Proteome Analysis of *Plasmodium falciparum* Extracellular Secretory Antigens at Asexual Blood Stages Reveals a Cohort of Proteins with Possible Roles in Immune Modulation and Signaling*§

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The highly co-evolved relationship of parasites and their hosts appears to include modulation of host immune signals, although the molecular mechanisms involved in the host-parasite interplay remain poorly understood. Characterization of these key genes and their cognate proteins related to the host-parasite interplay should lead to a better understanding of this intriguing biological phenomenon. The malaria agent *Plasmodium falciparum* is predicted to export a cohort of several hundred proteins to remodel the host erythrocyte. However, proteins actively exported by the asexual intracellular parasite beyond the host red blood cell membrane (before merozoite egress) have been poorly investigated so far. Here we used two complementary methodologies, two-dimensional gel electrophoresis/MS and LC-MS/MS, to examine the extracellular secreted antigens at asexual blood stages of *P. falciparum*. We identified 27 novel antigens exported by *P. falciparum* in the culture medium of which some showed clustering with highly polymorphic genes on chromosomes, suggesting that they may encode putative antigenic determinants of the parasite. Immunolocalization of four novel secreted proteins confirmed their export beyond the infected red blood cell membrane. Of these, preliminary functional characterization of two novel (Sel1 repeat-containing) parasite proteins, PISEL1 and PISEL2 revealed that they down-regulate expression of cell surface Notch signaling molecules in host cells. Also a novel protein kinase (PIEK) and a novel protein phosphatase (PIEP) were found to, respectively, phosphorylate/dephosphorylate parasite-specific proteins in the extracellular culture supernatant. Our study thus sheds new light on malaria parasite extracellular secreted antigens of which some may be essential for parasite development and could constitute promising new drug targets. *Molecular & Cellular Proteomics* 8:2102–2118, 2009.

*Plasmodium falciparum* is a wide spread protozoan parasite responsible for over a million deaths annually mainly among children in sub-Saharan Africa (1). Like other apicomplexan parasites such as *Leishmania*, *Trypanosoma*, and *Toxoplasma*, *Plasmodia* depend on a series of intricate and highly evolved adaptations that enable them to evade destruction by the host immune responses. These protozoan parasites have provided some of the best leads in elucidating the mechanisms to circumvent innate immunity and adaptive humoral and cellular immunity (2). Ingenious strategies to escape innate defenses include subversion of attack by humoral effector mechanisms such as complement lysis and lysis by other serum components (3), remodeling of phagosomal compartments in which they reside (4), modulation of host cell signaling pathways (5), and modification of the antigen-presenting and immunoregulatory functions of dendritic cells, which provide a crucial link with the adaptive immune response (6). Malaria parasites also predominantly use antigenic diversity and clonal antigenic variation to evade adaptive immunity of the host (7). Surface-associated and secreted parasite proteins are major players in host-parasite cross-talk and are advantageously used by the parasite to counter the host immune system. Proteins secreted by a wide range of parasitic pathogens into the host microenvironment result in symptomatic infections. For example, the excretory-secretory (ES)† products of the parasitic fluke *Fasciola hepatica* are key players in host-parasite interactions (8). Among the apicomplexans, proteomics analyses of rhoptry organelles of Toxo-
Extracellular Secreted Antigens of Asexual P. falciparum

**Experimental Procedures**

**Parasite Culture, Metabolic Labeling of Parasites, and Preparation of P. falciparum ESAs—**P. falciparum (3D7 line) parasites were synchronized with sorbitol and cultured at 10−15% parasitemia according to standard procedures. The culture was washed thrice in incomplete RPMI 1640 medium to remove intracellular proteins released by rupture of infected RBCs. The parasites were then grown from late trophozoites to schizonts in serum-free RPMI 1640 medium at 37 °C for 8 h. Simultaneously specific metabolic labeling of parasite proteins was achieved by incubating ~1 × 10^10 parasite-infected erythrocytes with 0.5mCi/ml 35S-Express Promix (methionine + cysteine) in methionine-, cysteine-free modified RPMI 1640 medium supplemented with 2 mM l-glutamine and 1% serum at 37 °C until the parasites matured to schizonts. Supernatants from 100 ml of both the labeled and unlabeled parasite cultures were prepared after pelleting the iRBCs, first at 1500 rpm for 5 min and then at 3300 × g for 15 min to remove any debris. These were desalted and concentrated using a 3-kDa-cutoff filter (Centricon YM3, Millipore), supplemented with protease inhibitors, and further concentrated in a SpeedVac.

**Two-dimensional Gel Electrophoresis—**Isoelectric focusing, for both unlabeled and labeled samples, was carried out on wide range immobilized pH gradients (7-cm-long pH 3–10 Immobiline DryStrip gels, Amersham Biosciences) using the Protean IEF Cell System (Amersham Biosciences). Both the supernatant samples were precipitated by a standard aceton precipitate protocol. For each, ~150 μg (quantified using BCA protein assay reagent (Pierce)) of aceton precipitate was redisolved in 140 μl of rehydration/sample buffer (7 M urea, 2 M thiourea, 100 mM DTT, 0.4% ampholytes). Samples were loaded by passive rehydration for 12 h and focused at a current limit of 50 μA/IPG strip using a step voltage gradient (500 V for 6 h stepped up to 5000 V maximum for 8 h; 16,000 V-h total) at 20 °C. The second dimension was carried out on 10% polyacrylamide gels (10 cm × 10 cm × 1.5 mm) using a Hoefer SE 600 system at 20-μA constant current and at 20 °C until the dye front reached the bottom of the gel. Both the gels were vacuum-dried, and the protein spots on the 35S-labeled gel were visualized by 2–4-day exposure on and subsequent development of Kodak BioMax MR films. The gel with unlabeled sample was silver-stained (using protocols compatible with mass spectrometry) and analyzed by Image Master 2D Platinum Software Version 5.0 (Amersham Biosciences). Silver-stained spots, which correlated well with corresponding spots in the autoradiogram of the duplicate gel, were excised and subjected to in-gel trypsin digestion for further analysis.

**Trypsin Digestion, Liquid Chromatography, and Mass Spectrometry Analyses—**The excised gel slices were digested with trypsin as follows. The gel pieces were washed thrice in deionized water, twice in 0.1 M NH₄HCO₃, and twice in 50% acetonitrile. The gel pieces were shrunk using 100% acetonitrile, and proteins were reduced by addition of 0.1 M dithiothreitol followed by an incubation step at 56 °C for 45 min. The washing procedure described above was repeated, and proteins were alkylated by adding 55 mM iodoacetamide and incubating for 30 min at room temperature in the dark. After an additional wash and shrinkage, 10 ng/μl trypsin in 0.1 M NH₄HCO₃ sufficient to cover the gel pieces was added followed by incubation on ice for 20 min. When the gel pieces were completely rehydrated, any excess trypsin solution was removed and replaced by 0.1 M NH₄HCO₃, and samples were incubated overnight at 37 °C. The digestion was stopped by adding 10 μl of glacial acetic acid, and the supernatant containing the tryptic peptides was harvested. An extraction step was carried out to recover the peptides from the gel slices by adding 50% acetonitrile and incubating at room temperature for 30 min. The supernatant was harvested again and pooled. The pooled peptide extracts were desalted, dried down to ~10 μl, and subjected to MALDI-MS/MS analysis (Bruker Ultraflex MALDI-TOF/TOF mass spectrometer). In-solution trypsin digestion of serum-free concentrated sample was similarly done, and peptide extracts were subjected to LC-MS/MS analysis as follows.

The LC-MS/MS analysis was done on an Agilent 1100 series 2D NanoLC system coupled to an ion trap mass spectrometer (LC/MSD Trap XCT, Agilent Technologies) for automated MS/MS analysis of individually isolated peptide ions (Chemstation 01 software). The complex peptide mixture was loaded on strong cation exchange columns (3.5–μm Zorbax), and peptide fractions were collected by an injected salt step gradient of increasing salt concentration. Fractions were concentrated using enrichment columns (Zorbax C₁₈, 5 μm), then allowed to enter C₁₈ reverse phase columns (3.5-μm Zorbax), and developed with a linear 70-min gradient from 0 to 100% Solvent B where Solvent A was 0.1% (v/v) aqueous formic acid and Solvent B was 0.1% aqueous formic acid, 60% (v/v) ACN with a flow rate of 0.8 μl/min. The peptides were then subjected to on-line MS/MS using an ion trap mass spectrometer.

**Database Search and in Silico Analysis of Protein Sequences—**The mass spectrometry data files from individual LC-MS/MS experiments were merged and then searched against the mass spectrometry protein sequence database (MSDB) 20060831 (P. falciparum, 10,980...
sequences, September 20, 2007) using Mascot version 2.1 (Matrix Sciences Ltd., London, UK). The search parameters were as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (Cys); variable modifications, oxidation (Met); mass values, monoisotopic; peptide mass tolerance, ±2 Da; fragment mass tolerance, ±1 Da; maximum missed cleavages, 1. Peptide identities were chosen to be correct with Mascot scores more than 33 (p < 0.05), and all peptides with scores below this were discarded. Only fully tryptic peptide matches were allowed. At least two unique peptides were required to identify a protein (Table I). Supplemental Fig. 1 provides the MS/MS spectra of the only protein that was identified on the basis of a single peptide. The spectra were manually validated for the following criteria: 1) several consecutive y-ions although absence of y-ions after proline and glycine, 2) none or few unassigned fragment ions, and 3) a charge state of the precursor ion and fragment ions that is in accordance with basic amino acids in the assigned peptide sequence.

Proteins identified by the two proteomics approaches were subjected to in silico analyses using algorithms for detecting the signal peptide (SignalP 3.0), transmembrane domains (transmembrane helices by hidden Markov model), and PEXEL motif. The transcription levels of cognate genes were derived from the DeRisi and co-worker (15) transcriptome data, orthologs were from the Plasmodium database (16), and genome-wide polymorphism data were obtained from a study by Mu et al. (17). Protein function was assigned based on published results. Novel proteins were assigned putative function (a) if strong homology over long stretches of the protein (based on BLASTP with default settings against NCBI nonredundant database) was found to a protein of known function, (b) by protein sequence analysis in Pfam and NCBI conserved domain database, and (c) by database annotation or literature references.

Expression of Recombinant Proteins and Production of Specific Antibodies—Different fragments of the selected proteins were amplified from P. falciparum 3D7 genomic DNA using the corresponding primers (supplemental Fig. 2 and Table II). The resulting PCR products were ligated into either pQE30 (Qiagen) or pMALc (New England Biolabs) vectors and expressed in Escherichia coli M15/BL21 cells as His$_6$-tagged (PISEL1, PISEL2, and PIEF (extracellular phosphatase)) and maltose-binding protein (MBP) (PIEK (extracellular kinase)) fusion proteins, respectively. These recombinant proteins were then purified on nickel-nitrilotriacetic acid matrix (Qiagen) and amylose resin (New England Biolabs), respectively (supplemental Fig. 3). Recombinant PfSEL1 and PfSEL2 protein fragments and against synthetic peptides unique to PIEK (CYEQVHLSKKKYIEDKY) and PIEF (CRKKKKKKNCRLKCHFM) were used to capture cross-reactivity with other kinases and phosphatases.

Semiquantitative RT-PCR of the Selected Genes—Total RNAs were isolated from synchronized P. falciparum 3D7 parasite cultures using a mini RNA isolation kit (Qiagen) at 16, 30, 40, and 48 h postinvasion (hpi). An aliquot of 10 ng of total RNA was used to synthesize cDNA using a cDNA synthesis kit (Invitrogen) and analyzed by 18 cycles of PCR using gene-specific primers (Table II). Genomic equivalents of each gene were normalized using that of 18 S rRNA for all the RNA samples.

Protein Immunodetection—Immunoblotting assays were performed with parasite lysate (isolated from 1 × 10$^8$ HRBCs), infected RBC cytosol (obtained by streptolysin O permeabilization of 1 × 10$^8$ IRBCs), and the extracellular culture supernatant of intact iRBCs (concentrated supernatant from 100 ml of parasite culture), and blots were developed by ECL Western blotting detection reagents (Amersham Biosciences). The new specific sera were used at a 1:100 dilution, whereas sera collected from P. falciparum-infected patients were used at a 1:50 dilution. Indirect immunofluorescence assays were performed on air-dried samples as described previously (18) for which sera were used at a 1:100 dilution. Images were captured by the Nikon A1-R confocal microscope. Immunoelectron microscopy was carried out on P. falciparum trophozoite/schizont stage parasites using gold-conjugated mouse IgG (19).

Microarray Experiments and Analyses—Four different groups of mice were independently immunized subcutaneously with (i) 20 mg of PISEL1 in CFA, (ii) 20 mg of PISEL2 in CFA, (iii) 20 mg of PIMSP1 in CFA, and (iv) CFA in PBS, which served as control. Each mouse was boosted 2 weeks later with corresponding recombinant proteins emulsified in incomplete Freund’s adjuvant. Splenocytes from immunized mice were stimulated in vitro with corresponding PISEL1, PISEL2, PIMSP1, and medium only (control) for 24 h, and total RNA from the cells was enriched. Different sets of cDNA prepared from stimulated and unstimulated cells were normalized (prior to hybridization) with respect to mouse β-actin to eliminate any variation resulting from differences in amount of starting material (supplemental Fig. 4a). Oligo GEArray Mouse Notch Signaling Pathway gene array from SuperArray was used to analyze differences in gene expression of PISEL1/PISEL2-stimulated cells versus PIMSP1-stimulated/unstimulated cells. These are cDNA arrays containing a total of 113 genes (involved in Notch binding and receptor processing and putative Notch target genes) along with the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. All steps were carried out strictly following the manufacturer’s instructions. Blots were developed using the Chemiluminescent Array Detection kit (SuperArray), and spots were quantified using the ImageMaster software (Amersham Biosciences). All the experiments were repeated twice independently with consistent results obtained each time. Intensity values for each spot were generated (by densitometry scan) using Image Master 2D Platinum Software Version 5.0 (Amersham Biosciences).

Change was measured as -fold ratios between individual spots of SEL1/SEL2-stimulated arrays versus the control/MSP1-stimulated array (supplemental Fig. 4b). For better visualization of gene expression changes, log values of these -fold ratios were tabulated (supplemental Table 1). The data shown (-fold ratios as in supplemental Table 1 and Fig. 6) are the average (mean) of replicates.

Kinase and Phosphatase Assays—Kinase assays were performed in 20-μl reactions in buffer containing 100 mM Tris (pH 8.0), 10 mM MgCl$_2$, 10 mM MnCl$_2$, 10 mM CaCl$_2$, [γ-32P]ATP, 0.1 mM orthovanadate, and 2 μg of substrate (myelin basic protein and Histone2A) incubated with 0.1 μg of PIEK. Also recombinant PIEK was added to in vitro P. falciparum culture in the presence of cell-impermeable [γ-32P]ATP to check phosphorylation of proteins in the extracellular culture supernatant of infected and infected RBCs. Culture supernatants were collected as described above. The samples were resolved by SDS-PAGE, dried, and visualized by autoradiography. The activity of purified PIEK was assayed by incubating the substrates (protein kinase C-phosphorylated myelin basic protein (0.5 μg), Src-phosphorylated myelin basic protein (0.5 μg), and protein kinase C-phosphorylated concentrated culture supernatant from P. falciparum culture) for 60 min at 37 °C in a Tris buffer (100 mM, pH 8.0). The reactions were terminated by the addition of SDS sample buffer, analyzed by SDS-12% PAGE, dried, and visualized by autoradiography.

RESULTS

2DE/MS and LC-MS/MS of P. falciparum Extracellular Secreted Antigens—To identify the proteins of P. falciparum that are secreted out of the infected erythrocytes, tightly synchronized 3D7 parasites were grown from late trophozoite to schizont stage (in 35S radioisotope-containing medium and in serum-free unlabeled medium), and the respective radiola-
beled and unlabeled culture supernatants were collected. Before this, the parasite cultures were microscopically examined to rule out any possibility of schizont rupture. To confirm the intactness of infected erythrocytes during sample collection, extracellular supernatant was identically prepared (as a control) from a tightly synchronized GFP-expressing transgenic parasite line (that traffics KAHRP signal sequence-fused GFP to the iRBC cytosol) and analyzed for the presence of GFP as determined by GFP antibody (Fig. 1).

The same supernatant was also analyzed for the presence of PfHRPII protein. As shown in Fig. 1, no PfHRPII was detected in the supernatant as suggested earlier by Haldar and co-workers (20). This confirmed the absence of intracellular proteins from the prepared extracellular culture supernatant.

To identify parasite ESAs, both the radiolabeled and the unlabeled supernatants were resolved on separate broad pH range IEF strips followed by 10% SDS-polyacrylamide gel electrophoresis. The gel with unlabeled proteins was visualized by silver-staining, whereas the radiolabeled protein gel was visualized by autoradiography. Forty well-defined spots were observed in the silver-stained gel, whereas a comparatively lesser number (28) of specific parasite-derived spots was seen in autoradiogram. These 28 spots correlated well with corresponding spots on the silver-stained gel run in parallel, were picked for identification by MALDI-MS. Eight spots that could be successfully matched to distinct proteins in the Plasmodium database (PlasmoDB) are numbered (also listed in Table I).
As a complementary approach to two-dimensional gel electrophoresis/mass spectrometry, LC-MS/MS analysis of the serum-free culture supernatant identified a total of 25 proteins (Table I). This approach resulted in the identification of not only the eight proteins identified previously by 2DE/MS but also additional proteins that were not detected on two-dimensional gels.
sional electrophoresis gels such as a 41-kDa antigen, a DegP-like serine protease 1 (25), a putative protein kinase, a putative protein phosphatase, two proteins containing Sel1 repeats (26, 27), and a putative serine esterase (28) along with some hypothetical proteins. Again a few known/probable plasma membrane-associated proteins of infected erythrocytes were observed; for example, the malarial adhesion protein PfEMP1; an acidic phosphoprotein precursor, PCEMA1 (29); a P-loop-containing NTP hydrolase; and a major facilitator superfamily transporter having a signaling module called the GAF domain (30). However, LC-MS/MS also detected a female gametocyte-specific protein, PFG377 (31), along with some merozoite surface proteins (Pf12, PFSUB2, GBP130, and PfRh2) that could have been released due to inadvertent parasite lysis. Overall proteins with possible immunomodulatory (41-kDa antigen, PCEMA1, and CCP1) and signaling (kinase, phosphatase, and GAF domain-containing transporter) functions were detected. Proteome identification of ESAs thus suggested quite a few players involved in the intricate host-pathogen cross-talk. Supplemental Fig. 3 shows the schematic representation of the major functional classes of the "extracellular secretome" identified. The pie chart clearly shows that immunomodulatory (27%) and signaling proteins (15%) predominate in the prepared culture supernatant and constitute major classes of parasite effector molecules in the extracellular milieu.

In Silico Analysis of ES Antigens—Table I gives the number and sequence of unique peptides identified and the percent sequence coverage for each protein. The ES protein sequences were analyzed by a series of algorithms designed to detect targeting signals. Some proteins (for e.g. CCP1, the two Sel1 repeat-containing proteins, PFG377, DegP protease, etc.) have the canonical amino-terminal signal sequence, whereas some (for e.g. PCEMA1, the protein kinase, the protein phosphatase, etc.) do not. Most proteins (except PfEMP1 and the FIKK kinase) do not possess the host targeting/PEXEL motif that is important but not critically essential for export beyond the parasite plasma membrane. Transmembrane sequences are present in membrane transporter proteins, PCEMA1, PfEMP1, and some other hypothetical proteins.

Transcript Levels, Genetic Polymorphism, and Ortholog Identification of Genes of ES Antigens—The transcript levels of cognate genes were derived from the DeRisi and co-worker (15) transcriptome data that clearly showed that most of them peak at the trophozoite and schizont stages when the ES culture supernatant had been prepared. Data obtained from a genome-wide variation study of four *P. falciparum* isolates (Dd2, Hb3, D10, and 7G8) (17) showed that genes coding for almost half of the ES antigens (for e.g. the DegP protease, 41-kDa antigen, etc.) are more variable (having more single nucleotide polymorphisms (SNPs), especially non-synonymous SNPs that cause changes in amino

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**Table I—continued**

| Protein ID | Protein Description | Unique peptides identified | Unique peptides/Sorting (No. of SNPs) | Protein Level | Ortholog |
|------------|---------------------|---------------------------|---------------------------------------|---------------|----------|
|            |                     |                           |                                       |               |          |

**Notes:**

- **pl** and **RSPEXEL** refer to the number of peptides identified at the plasma and PEXEL motifs, respectively.
- **Identified** indicates the number of unique peptides identified for each protein.
- **Extracellular** refers to the number of unique peptides identified for each extracellular protein.
- **Intracellular** refers to the number of unique peptides identified for each intracellular protein.
- **host** and **parasite** refer to the number of unique peptides identified for each host and parasite protein, respectively.
- **Parasite** refers to the number of unique peptides identified for each parasite protein.
- **SNL** refers to the number of unique peptides identified for each SNL protein.
- **TMD** refers to the number of unique peptides identified for each TMD protein.
- **g. pos.** refers to the number of unique peptides identified for each g. pos. protein.
- **E. uncult** refers to the number of unique peptides identified for each E. uncult. protein.
- **E. bact** refers to the number of unique peptides identified for each E. bact. protein.
- **E. bact** refers to the number of unique peptides identified for each E. bact. protein.
Extracellular Secreted Antigens of Asexual P. falciparum

**Table II**

| Serial no. | Locus     | Protein name | Sequence of primers/peptides | Expressed region |
|------------|-----------|--------------|------------------------------|-----------------|
| 1          | PFB0190c  | PfB190 (PISEL1) | 5'-ggg CCA ACC TTA CTC gAg TCC gTA AAA TTA gCT gAT | Glu\(^{1080}\)–Gly\(^{1238}\), includes Sel1 repeats |
| 2          | PF14_0462 | PfN462 (PISEL2) | 5'-ggg CCA ACC TTA CTC gAg TCC ATT CCT TCC CAT TgA TTT TAC | Ala\(^{382}\)–Ala\(^{525}\), includes Sel1 repeats |
| 3          | PF11_0220 | PfK22 (PIEK)  | 5'-gg CCA ACC TTA CTC gAg TCC TTT ATT TCT ACC TTT TTT | Lys\(^{201}\)–Glu\(^{577}\), includes kinase activity domain |
| 4          | PF11_0139 | PfK139 (PIEP) | 5'-ggg CCA ACC TTA CTC gAg TCC TTG gAg AAT AAC gAA TTT ATT TCT ACC TTT TTC | Lys\(^{2}\)–Met\(^{218}\), includes phosphatase activity domain |

acids) than others, and they clustered with other highly polymorphic genes on chromosomes (refer to Table I). Most of the genes had orthologs in the rodent malarial genomes (Plasmodium yoelii yoelii, Plasmodium chabaudi chabaudi, and Plasmodium berghei) showing that these proteins are conserved across Plasmodia and hence might have important roles in the parasite life cycle (Table I).

Expression of Selected Gene/Gene Fragments as Recombinant Proteins—To validate expression of ES antigens at asexual blood stages of the parasite, we selected four hypothetical proteins with possible signaling/immunomodulatory roles (based on *in silico* predictions and data from existing literature) (26, 27). The selected proteins PfB190 (PISEL1), PfN462 (PISEL2), PfK22 (PIEK), and PfK139 (PIEP) correspond to the PlasmoDB accession numbers PFB0190c, PF14_0462, PF11_0220, and PF11_0139, respectively (Table II). Recombinant His\(_6\)-tagged PISEL1 and PISEL2 contain Sel1 repeats (structural and protein–protein interaction module), whereas recombinant MBP-tagged PIEK and His\(_6\)-tagged PIEP include the kinase and phosphatase catalytic domains, respectively (Table II and supplemental Fig. 2). Polyclonal sera were raised in mice against purified PISEL1 and PISEL2 protein fragments and against PIEK and PIEP by using synthetic peptides unique to these proteins to avoid cross-reactivity with other kinases and phosphatases.

Stage-specific Transcription of the Selected Genes in Asexual Blood Stage Parasites—To ascertain the expression pattern of the selected genes during the asexual blood stage life cycle of the parasite, cDNAs were prepared from synchronized parasite cultures at 16, 30, 40, and 48 hpi and analyzed by semiquantitative RT-PCR using gene-specific primers (Table II). Genomic equivalents of each gene were normalized using that of 18 S rRNA for all the RNA samples. PISEL1 peaked at the early trophozoite stage (30 hpi), whereas PISEL2 peaked a little later at the late trophozoite stage (40 hpi) when the parasite is metabolically most active. However, PIEK and PIEP showed almost similar levels in all the stages (with a slight increase in the trophozoite/schizont stages) suggesting their near constitutive expression (Fig. 2a).

Detection of Selected ES Antigens in Parasite Culture Supernatant by Western Blotting—To confirm that the selected proteins are exported into the extracellular medium, culture supernatant was analyzed by Western blot using sera specific against PISEL1, PISEL2, PIEK, and PIEP (Fig. 2b). Preimmune serum was used as a negative control. Proteins of approximately the expected size (except for a slight increase in the observed molecular mass of PIEP; Table III and Fig. 2b) were detected in the three fractions, namely parasite lysate, infected RBC cytosol, and the concentrated extracellular culture supernatant (lanes 1, 2, and 3, respectively, in each blot in Fig. 2b). The same set of sera did not show reactivity with similar fractions prepared from uninfected erythrocytes. As a control, oPIMIF antibody (a kind gift from A. P. Waters) was used to probe the same fractions of parasite culture. It detected the 12-kDa protein in parasite lysate and in infected RBC cytosol but detected much less in the concentrated extracellular culture supernatant (probably because of inadvertent schizont rupture) consistent with a previous study (32). Thus the four selected ES proteins were observed to be exported by the parasite through the infected RBC cytosol beyond the iRBC plasma membrane.

Localization of the Selected ES Antigens, en Route in Infected Erythrocytes, by Confocal Microscopy and Immunoelc-
tron Microscopy—Immunofluorescence assay and immunoelectron microscopic studies were performed to determine the cell-specific localization of the four malaria parasite ESAs at trophozoite/schizont stages using their respective antibodies. None of the sera reacted with uninfected erythrocytes, and even preimmune sera did not show any reactivity with iRBCs. Images by confocal microscopy showed punctate vesicle-like staining of the infected RBCs with all four (\(Pf\)SEL1, \(Pf\)SEL2, \(Pf\)EK, and \(Pf\)EP) antibodies with \(Pf\)EK also showing specific rimlike staining of the plasma membrane of the infected RBC. \(Pf\)MIF antibody was used each time as a marker for co-localization as \(Pf\)MIF is exported via the Maurer clefts to the extracellular medium after schizont rupture. A partial co-localization of \(Pf\)MIF was seen with each of the four proteins, suggesting a distinct pathway for the release of these ESAs (Fig. 3).

To rule out the possibility of the antibodies cross-reacting with other proteins, we preincubated the antibodies with the respective recombinant proteins before incubating with the fixed infected RBC. This treatment abolished the staining observed with these antibodies, confirming the specificity of the immunofluorescence signal. Notably preincubation with the anti-\(Pf\)EK antibody could not abolish the staining observed because the peptide epitope in \(Pf\)EK lay outside the catalytic domain that was cloned and expressed as the recombinant protein (CONTROL column in three panels in Fig. 3). However, anti-\(Pf\)EK and anti-\(Pf\)EP antibodies were specific to the respective proteins as they were raised by using synthetic peptides unique to these proteins.

The extracellular secretion of these four selected ES antigens was further confirmed by immunoelectron microscopic studies (at trophozoite/schizont stages) using the same antibodies. As in Fig. 4a, \(Pf\)SEL1 showed a secreted/extracellular staining pattern, localizing in the iRBC cytosol close to the erythrocyte plasma membrane. The gold particle staining

Fig. 2. a, stage-specific transcript levels of selected genes in asexual blood stage parasites. Total RNA isolated from synchronized \(P. falciparum\) 3D7 parasite cultures at 16 h (ring (R)), 30 h (early trophozoite (ET)), 40 h (late trophozoite (LT)), and 48 h (schizont (S)) postinvasion were used to synthesize cDNA (10 ng each) and analyzed by 18 cycles of PCR using gene-specific primers. Genomic equivalents of each gene were normalized using that of 18 S rRNA for all the RNA samples. 1-kb DNA ladder (Fermentas) was used to read the size of PCR products on the 1% agarose gel. b, sub- and extracellular allocation of the selected ES antigens. Western blot analyses were performed using mouse antibodies (1:100 dilution) raised against recombinant \(Pf\)SEL1 and \(Pf\)SEL2 protein fragments and against synthetic peptides unique to \(Pf\)EK and \(Pf\)EP. Lanes 1, parasite lysate (isolated from \(1 \times 10^{10}\) iRBCs); lanes 2, infected RBC cytosol (obtained by streptolysin O permeabilization of \(1 \times 10^{9}\) iRBCs); lanes 3, the extracellular culture supernatant of intact iRBCs (concentrated supernatant from 100 ml of parasite culture). As a control, rabbit \(\alpha\)PfMIF antibody was used to probe the same fractions of parasite culture. The specifically detected proteins are marked by arrows. Molecular mass markers are indicated (kDa).
TABLE III

Summary of the data for PfMIF and the data obtained for the four selected hypothetical proteins

The predicted and observed (Western blot) molecular masses and signal peptides (SP) are indicated. Stage-specific transcript levels were determined by semiquantitative RT-PCR (R, ring; ET, early trophozoite; LT, late trophozoite; S, schizont). The specific sera were used for the detection of proteins (by Western blot) in extracellular supernatant of intact iRBCs as well as for immunofluorescence and immunoelectron micrography for their localization in intact iRBCs. PfMIF, released after rupture of mature schizonts, was used for comparison of localization within infected RBCs. Western blot analysis showed reactivity of the recombinant proteins with sera from P. falciparum-infected patients as compared with sera from healthy controls. PfSEL1 and PfSEL2 were characterized as immunomodulators of the Notch signaling pathway in mice, whereas recombinant PIEK and PIEP were shown to be active extracellular kinase and phosphatase, respectively.

| Serial no. | Parasite protein | Molecular mass (kDa) | Signal peptide | Stage-specific transcript level (R/ET/LT/S) | Detection in extracellular supernatant of intact iRBCs | Immunolocalization in iRBCs | Reactivity with sera of malaria patients | Functional role |
|------------|------------------|----------------------|----------------|-------------------------------------------|------------------------------------------------------|-----------------------------|----------------------------------|----------------|
| 1          | PfB190 (PfSEL1)  | 272                  | SP             | ET > LT > S > R                          | +                                                    | iRBC cytosol/extracellular  | +                                | Immunomodulator   |
| 2          | PfN462 (PfSEL2)  | 100                  | LT > S > ET > R| +                                        | +                                                    | +                           | +                                | Immunomodulator   |
| 3          | PfK22 (PIEK)     | 202                  |               | LT > S > ET > R                          | +                                                    | +                           | +                                | Extracellular kinase |
| 4          | PfK139 (PIEP)    | 25                   | LT > S > ET > R| +                                        | +                                                    | +                           | +                                | Extracellular phosphatase |
| 5          | PfMIF            | 12                   |               | LT > S > ET > Ra                         | -                                                    | -                           | +                                | Extracellular cytokine |

*As studied by Augustijn et al. (32).*

Functional Characterization of the Selected ES Antigens—To get an insight into the functions of the selected ES antigens, we studied possible signaling/immunomodulatory roles (based on in silico predictions) of PfSEL1 and PfSEL2 proteins. Earlier studies in Caenorhabditis elegans have shown that C. elegans homologs of C. elegans SEL1, a secreted or membrane-associated protein, are negative regulators of LIN-12 and GLP-1 receptors of the Notch signaling pathway (26, 27). As PfSEL1 and PfSEL2 are secreted proteins, we studied their possible role in Notch pathway modulation. The main aim of the microwell-based assay was to investigate whether PfSEL1 and PfSEL2 proteins might have immunomodulatory effects in vivo. This was done by analyzing the expression of Notch signaling pathway components in PBS/CFA-stimulated and PfSEL1/PfSEL2-stimulated ex vivo spleen cells from mice inoculated with recombinant PfSEL1 and PfSEL2 proteins.

Change was measured as fold ratios between experimental (PBS/CFA-stimulated) and control (PBS/CFA only) conditions. The experiment was repeated in triplicate, and data from one representative experiment are shown. The four selected ES antigens were also probed to investi- gate their expression pattern and localization in the training ex vivo spleen cells. The expression levels of PfSEL1 and PfSEL2 were significantly higher in PBS/CFA-stimulated ex vivo spleen cells than in PBS/CFA only (as control). Preimmune sera did not react with these antigens (Fig. 4).
**DISCUSSION**

The success of protozoan parasites in host organisms depends on a series of intricate and highly evolved adaptations that enables them to evade destruction by the host immune system. The efficiency with which these parasites avoid clearance and persist in the host cells/tissues is determined largely by surface and secreted antigens of the parasite (34). Furthermore, secretion of effectors is a major component of parasite virulence. Recent proteomics studies have identified about 70 excreted/secreted proteins of *Toxoplasma* tachyzoites implicated in host-parasite interactions (35). Transcriptomics studies of *P. falciparum* have identified many proteins that show a profile of major surface antigens, and many of these proteins have been shown to be required for virulence and rigidity of *P. falciparum*...
Fig. 4. a–d, localization of the selected ES antigens by immunoelectron microscopy. Ultrathin sections of *P. falciparum*-infected erythrocytes (at late trophozoite/schizont stages) were labeled with specific sera (against the four selected proteins) and gold-conjugated secondary antibody. Localization is depicted as black dots (of gold particles) for PfSEL1 (a, i and ii), PfSEL2 (b), PfEK (c), and PfEP (d). Enlarged panels and arrows show detailed images of the intracellular staining pattern. Scale bar, 250 nm.
falciparum-infected human erythrocytes (15, 36). However, little is known about ESAs among these candidate genes. In this study, we identified 27 novel ESAs exported by *P. falciparum* in the culture medium; some of these possess extracellular domains involved in protein-protein interactions and could have potential roles in crucial host-parasite interactions.

To identify *P. falciparum* extracellular secreted antigens, we applied two complementary approaches, 2DE/MS and LC/MS-MS, on culture supernatants collected at asexual blood stages of *P. falciparum*. Broad pH range IEF strips (pH 3–10) were used despite the skewed migration of the ES antigens toward the acidic region of the gels. This was done to ensure a complete representation of secretory proteins. Passive release of intracellular proteins by rupture of infected erythrocytes was carefully avoided by allowing tightly synchronized parasites to grow from trophozoites to schizonts and by microscopically examining the parasites before collecting the supernatant. As controls, culture supernatant prepared (before schizont rupture) from a tightly synchronized GFP-expressing transgenic parasite line (that traffics KAHRP signal sequence-fused GFP to the iRBC cytosol) showed absence of PfHRPII and GFP, confirming the robustness of the protocol used for preparation of supernatant samples (20).

To avoid serum contaminants, tightly synchronized parasites (precultured in serum-supplemented medium) were washed and then grown to schizonts in serum-free medium before collection of the supernatant sample. Also to distinguish parasite-derived secreted proteins from host RBC proteins, we simultaneously prepared supernatant from 35S-labeled parasite culture (in serum-supplemented medium). Both labeled and unlabeled samples were analyzed by two-dimensional gel electrophoresis, and protein spots were identified by autoradiography and silver staining, respectively. Comparatively fewer spots were observed in the gel with the metabolically labeled sample in comparison with that with unlabeled sample. Additional spots in the gel with unlabeled sample can be attributed to the presence of some unwashed serum proteins left in the supernatant fraction. Nevertheless the labeled spots matched well with corresponding spots on the silver-stained gel. Analysis of 28 such spots (from the silver-stained gel) by MALDI-MS successfully identified eight parasite proteins in PlasmoDB using Mascot search. Thus, by using correlation between the two gels, we could detect parasite-specific proteins by avoiding the drawbacks of ionization-suppression and masking by contaminating serum proteins.

This gel-based approach provided the advantage of visualizing the heterogeneity of the extracellular antigens present.

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**Fig. 5.** *a* and *b*, reactivity of parasite ES antigens with malarial patient sera. Western blot analyses were performed using sera collected from *P. falciparum*-infected patients (PS) and from control subjects (CS) at 1:50 dilution. *a*, lanes 1 and 3, extracellular culture supernatant of intact iRBCs before merozoite egress; lanes 2 and 4, extracellular culture supernatant of iRBCs after merozoite egress. Both the samples correspond to supernatants concentrated from 100 ml of parasite culture. *b*, recombinant PISEL1, PISEL2, PIEK, PFEK, and PIMIF (positive control) were also detected by the same set of sera. The specifically detected proteins are marked by arrows. Molecular mass markers are indicated (kDa).
in the culture supernatant but was limited by relatively low sensitivity. Hence as a complementary approach, LC-MS/MS was further applied to the analysis of the serum-free culture supernatant. The combination of the two proteomics approaches promoted maximum representation of sample complexity and provided validation for those products identified by more than one strategy. Twenty-five proteins were identified in the serum-free culture supernatant by LC-MS/MS. Hence a total of 33 proteins were obtained of which 27 were novel extracellular antigens of *P. falciparum*.

Overall soluble proteins as well as some known/probable plasma membrane-associated proteins of infected erythro-

| Gene name | Description | Position of Spot | Log2(Fold Ratio SEL1) | Log2(Fold Ratio SEL2) |
|-----------|-------------|------------------|-----------------------|-----------------------|
| Adam17    | ADAM20A12   | A3               | -6.89                 | -6.74                 |
| Cathepsin | alpha-1     | A7               | -6.73                 | -8.33                 |
| CD44      | CD44        | B3               | -6.79                 | -8.96                 |
| Chik      | Chik        | B8               | -7.96                 | -7.38                 |
| Dlx2      | Delta-2     | C5               | -2.13                 | -9.96                 |
| EGF       | EGF         | D1               | -2.13                 | -6.50                 |
| Fos       | Fos         | D2               | -7.15                 | -7.64                 |
| Pad10     | Przized homolog 10 (Drosophila) | D5 | -6.50 | -4.75 |
| Pad4      | Przized homolog 4 (Drosophila) | D8 | -6.64 | -4.60 |
| Pad5      | Przized homolog 5 (Drosophila) | E1 | -6.25 | -5.21 |
| Pad6      | Przized homolog 6 (Drosophila) | E2 | -4.64 | -7.64 |
| Hes6      | Hairy and enhancer of split 6 (Drosophila) | F5 | -5.96 | -7.96 |
| Heyl      | Hairy/Enhancer-of-split related with TRFW motif-like | F8 | -4.64 | -6.15 |
| Hve        | Hairless    | G2               | -8.38                 | -5.44                 |
| Jag1      | Jagged 1    | G8               | -3.73                 | -7.38                 |
| Lmo2      | Lim domain only 2 | H7 | -8.96 | -6.96 |
| Mas1/1    | Mastermind like 1 (Drosophila) | J3 | -6.23 | -5.00 |
| Nec1/2    | Nuclear receptor co-repressor 2 | J1 | -8.89 | -4.64 |

FIG. 6. Microarray experiments and analyses to detect change in expression of genes involved in Notch signaling in mice. Total RNA was enriched from splenocytes from primed mice following stimulation with PISEL1, PISEL2, and medium only (control) for 24 h. 1 ug of RNA was processed for microarray analyses using the GEArray Mouse Notch Signaling Pathway Gene array from SuperArray strictly following the manufacturer’s instructions. The table depicts genes that are predominantly down-/up-regulated, their description and position on the arrays, and the log2 (-fold ratio) values representing the change in expression of these genes following the two stimulations (with respect to control). LIM, Lin11, Isl-1 and Mec-3 proteins; FBJ, Finkel-Biskis-Jenkins.

**Extracellular Secreted Antigens of Asexual *P. falciparum***

2114 Molecular & Cellular Proteomics 8.9
heat-inactivated PfEP.

d

concentrated culture supernatant from
PfEK was added to
0.1 mM orthovanadate.

Asterisks
protein kinase C-phosphorylated concentrated culture supernatant

started the buildup of its repertoire of proteins required for
maturation (31). Hence the parasite might have already
mitment to sexual differentiation occurs prior to schizont
earlier studies that have suggested that the parasite com-
promised and accumulated in the extracellular fraction during

polarized and accumulated in the extracellular fraction during

��ness of parasite development in dif-
ferent stages. Forexample, transcription of bir genes was
detected in both the asexual blood stage and gametocytes of
P. berghei suggesting that these proteins may have multiple
functions in different stages (37). Another such example of
multiple stage expression is the Apicomplexa-specific LCCL
domain-containing family of proteins. The protein CCP1, a
member of this LCCL family, has been reported to be a
secreted extracellular protein (23, 24). Hence its detection in
the ES fraction provided significant support to the definition of
the extracellular nature of the prepared sample. Also there
was a marked absence of many intracellular parasite pro-
tases like PISUB1 and serine repeat antigen protein that are
released in the extracellular milieu just as the schizont-in-
fected RBCs rupture (38). DegP-like serine protease, the hom-
olog of a bacterial ectoprotease, was an interesting exception
(25). These observations strengthen the validity of this pro-
teomics study.

Validation of Extracellular Nature of P. falciparum ES Anti-
gen—Immunoblot and immunofluorescence assays were
done to study the localization of the four malaria parasite
ESAs in the infected RBCs using their respective antibodies.
As expected, each assay showed release of the four ES
antigens by the parasite through the iRBC cytosol into the
extracellular medium. Confocal microscopy indicated punc-
tate vesicle-mediated export of PISEL1, PISEL2, PfEK, and
PfEP in the iRBC cytosol. As a step further, immunoelectron
microscopic studies were done to reconfirm their extracellular
localization. Interestingly in the case of PISEL1, a large num-
ber of gold particles could be seen on the surface of iRBCs
and being secreted in the extracellular milieu (Fig. 4), thus
confirming the release of identified proteins beyond the iRBC
plasma membrane. It would be interesting to study the traf-
ficking of these proteins in the infected erythrocyte as they
lack the host cell targeting/PEXEL motif shown to target pro-
teins to the host erythrocyte. In addition to immunolocaliza-
tion studies, we also observed that all four ESAs were strongly
recognized by sera from P. falciparum-infected patients. To-
gether these results provided strong support for the extracel-
lar nature of the identified P. falciparum proteins.

Potential Roles of P. falciparum ES Antigens—The excreto-
y/secretory proteins produced by parasites are key players in
host-parasite interactions. Many parasites use genetic varia-
bility to defeat host immunity and drug treatments. For exam-
ple, secreted proteins of the parasitic nematode Haemonchus
contortus reveal extensive sequence variation and differential
immune recognition (39). Likewise two secreted polymorphic
kinases of T. gondii have been recently shown to be key
virulence determinants (40, 41). As a consequence, there is a
reduction in genetic diversity around loci under positive nat-

Fig. 7. a–d, catalytic activity of PIEK and PFEP. Kinase assays
were performed in 20–μl reactions in buffer containing 100 mM Tris
(pH 8.0), 10 mM MgCl2, 10 mM MnCl2, 10 mM CaCl2, [γ-32P]ATP, and
0.1 mM orthovanadate. a, 2 μg of substrate myelin basic protein
(MBP) (lane 1, with 0.1 μg of PIEK; lane 2, with protein kinase C; lane
3, with 0.1 μg of heat-inactivated PIEK), and 2 μg of substrate
Histone2A (H2A) (lane 4, with 0.1 μg of PIEK; lane 5, with protein
kinase C; lane 6, with 0.1 μg of heat-inactivated PIEK). b, recombinant
PIEK was added to in vitro P. falciparum culture in the presence of
cell-impermeable [γ-32P]ATP in the extracellular culture supernatant
of uninfected (lane 1) and infected RBCs (lane 3). Lane 2 depicts the
basal phosphorylation by host erythrocytic kinases or by other extracel-
ular kinases of the parasite. Activity of purified PIEK was assayed
by incubating with the substrate for 60 min at 37 °C in a Tris buffer
(100 mM, pH 8.0). c, lane 1, protein kinase C-phosphorylated myelin
basic protein substrate (0.5 μg); lane 2, substrate with PIEK; lane 3,
with heat-inactivated PIEK; lane 4, Src-phosphorylated myelin basic
protein substrate (0.5 μg), lane 5, substrate with PIEK; lane 6, with
heat-inactivated PIEK. d, lane 1, protein kinase C-phosphorylated
centredature supernatant from P. falciparum culture; lane 2, protein
kinase C-phosphorylated concentrated culture supernatant incubated
with PIEK. Molecular mass markers are indicated (kDa). Asterisks show phosphorylated proteins in panel B whereas dephos-
phorylated proteins in panel D.
ural selection (for example known drug resistance loci) as opposed to an increase in polymorphisms around loci coding for antigenic determinants (42). Data extracted from a genome-wide study of four *P. falciparum* isolates (Dd2, Hb3, D10, and 7G8) (17) showed that genes coding for almost half of the extracellular proteins are more variable than others. These clustered with other highly polymorphic genes on chromosomes (refer to Table I) suggesting that they may encode important putative antigenic determinants of the parasite. In this respect, proteins like the 41-kDa antigen and some hypothetical proteins (PFE0440w, PF11_0369, PFB0765w, and PF07_0113) could be studied in detail. Here we attempted to characterize two such proteins, PF14_0462 (PfSEL2) and PF11_0220 (PfEK), functionally. Also the *P. falciparum* homolog of a bacterial DegP-like serine protease emerges as an interesting polymorphic candidate as the bacterial protein has been reported to be an ectoprotease and hence a virulence determinant (25). On the contrary, some genes like the protein-tyrosine phosphatase, membrane-bound transporter, etc. showed more sequence conservation among the *P. falciparum* isolates suggestive of their positive selection by the host immune system. The genetic variability of these genes could be a consequence of the interactions between the encoded proteins and the host immune system. Indeed sera from malarial patients showed reactivity with many proteins in the prepared extracellular secretory fraction.

Parasites have evolved with the host immune system, and a critical step in their interaction is the evasion of innate and adaptive immune defenses. Parasite factors such as surface proteins and secretory molecules can directly suppress the function of certain subsets of immune cells as well as stimulate other cell populations that have suppressive activity like the regulatory T cells (43). A previous study has reported that *P. falciparum* and *Plasmodium vivax* gametocyte-specific exoantigens stimulate proliferation of T cell receptor γδ lymphocytes (44). The LCCL family of Apicomplexa-specific proteins (PICCP1 in our data) has also been reported to play a role in host immune evasion (23). Similarly in *C. elegans* SEL1 is a secreted or membrane-associated protein that negatively regulates LIN-12 and GLP-1 receptors of the Notch signaling pathway by controlling their turnover (26, 27). The Notch proteins comprise a family of epidermal growth factor-like transmembrane receptors that function in highly conserved intercellular signaling pathways and direct cell fate decisions, proliferation, and apoptosis in metazoans. Signals transduced by Notch receptors are indispensable for T cell specification and differentiation of αβ T lineage cells (45). As PfSEL1 and PfSEL2 are homologs of *C. elegans* SEL1, we studied their possible role in Notch pathway modulation. Array analysis showed down-regulation of particularly some of the 113 Notch signaling genes on the array (Fig. 6). Among these, Notch ligands (Delta1/3/4 and Jag1/2) especially Jagged1 are prominently down-regulated. It has been shown that Jagged1 inhibits the differentiation of thymocytes into B cell lineage and also influences their differentiation along the natural killer and γδ T cell lineages during late stages of thymic development (46). Expression of most Notch receptors (Notch1/3/4) went down with the exception of Notch2 that went up. Although Notch1 favors T cell lineage commitment during lymphoid development (47), the distinctive functions of different Notch receptors are related to the activation of different downstream signals or to a variable binding efficiency with different Notch ligands (45). Ligand binding initiates two proteolytic cleavage events that release intracellular Notch from the membrane. Here some Notch receptor processing genes (Adam10/17, Aph1a, and Ncstn) were down-regulated, whereas some (Psen2 and Psenen) were found to be up-regulated compared with that of control. Downstream effects are shown by modulation of expression of transcription factors. It is interesting to speculate from our results how down-regulation of Notch signaling genes by PfSEL1/PfSEL2 might confer a survival advantage to the parasite. One possible explanation could be that the binding or uptake and presentation of PfSEL1/PfSEL2 by host antigen-presenting cells (macrophages/dendritic cells) could induce down-regulation of Notch ligands in these cells. Consequently interaction of antigen-presenting cells with T cells would result in skewing of host CD4+ T cells to differentiate to Th1 cells rather than Th2 type cells. The absence of a strong Th2 response would then result in a weak antibody response by the host, thereby favoring survival of the parasite. We have also shown that PfSEL1 and PfSEL2 bind to mouse antigen-presenting cells and induce subsequent polarization of the CD4+ T cell population to differentiate into predominantly Th1 effector cells, thereby capable of regulating an in vivo immune response.2 This hypothesis is consistent with the classical function of Notch as a determinant of T cell fate decisions (46, 47). However, detailed studies (with full-length proteins) are needed to confirm these preliminary observations and to study other possible functions of these proteins (48). We also observed sequence similarity of some *P. falciparum* ES antigens (PFB0765w, PF11_0168a, and MAL13P1.39) with those of known viral/bacterial immunoreactive ecto/exoantigens (Table I), suggesting immune evasion as an important component of host-parasite interaction. Such extracellular immunomodulatory parasite proteins are particularly intriguing given the fact that maturation of dendritic cells and their subsequent ability to activate T cells are profoundly modulated by their interaction with intact infected RBCs (49, 50). Extracellular phosphorylation/dephosphorylation is emerging as a novel mechanism in the regulation of many biological processes such as cell adhesion, cell proliferation, and modulation of immune responses by various cell types (51). Antigenic variation and cytoadhesion properties of *P. falciparum*-infected eryth-

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2 M. Singh, P. Mukherjee, and P. Malhotra, unpublished data.
cytes have been reported to be modulated by active signaling between host and parasite (52). An interesting clinical study of prostate cancer has reported the presence of a free extracellular kinase activity of protein kinase A in the growth medium of cultured prostate and other cancer cells as well as in plasma samples from prostate cancer patients (53). A secreted serine-threonine kinase of *T. gondii* has been shown to determine virulence in the host (40, 41). As such, the identified kinase and phosphatase (PIEK and PIFP from Table III) were analyzed for functional activity, and both were found to, respectively, phosphorylate/dephosphorylate parasite-specific proteins in the prepared extracellular culture supernatant. PIEK and PIFP might have other potential intracellular substrates as well and thus could have important functions in intracellular compartments also. Similar activity by respective immunoprecipitates from in vitro *P. falciparum* culture should further consolidate these observations. An interesting possibility would be to study whether they regulate signaling between the host and the parasite.

In conclusion, we identified and functionally validated some of the proteins in the extracellular proteome of intact *P. falciparum*-infected erythrocytes. Among the 33 proteins obtained, almost half showed clustering with highly polymorphic genes on chromosomes and reacted with sera from malarial patients, thus suggesting that they may encode putative antigenic determinants of the parasite. Immunolocalization of four novel secreted proteins confirmed their export beyond the IRBC membrane. Preliminary functional characterization of these four proteins (along with the predicted functions of some others) suggests immune evasion and signaling as important roles of these proteins in host-pathogen interactions. Some of these proteins may be essential for parasite development, and because they are parasite-specific, they could constitute promising new drug targets.

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