>
<td>16.9 ± 3.1</td>
<td>Hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFC0185w</td>
<td>3.1</td>
<td>2.8 ± 0.2</td>
<td>1.9 ± 0.7</td>
<td>Hypothetical protein</td>
<td>Identified as type 1 signal peptidase [30]</td>
</tr>
<tr>
<td>PFE0255w</td>
<td>3.0</td>
<td>3.8 ± 1.0</td>
<td>12.4 ± 2.1</td>
<td>Actin-related protein, putative</td>
<td>Shows homology with articulin membrane skeletal protein family</td>
</tr>
</tbody>
</table>

**NOTE.** Quantitative RT-PCR (RT-qPCR) A/NA ± SD are the gene expression ratios in the pooled apoptogenic (A) field isolate group versus nonapoptogenic (NA) group ± SD. RT-qPCR 3D7/NA ± SD are the gene expression ratios in 3D7 strain versus NA field-isolate group ± SD. Additional in silico analysis results are indicated in the comment column. pRBC, parasitized red blood cell.

* Gene with transmembrane domain(s) predicted by TMAP.

* Indicates genes targeted by use of long dsRNA.
isolates relative to that in nonapoptogenic isolates, depending on their apoptogenicity (table 1). In contrast, the moderately apoptogenic A2 isolate exhibited marked expression levels of 10 genes (indicated by asterisks in figure 1), with no clear correlation with its apoptogenicity.

RT-qPCR analysis of the 25 aforementioned genes was also performed on the apoptogenic 3D7 laboratory strain [6] incubated with HLECs. Twenty-one of 25 genes were identified as being significantly up-regulated (>2-fold magnitude), both in 3D7 parasites and in the apoptogenic isolates, compared with the nonapoptogenic isolates (table 4), allowing us to use these genes and strain 3D7 in our functional assays.

Identification of pathogenicity factors associated with the induction of apoptosis in human ECs. Functional assays were performed with the apoptogenic strain 3D7 [6], using long dsRNA to knock down specific gene expression [32, 33]. Eight genes selected from those up-regulated in both the apoptogenic isolates and strain 3D7 were analyzed (footnote b in table 4); PFE0255w was annotated as actin-related protein, whereas the other genes encoded proteins with predicted transmembrane domain(s) and, for several of them, a signal sequence/anchor. Five of these genes encoded hypothetical proteins without any recognized function; PF07_0008 and PF13_0032 proteins have been previously identified in pRBC membrane fractions [28]. The last 2 proteins, PF13_0118 and MAL13P1.206, were annotated as type 1 signal peptidase [30] and phosphate transporter, respectively.

Highly synchronized 3D7 schizont and young-trophozoite parasite cultures were incubated for 3 and 16 h, respectively, with the relevant long dsRNA until the mature schizont stage was reached. Besides the untreated parasite control, 2 irrelevant dsRNAs were used as negative controls. One was derived from the *Drosophila nautilus* gene (AN: M68897), and the second one was derived from the *P. falciparum* thrombospondin-related adhesive protein (*PfTRAP*) gene, which is known to not be expressed in *Plasmodium* blood stages [34].

Three types of functional assays were performed in triplicate using the same parasite culture. First, the ability of the dsRNA to specifically knock down gene expression was evaluated. Figure 2A shows the data obtained using RT-qPCR for each *Plasmodium* transcript after a 3- or 16-h treatment with the specific dsRNA. Except for PF13_0032 dsRNA, the transcript level decreased significantly, from 25% to 73% (*P* < .05), when parasites were incubated with the relevant dsRNA, with a slightly more potent silencing effect when schizont cultures were treated for 3 h. PFI0130c dsRNA, which leads to a 38% decrease after a...
Figure 2. Gene expression analysis of *Plasmodium* 3D7 cultures treated with long dsRNA. Eight genes overexpressed in apoptogenic field isolates were analyzed by reverse-transcriptase quantitative polymerase chain reaction after a 3-h (left) or 16-h (right) dsRNA treatment in strain 3D7 culture (A). Black bars, untreated culture; dark gray bars, irrelevant *Drosophila nautilis* dsRNA–treated culture; light gray bars, irrelevant *PfTRAP* dsRNA–treated culture; white bars, relevant dsRNA–treated culture. The steady-state levels of 2 transcripts, PFD0875c (left) and PF07_0032 (right), were analyzed by Northern blotting after a 3-h incubation with dsRNA (B). The decreases in transcript levels were estimated after normalization to the *P. falciparum* GAPDH gene and are indicated under the blots. Expression of all putative pathogenesis factors was also evaluated after dsRNA invalidation of the same 2 transcripts (C), using the same experimental approaches and illustration as those presented in panel A. For all experiments, levels of gene expression in dsRNA-treated cultures were evaluated against the average expression levels (assumed to be 1) of both untreated and irrelevant dsRNA–treated cultures. Gene expression decrease is expressed as a percentage of average expression levels. Parasite growth was evaluated in 3 independent experiments using parasitized red blood cell (pRBC) counting of Giemsa-stained blood smears (D, left). Late-ring and late-trophozoite 3D7 cultures were respectively incubated for 3 h (gray bars) or 16 h (black plots) with dsRNA to reach schizont stage. At right is the evaluation of parasite growth in the presence of 100 μg/mL dsRNA by a [3H]-hypoxanthine–uptake assay, following the protocol of Desjardins et al. [26]. Experiments were repeated twice, with 2 replicates. *P < .05, compared with irrelevant dsRNA–treated parasites (1-way analysis of variance followed by the Tukey multiple-comparisons test).
3-h treatment, was no longer able to knock down gene expression after 16 h. The gene expression silencing effects were corroborated by Northern blotting. Only PFD0875c and PF07_0032 transcripts gave a sufficient signal after hybridization. For these 2 genes, a 40% and 80% decrease in cognate transcripts, respectively, was observed in relevant dsRNA-treated cultures compared with control cultures (figure 2B). In addition, treatment with the 2 aforementioned dsRNAs did not modify expression of untargeted mRNA, in contrast to the cognate transcripts (figure 2C). Similar experiments were performed for all dsRNAs used, and no unspecific effect was observed (data not shown).

Second, the ability of parasites to induce apoptosis was then evaluated in dsRNA-treated cultures showing significant gene silencing. As shown in figure 3A, the addition of PF13_0118 and PF07_0008 dsRNA did not significantly affect parasite apoptogenic effects (<12% inhibition), as was also observed for both irrelevant dsRNAs. In contrast, 5 dsRNAs (i.e., PFI0130c, PFD0875c, MAL13P1.206, PFE0255w, and PF07_0032) were able to inhibit apoptosis (ranging from 25% to 40%; P < .01) after a 3-h (black bars) or 16-h (gray bars) incubation period. As for gene silencing, inhibition of apoptosis was not observed with PFI0130c dsRNA after 16 h of incubation (figure 3A).

Third, parasite viability was evaluated using Giemsa-stained blood smears and [3H]-hypoxanthine–uptake assays in culture treated with the 5 dsRNAs showing a significant inhibition of apoptosis. None had any effect on parasite asexual growth (figure 2D). Moreover, treatment had no effect on parasite morphology (data not shown).

Silencing of the PFI0130c, PFD0875c, MAL13P1.206, PFE0255w, and PF07_0032 genes was coupled with significant apoptosis reduction without affecting parasite growth. On the basis of our results, we named these genes “Plasmodium apoptosis–linked pathogenicity factors” (PALPF-1 to -5, respectively).

**PALPF-2 and PALPF-3 are associated with parasite cytoadherence.** To determine whether the pathogenicity factors identified above could be linked to parasite cytoadherence, the cytoadherence status of highly synchronized 3D7 parasites treated for 3 or 16 h with relevant or irrelevant dsRNA was evaluated and compared with that of untreated parasites. Of the 5 dsRNAs assayed, PALPF-2 (PFD0875c) and PALPF-3 (MAL13P1.206) dsRNA induced a significant decrease in cytoadherence (~65% and 45%, respectively; P < .01) after both 3 h (black bars) and 16 h (gray bars) of treatment, whereas irrelevant dsRNA did not affect parasite cytoadherence (figure 3B). These results suggest that PALPF-2 and PALPF-3 might be involved in Plasmodium cytoadherence.

**DISCUSSION**

The nature of the parasite molecules involved in the pathogenesis of severe malaria remains elusive. To avoid possible artifacts linked to in vitro culture of parasites, we used clinical isolates with a minimum of ex vivo manipulation. Consequently, we obtained only scarce amounts of cytoadherent Plasmodium total RNA substantially contaminated with human RNA (data not shown). To overcome these difficulties, 2 approaches were used: (1) an optimized [3H]-dATP radiolabeling protocol and (2) addition of human total RNA and genomic DNA to the hybridization assays to reduce the cross-hybridization signals to negligible levels. In previous studies with thematic microarrays, the use of a radiolabeling protocol with [3H]-dATP and [35S]-dATP required substantial amounts of RNA [13, 14]. Applications of this sensitive approach appeared to be difficult with high-density mi-
croarrays. Indeed, signal diffusion and saturation of high energy \[^{35}\text{S}\]-dATP signals can bias the algorithmic filtration used to discriminate the 2-radiolabeled cDNA (data not shown). In contrast, with a single-labeling system based on low-energy \[^{3}\text{H}\]-dATP, no algorithmic filtration was required. No signal diffusion and low background were observed when \[^{3}\text{H}\]-dATP cDNAs were hybridized.

Applying this approach, we were able to identify 59 genes that were up-regulated in apoptogenic field isolates compared with nonapoptogenic isolates; these putative \textit{Plasmodium} apoptogenic genes might play an important role in CM. Gene down-regulation was also observed and deserves to be analyzed as well; however, lower expression of some of these genes might in part be the result of genetic polymorphism. In the same way, 7 up-regulated genes (i.e., \textit{var} and \textit{rifin}) that are known to be polymorphic, expressed at the surface of pRBCs, and linked to parasite pathogenesis were not explored in this study. Indeed, hybridization of field strain cDNA containing hypervariable genes might be unreliable because of sequence variation between isolates. We also took into account the possible polymorphism between field isolates and strain 3D7 to validate the microrarray data, using RT-qPCR primers and probes located externally to the microarray’s oligonucleotides. The explanation for the over-representation of transmembrane proteins remains at present speculative, whereas one can assume that some of the genes identified might encode surface proteins.

For each individual isolate, we determined the expression levels of 25 genes identified as being up-regulated in pooled apoptogenic isolates. Despite the diversity of the field-isolate transcriptome, these genes were found for the most part to be overexpressed in every apoptogenic field isolate, with the expression level for 20 of the 25 genes globally correlated to the apoptogenicity of the isolates except for A2. This observation strengthens the proposal of the participation of these genes in pathogenicity. The moderately apoptogenic A2 isolate, obtained from a child with symptoms of CM, exhibited marked expression levels for 10 genes, with no clear correlation with its apoptogenicity. The potential link between these 10 up-regulated genes and CM will have to be investigated using a larger sample size.

To unravel and screen rapidly the function of these 59 genes, we decided, as a first approach, to knock down a set of selected genes via dsRNA silencing, even though this technique remains controversial because of the lack of identified protein involved in RNA interference [35]. The characterization of such a mechanism, whether involving not-yet-described classic dsRNA interference machinery or an antisense effect as shown by Noonpakee et al. [36], is beyond the scope of the present study, all the more because most of the \textit{Plasmodium} genes remain unannotated. Moreover, the existence of antisense transcripts in the parasite has been extensively demonstrated [37, 38]. It has been suggested that antisense transcripts could be a key element in gene-transcription regulation [39]. These studies might imply that the machinery required to process these antisense transcripts and the resulting dsRNA is present in \textit{P. falciparum}. On the basis of studies showing a specific and genuine gene silencing with the use of long dsRNA [32, 33], we took advantage of this technique to perform the screening of 8 genes. To identify the parasite proteins involved in triggering the apoptogenic signal activated by parasite-EC contact, we chose to focus essentially on genes encoding proteins with predicted transmembrane domain(s). In return, all genes potentially implicated in parasite growth—such as PF13_0080, a telomerase RT gene for which a knockout parasite is not attainable [29]—were excluded to avoid an apoptogenic phenotype linked with parasite viability. Inter-

![Figure 4](image-url)
estingly, gene silencing appeared to be more potent when parasites were treated for 3 h than for 16 h with the corresponding dsRNA (figure 2A). Moreover, under our experimental conditions, gene silencing was also less effective when ring parasites were treated, until they reached the schizont stage (data not shown). These results, similar to those described recently [40], suggest that RNA silencing is rapidly reached in *Plasmodium* species.

A significant reduction in apoptosis was observed after dsRNA silencing of the 5 PALPFs (figure 3). Furthermore, inhibition of cytoadherence was induced only when the levels of PALPF-2 and -4 were decreased (figure 3B). It is possible to predict additional features for these 5 parasite proteins by means of in silico tools (figure 4): (1) PALPF-1 was characterized by the presence of a signal peptide, a PEXEL/VTS motif, and a PHISTb domain [27, 31, 41]; (2) PALPF-2 showed slight similarity with the SAPS/SIT4 phosphatase-associated protein family; (3) PALPF-3 was previously reported to be a protein belonging to the Na⁺-dependent inorganic phosphate transporter (Pi:Na⁺ symporter) family [42] and is known to be located on the eu-karyotic cell surface [43]; (4) PALPF-4 was predicted to be a putative actin-related protein, with 3 domains highly homologous to eukaryotic actin; and (5) PALPF-5 was annotated as cg8, a protein belonging to a 36-kb segment of *Plasmodium* chromosome 7 initially predicted to be associated with chloroquine resistance [44].

A recent study showed that skeleton-binding protein (SBP)-1, a parasite transmembrane protein that is located in Maurer’s clefts and that lacks a signal peptide and a PEXEL/VTS trafficking motif, is essential for transport of *P. falciparum* erythrocyte membrane protein (PfEMP1) to the infected RBC membrane [45]. With a SBP-1 knockout parasite line, presentation of PfEMP1 on the infected RBC membrane was abolished, and the parasites lost their cytoadherent phenotype [45]. Hence, on the basis of the results shown in the present study, we propose that, among the genes identified here, several—such as PALPF-2 and -3, two potential transmembrane proteins lacking (as does SBP-1) a PEXEL/VTS trafficking motif—might be involved in parasite apopptogenicity via cytoadherence. Furthermore, other pathogenicity factors might encode transmembrane proteins expressed on the pRBC surface, independently of parasite cytoadherence. Finally, as shown with PALPF-3, factors that are not exclusively transmembrane proteins also appear to be involved in the triggering of parasite apopptogenicity. These factors could be secreted by the pRBC as the trypanosome apoptotic factor involved in human brain EC apoptosis [46, 47].

We report here the first attempt to unravel the parasite pathogenic mechanisms related to human EC apoptosis, via an analysis of whole-genome-transcriptome profiling of *P. falciparum* field isolates. Additional functional analyses are presently under way (using gene knockouts and antibodies) to ascertain the role played by these proteins. Progress in the fight against severe malaria relies on a better understanding of the complex host-pathogen interaction that leads to multisystem disorder, cerebral manifestation, and death.

**Acknowledgments**

We express our gratitude to Jean François Franetich and Dr. Olivier Silvie for critical reading of the manuscript and Dr. Philippe Ravassard for his help.

**References**


