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*[S]

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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, PfSEL1 and PfSEL2 revealed that they down-regulate expression of cell surface Notch signaling molecules in host cells. Also a novel protein kinase (PfEK) and a novel protein phosphatase (PfEP) 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)[1] 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-*

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[1] The abbreviations used are: ES, excretory-secretory; ESA, extracellular secreted antigen; 2DE, two-dimensional gel electrophoresis; LCCL, *Limulus* clotting factor C, Coch-5b2, and Lgl1; Pf, *P. falciparum*; EK, extracellular kinase; EP, extracellular phosphatase; MIF, macrophage migration-inhibitory factor; HRPII, histidine-rich protein II; SNP, single nucleotide polymorphism; RBC, red blood cell; iRBC, infected RBC; PEXEL, *Plasmodium* export element; NCBI, National Center for Biotechnology Information; MBP, maltose-binding protein; hpi, h postinvasion; CFA, complete Freund's adjuvant; GFP, green fluorescent protein; KAHRP, knob-associated histidine-rich protein; PlasmoDB, *Plasmodium* database.

plasma gondii have revealed many novel constituents of host-parasite interactions (9).

The identification and trafficking of *Plasmodium* proteins exported into the host erythrocyte have been subjects of recent detailed investigations. A number of studies have identified *Plasmodium* proteins that contain signature sequence motifs, the host cell targeting signal or the *Plasmodium* export element (PEXEL), that target these proteins into the infected erythrocytes (10, 11). Recent proteomics analyses have identified novel proteins in the raftlike membranes of the parasite and on the surface of infected erythrocytes (12, 13). *P. falciparum* translationally controlled tumor protein (PfTCTP), a homolog of the mammalian histamine-releasing factor, has been shown to be released into the culture supernatant from intact as well as ruptured infected RBCs and causes histamine release from human basophils and IL-8 secretion from eosinophils (14). However, the total spectrum of proteins actively exported by the asexual intracellular parasite beyond the host RBC membrane (before merozoite egress) has been poorly investigated so far.

In the present study, we used two complementary methodologies, two-dimensional gel electrophoresis (2DE)/MS and LC-MS/MS to examine the cohort of extracellular secreted antigens (ESAs) at asexual blood stages of *P. falciparum.* Our findings reveal that malaria parasites secrete a number of effector molecules such as immunomodulators and signaling proteins that are potentially involved in host-parasite interactions. Prominent among these are proteins with Sel1 domain, a protein of the LCCL family, a novel protein kinase, and a novel protein phosphatase. Secreted-extracellular/iRBC surface localization of some of these proteins was validated by immunolocalization studies. We also characterized the functions of some of these proteins in the culture supernatant, thus providing an insight into the nature of some of the malaria parasite extracellular antigens.

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 $\sim 1 \times 10^{10}$ parasite-infected erythrocytes with 0.5mCi/ml ^{35}S-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 acetone precipitation protocol. For each, ~150 μg (quantified using BCA protein assay reagent (Pierce)) of acetone precipitate was redissolved 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 ^{35}S-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 NH_4HCO_3, 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 NH_4HCO_3 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 NH_4HCO_3, 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 C_{18}, 5 μm), then allowed to enter C_{18} 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 transcript 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 (PfSEL1, PfSEL2, and PfEP (extracellular phosphatase)) and maltose-binding protein (MBP) (PfEK (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. 2). Specific antibodies were obtained by immunization of BALB/c mice according to standard schedules. Polyclonal sera were raised against purified PfSEL1 and PfSEL2 protein fragments and against synthetic peptides unique to PfEK (CYEQVHLSKKKYIEDKY) and PfEP (CRKKKKKKNCLRKCHFM) to avoid 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^{10} iRBCs), infected RBC cytosol (obtained by streptolysin O permeabilization of 1×10^9 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 PfSEL1 in CFA, (ii) 20 mg of PfSEL2 in CFA, (iii) 20 mg of PfMSP1 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 PfSEL1, PfSEL2, PfMSP1, 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 PfSEL1/PfSEL2-stimulated cells *versus* PfMSP1-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-^{32}P]ATP, 0.1 mM orthovanadate, and 2 μg of substrate (myelin basic protein and Histone2A) incubated with 0.1 μg of PfEK. Also recombinant PfEK was added to *in vitro P. falciparum* culture in the presence of cell-impermeable [γ-^{32}P]ATP to check phosphorylation of proteins in the extracellular culture supernatant of infected and uninfected RBCs. Culture supernatants were collected as described above. The samples were resolved by SDS-PAGE, dried, and visualized by autoradiography. The activity of purified PfEP 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 ^{35}S radioisotope-containing medium and in *serum-free* unlabeled medium), and the respective radiola-

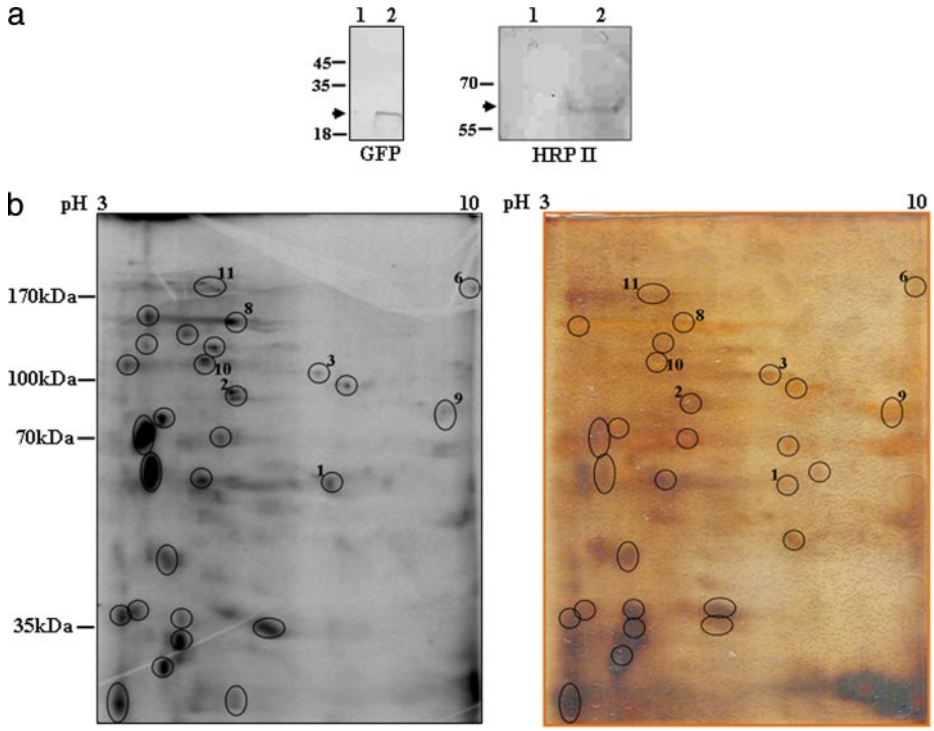

FIG. 1. *a*, absence of intracellular proteins from the prepared extracellular culture supernatant. Western blot analysis was performed using αGFP and αHRPII antibodies. *Lanes 1* and *3*, extracellular culture supernatant of intact RBCs (before merozoite egress) infected with a GFP-expressing transgenic +HIS 3D7 parasite line; *lanes 2* and *4*, extracellular supernatant of similar culture after merozoite egress. Molecular mass markers are indicated (kDa). 26-kDa GFP and 68-kDa HRPII are indicated by *arrows*. *b*, two-dimensional electrophoresis profile of ^{35}S-labeled and unlabeled ESAs from intact *P. falciparum*-infected erythrocytes. Samples were resolved on broad pH range (pH 3–10) IEF strips followed by 10% SDS-polyacrylamide gel electrophoresis. Molecular mass markers are indicated (kDa). The radiolabeled-protein gel was visualized by autoradiography, whereas the gel with the unlabeled sample was silver-stained. Twenty-eight *encircled* spots, correlating 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).

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 +His 3D7 parasite line (that traffics KAHRP signal sequence-fused GFP to the iRBC cytosol) and analyzed for the presence of GFP in an immunoblot assay. This culture supernatant did not show the presence of GFP as determined by αGFP antibody (Fig. 1*a*). The same supernatant was also analyzed for the presence of PfHRPII protein. As shown in Fig. 1*a*, no PfHRPII was detected in the supernatant as suggested earlier by Haldar and coworkers (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 (pH 3–10) 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 (Fig. 1*b*), and the latter were subsequently excised and subjected to in-gel trypsin digestion and identification by MALDI-MS. However, only eight spots could be successfully matched to distinct proteins in the *Plasmodium* database (PlasmoDB). Apart from soluble exoantigens, peptides corresponding to parasite-derived iRBC plasma membrane-associated proteins were also identified in the extracellular supernatant. For example, a vacuolar protein sorting component, VPS45; a membrane-bound putative monocarboxylate transporter; LCCL domain-containing protein CCP1; a FIKK kinase (21); and a hypothetical protein with similarity to viral A type inclusion proteins (22) were identified along with a few hypothetical proteins (Table I). Predicted functions of the observed proteins are indicative of their secretory and/or surface-associated nature. Notably the detection of LCCL domain-containing protein CCP1, a member of a previously reported family of Apicomplexa-specific secreted extracellular molecules, lent important support to the definition of extracellular secretory fraction of parasite-infected RBCs (23, 24).

TABLE I

Proteins identified from extracellular culture supernatant of intact P. falciparum-infected red blood cells by 2DE/MS and LC-MS/MS

Mascot analysis used a probability-based molecular weight search (Mowse) score >30 to assign significance ($p < 0.05$) to the peptides identified. The gene locus as annotated in the *Plasmodium* database is indicated for each identified protein (PlasmoDB ID). The protein description is based on BLASTP search, database annotation, or protein sequence analysis in Pfam and NCBI conserved domain database. The predicted molecular mass (MM), isoelectric point (pI), signal peptide (SP), PEXEL motif, and transmembrane domain (TMD) of the proteins along with the parasite stage (R, ring; T, trophozoite; S, schizont) with maximum transcript level are indicated. For each protein, unique peptides identified (periods indicate cleavage sites), percent sequence coverage, number of SNPs, number of non-synonymous SNPs (nsSNPs) (causing change in amino acid sequence), Watterson's θ (giving the genome-wide average population mutation rate), and orthologs in rodent malarial genomes have been indicated. Regions with consecutive polymorphic genes are highlighted in either light blue (with known antigen genes) or yellow (with unknown antigen genes). In blank cells are genes that were not sequenced or for which no good sequences were obtained. MFS, major facilitator superfamily.

Serial No.	PlasmoDB ID	Protein Description	Unique peptides identified	Unique peptide(s)/Coverage (%)	No. of SNPs	No. of nsSNPs	Watterson's Theta	Predicted						Orthologs		
								MM (kDa)	pI	SP	PEXEL motif	TMD	Maximum Transcript Level Stage (R/T/S)	P. y. yoelii	P.c. chabaudii	P. berghei
1	MAL13P1.39	Hypothetical a)Similarity to Con A-like lectins/glucanases b)Has Ehrlichia tandem repeat c)Similarity to Orthopoxvirus C10L protein family	K.DEENVQK.N K.MGKFYSSDNIK.Y K.DKNSSFLIMETVPSK.A K.DVKHNYISDGIYNNK.E K.YSAITYMLLCNILNRIDYDEYK.V K.NVEYYHVIK.Y K.GMNNKLLNMSINK.S	7/1.72				736.35	9.41	No	No	1	S	PY03155	PC000149.03.0	PB000072.03.0
2	PFI1150w	Hypothetical	MNNISDKNILK.Y K.SVYNSVGDK.N K.IKNSEINLK.K K.FVLNNQLDNKK.V	4/4.25	1	1	0.000388	136.2	6.52	No	No	No	R/T			
3	*Pf*EMP 1	Malarial adhesion protein, has Duffy binding domain	MVTGSGGEDKYK.S R.QMFYTLGDYR.D K.CIPSGDNSTTREGSESGLR.R	3/2.05				254.415	5.04	No	Yes	1	S			
4	**PFA0185w**	**P-loop containing nucleotide triphosphate hydrolase**	R.HFLKVINK.K K.TNKKPTSYNQIK.Y K.AIYLFLYFFVNIKK.E K.NINGNIFYPVFKK.S K.LFYNNTYNIIDSFKR.Q	5/14.2	**4**	**3**	**0.001481**	62.41	10.71	No	No	No	R/T	PY04150	PC000015.05.0	PB000210.02.0
5	PF11_0139	protein tyrosine phosphatase, putative	K.EMKNYNVTDLVR.T K.NYNVTDLVRTCER.T	2/13.30	1	0	0.000762	25.49	8.28	No	No	No	S	PY05564	PC000676.00.0	PB000082.01.0
6	**PFL2405c**	**PFG377 protein, female gametocyte specific protein**	K.TPHVHLKK.N K.HFIKVNIK.C R.FNEQQEQR.E K.KYVTYVQDEK.F K.DPNNLPPTYRHIK.Y K.MSNGEQPELNKLHK.Y K.YDERDISPHNVNQHK.K R.IVPYSEDIYDYITWVR.D K.NKPDISTSSHATDEQQVSDTLIR.G	9/4.26	**4**	**3**	**0.001268**	377.4	6.07	Yes	No	No	S			
7	PFE0245c	Hypothetical	K.YCTLK.L K.NEIEKIK.E K.LKDSIIFK.I K.WPNNNLIK.I K.INSIYDLNK.I K.MLVSVPLGLIR.Y K.NVDNLCGGYNK.K K.DYMKIIDDDK.E R.LLYLIINTLNYFLK.Y K.GIMNYLFMYFDKIK.N	10/3.93	3	2	0.000940	358	4.98	No	No	4	R/T	PY04880	PC000354.04.0	PB000078.01.0
8	MAL7P1.138	Hypothetical	K.HMLDLILK.H R.QLNVAKLVK.G K.LENVNENLSDK.V K.YIEELINKYEK.H K.DKHHNMNDISDIR.Y K.KYLFIINDEYIYK.E K.ENEDDDDEEEEEEKK.K K.LKMCTSNINDVINCAHMGNK.S	8/7.36				192.4	9.02	No	No	5	T	PY03675	PC000225.04.0	PB105232.00.0
9	PFB0190c	Hypothetical, Sel 1 repeats (related to tetratricopeptide (TPR) repeats	K.ANANVKANANVK.E K.ANANVKENANVK.E K.DLNDDLNKSLK.N K.SLKNAEEYFHK.A K.YNIVENLKLSLIK.E K.MSAQNGNIISLYNK.S R.NHDFLSDENRIIPR.K R.NLMRIHEVSNEGNNR.N K.DNTKNNEDVNYNVVLK.D K.FEKSEGTFNNISGGEDTFK.N	10/6.84	0	0	0.000000	272.4	8.59	Yes	No	No	S		PC000150.03.0	PB000240.02.0
10: Spot 1	PFB0465c	Membrane bound monocarboxylate transporter , putative	K.NFINKPNGLSNSLR.T R.IFWGLISDFTSFK.T	2/6.78	0	0	0.000000	52.5	8.37	Yes	No	12	T	PY00444	PC001062.02.0	PB001144.02.0
11	PF13_0198/ MAL13P1.176	Rh2:Normocyte/ reticulocyte binding protein2 (homolog a/b)	K.MDIHK.L K.SDAHNNNTQVDKLK.E	2/0.74	9	9	0.002564	370.44	5.28	No	No	1	S	PY01365	PC001072.02.0	PB000327.03.0
12	PF07_0086	Acidic phosphoprotein precursor PCEMA1	K.NVKVNK.N K.DEEEDIYINNK.I K.NFIIISSDIR.S K.MNEEVNKMNEEVNK.M	4/1.43	3	2	0.000907	421.33	8.69	No	No	4	S	PY01730	PC001084.02.0	PB000937.03.0
13	**PFE0440w**	**Hypothetical**	R.NTIDNENNK.L K.EYEKNNQLK.M K.KSENVSVHIMNK.N	3/1.15				372.63	5.29	No	No	No	S			
14	PF10_0318	Hypothetical, Has an uncharacterized highly conserved domain	K.GVNKIYTQK.K K.NMKNFLYFCK.S	2/6.57	0	0	0.000000	42.1	9.18	No	No	1	T/S	PY02602	PC000669.04.0	PB001619.02.0
15	PF11_0168a	Putative serine esterase	R.IVSNAR.N K.IINEIK.T K.NYSFLYPSK.N	3/1.5	2	2	0.000652	217.077	8.82	No	No	No	S			
16	**PF14_0462**	**Sel1 protein, putative**	R.NNELILK.I K.AHNYLIKASK.Y K.NTNLAFYYFELASK.N	3/4.35	**1**	**0**	**0.000344**	100.45	9.19	Yes	No	No	T/S		PC000360.02.0	PB000807.02.0
17	MAL13P1.24	Hypothetical	R.QNNLDMYGLK.N K.NHNDFEK.S	2/9.86	0	0	0.000000	25.74	9.67	Yes	No	No	R/T	PY07311	PC000080.03.0	PB000071.01.0

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-dimen-

TABLE I—*continued*

Serial No.	PlasmoDB ID	Protein Description	Unique peptides identified	Unique peptide(s)/Coverage (%)	No. of SNPs	No. of nsSNPs	Watterson's Theta	Predicted						Orthologs		
								MM (kDa)	pI	SP	PEXEL motif	TMD	Maximum Transcript Level Stage (R/T/S)	P. y. yoelii	P.c. chabaudii	P. berghei
18:Spot 11	PF14_0723	LCCL domain-containing protein CCP1	K.HGELGDNFLYGIR.S K.TYDEELK.E K.LAFLTNK.N K.VDENFLQVK.S K.HDPTTSK.E	5/3.27				184.88	4.9	Yes	No	No	S	PY05554	PC000402.02.0	PB000652.01.0
19	**PF10_0159**	**Glycophorin-binding protein 130 precursor**	K.STGVSNYK.N R.ILAEGEDTCAR.K	2/2.8	**1**	**0**	**0.000956**	95.85	4.77	No	Yes	1	T/S			
20	PF11_0467	MFS general substrate transporter, with GAF domain-like sequence	K.INSCLYHK.N K.ITHVNNIQSIDTK.G K.YSNILQTK.K	3/5.64	3	0	0.001048	32.96	9.69	No	No	3	T/S	PY07272	PC000069.00.0	PB000947.01.0
21	PF11_0324	Hypothetical	K.SNINMHK.N K.NILENDIR.Y K.NDDHNSIMDHHYIFK.I	3/2.11				206.85	9.67	No	No	3	S	PY06083	PC000188.01.0	
22	**PF11_0220**	**Protein Kinase; putative**	R.EENIFIPLK.I K.ECILHRMHK.D R.NISNILYECISR.K	3/2.12	**1**	**1**	**0.000308**	202.85	7.19	No	No	No	T/S	PY07273	PC302273.00.0	PB000021.00.0
23	**PF11_0369**	**Hypothetical**	K.GQLEELK.K K.SNNNNYSYFSK.L	2/13.8	**1**	**1**	**0.001194**	19.13	9.94	No	No	No	R	PY03008	PC000664.01.0	PB001093.02.0
24	**MAL8P1.126**	**DegP-like serine protease 1 precursor**	K.NKIGQIK.F K.KNDIILR.V K.IMLMIIMLISLR.T	3/3.68	**5**	**5**	**0.001165**	101.9	9.84	Yes	No	No	S			
25	PF11_0381	Subtilisin-like protease 2	K.EILNFLPK.E K.HDFTNESISNSR.K	2/1.8				155.22	8.58	Yes	No	1	S	PY01222	PC000550.00.0	PB000680.03.0
26:Spot 3	**PFB0750w**	**Vacuolar protein sorting protein VPS 45, putative; Sec 1 family;**	K.VLVLDDETK.S K.EQTFLNLAK.T	2/3.05				86.6	6.98	No	No	No	T/S	PY01251	PC301441.00.0	PB000477.03.0
27	PFF0615c	*Plasmodium falciparum* membrane protein Pf12 precursor, Sexual stage antigen; contains 6-Cys motif	K.KDIINR.G K.LTNIIMDHYNNTFYSR.L	2/7.49				40.26	8.46	Yes	No	GPI anchor	S			
28	**PFB0315w**	**41 kDa antigen**	K.NVQSSKK.K K.YVNNSNINKIK.I K.ELQEEKMK.K	3/1.84	**2**	**1**	**0.000809**	205.94	9.88	No	No	No	S	PY04357	PC000666.00.0	PB000270.00.0
29:Spot 6	PF07_0074	Hypothetical	R.RTQANVR.S K.RSVSWESTR.S K.CVDLSMIKGK.Y	3/2.09	0	0	0.000000	183.1	9.9	No	No	No	S	PY03294	PC300736.00.0	PB000710.02.0
30:Spot 2	PFB0655c	Hypothetical	K.EKLSESEK.K K.EEDQNGSNSK.E K.QDIFIHDNIIHMNDNIKK.E	3/6.78	0	0	0.000000	73.96	5.4	No	No	No	S/R		PC301157.00.0	PB000194.00.0
31:Spot 8	**PFB0765w**	**48% similarity to Viral A type inclusion proteins**	K.SIENLLNDK.N K.EYEIQIKEK.E K.IFSVEK.Y K.EINMIIEQYNKK.I	4/3.2	**3**	**2**	**0.001353**	167.87	6.5	No	No	No	S		PC000598.00.0	PB000254.00.0
32:Spot 9	**PF07_0113**	**Hypothetical**	K.IYGNIYK.N K.IDNTILGK.K R.ICHLFEKNNNDK.Y	3/4.67	**4**	**3**	**0.001325**	86.93	9.95	Yes	No	1	T/S	PY02093	PC302169.00.0	PB300563.00.0
33:Spot 10	PF10_0380	Kinase(Apicomplexa-specific FIKK family),FIKK 10.2, Trophozoite antigen R45	K.IELVNGNSK.D	1/1.20				108.47	5.17	No	Yes	No	R			

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. Proteomics 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

TABLE II

Sequence of the primers used for the PCR amplification of selected genes/gene fragments to express His_6/MBP fusion proteins

The proteins have been named according to the locus annotated in the *Plasmodium* database. The added restriction sites are underlined in the primer sequence. The protein region (comprising functional stretches) expressed as a His/MBP fusion protein is indicated by its amino- and carboxyl-terminal amino acids.

Serial no.	Locus	Protein name	Sequence of primers/peptides	Expressed region
1	PFB0190c	PfB190 (PfSEL1)	5′-ggg ggA TCC ATg gAA TAT TTT gTA AAA TTA gCT gAT 3′-CCg TCg ACC TTA CTC gAg TCC ATT CCT TCC CAT TgA TTC ATC	Glu^{1080}–Gly^{1238}, includes Sel1 repeats
2	PF14_0462	PfN462 (PfSEL2)	5′-ggg ggA TCC ATg gCA TCT AAA TAT AAT AAT TCA gAA 3′-CCg TCg ACC TTA CTC gAg TgC TTC TTT ATT TCT ACC TTT TTC	Ala^{382}–Ala^{553}, includes Sel1 repeats
3	PF11_0220	PfK22 (PfEK)	5′-ggg ggA TCC ATg ggg AAg ATT TTA TCT AAT AAA TAT gAT 3′-CCC CCT Agg gTC gAC TTC TAA TAA TAT TAA ATT TAC Tag	Lys^{201}–Glu^{577}, includes kinase activity domain
4	PF11_0139	PfK139 (PfEP)	5′-ggg ggA TCC ATg ggg AAg AgT TTg gAg AAT AAC gAA 3′-CCC CCT Agg gTC gAC CAT AAA ATg ACA TTT CCT AAg ACA	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 (PfSEL1), PfN462 (PfSEL2), PfK22 (PfEK), and PfK139 (PfEP) correspond to the PlasmoDB accession numbers PFB0190c, PF14_0462, PF11_0220, and PF11_0139, respectively (Table II). Recombinant His_6-tagged PfSEL1 and PfSEL2 contain Sel1 repeats (structural and protein-protein interaction module), whereas recombinant MBP-tagged PfEK and His_6-tagged PfEP include the kinase and phosphatase catalytic domains, respectively (Table II and supplemental Fig. 2). Polyclonal sera were raised in mice against purified PfSEL1 and PfSEL2 protein fragments and against PfEK and PfEP 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. *PfSEL1* peaked at the early trophozoite stage (30 hpi), whereas *PfSEL2* peaked a little later at the late trophozoite stage (40 hpi) when the parasite is metabolically most active. However, *PfEK* and *PfEP* showed almost similar levels in all the stages (with a slight increase in the trophozoite/schizont stages) suggesting their near constitutive expression (Fig. 2*a*).

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 PfSEL1, PfSEL2, PfEK, and PfEP (Fig. 2*b*). 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 PfEP; Table III and Fig. 2*b*) were detected in the three fractions, namely parasite lysate, infected RBC cytosol (obtained by streptolysin O permeabilization of iRBCs), and the concentrated extracellular culture supernatant (*lanes 1*, *2*, and *3*, respectively, in each blot in Fig. 2*b*). The same set of sera did not show reactivity with similar fractions prepared from uninfected erythrocytes. As a control, αPfMIF 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 Immunoelec-

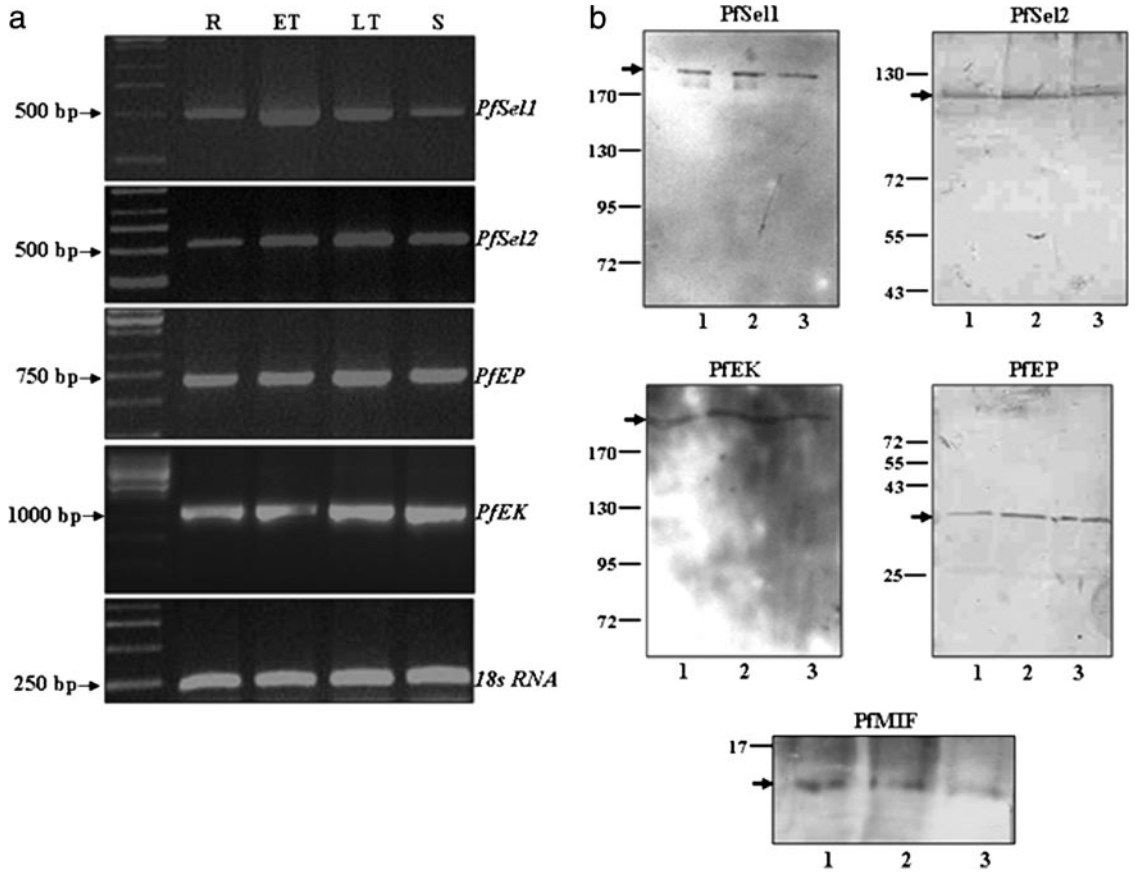

FIG. 2. *a*, stage-specific transcript levels of selected genes in asexual blood stage parasites. Total RNA isolated from synchronized *P. falciparum* 3D7 parasite cultures at 16 h (ring (*R*)), 30 h (early trophozoite (*ET*)), 40 h (late trophozoite (*LT*)), and 48 h (schizont (*S*)) postinvasion were 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 PfSEL1 and PfSEL2 protein fragments and against synthetic peptides unique to PfEK and PfEP. *Lanes 1*, parasite lysate (isolated from 1×10^{10} iRBCs); *lanes 2*, infected RBC cytosol (obtained by streptolysin O permeabilization of 1×10^{9} iRBCs); *lanes 3*, the extracellular culture supernatant of intact iRBCs (concentrated supernatant from 100 ml of parasite culture). As a control, rabbit αPfMIF 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).

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 (αPfSEL1, αPfSEL2, αPfEK, and αPfEP) antibodies with αPfEK also showing specific rimlike staining of the plasma membrane of the infected RBC. αPfMIF antibody was used each time as a marker for co-localization as PfMIF is exported via the Maurer clefts to the extracellular medium after schizont rupture. A partial co-localization of PfMIF 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-PfEK antibody could not abolish the staining observed because the peptide epitope in PfEK lay outside the catalytic domain that was cloned and expressed as the recombinant protein (*CONTROL column* in three panels in Fig. 3). However, anti-PfEK and anti-PfEP 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. 4*a*, PfSEL1 showed a secreted/extracellular staining pattern, localizing in the iRBC cytosol close to the erythrocyte plasma membrane. The gold particle staining

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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 PfEK and PfEP were shown to be active extracellular kinase and phosphatase, respectively.

Serial no.	Parasite protein	Molecular mass		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
		Predicted	Observed						
		kDa							
1	PfB190 (PfSEL1)	272	>200	SP	ET > LT > S > R	+	iRBC cytosol/extracellular	+	Immunomodulator
2	PfN462 (PfSEL2)	100	100	SP	LT > S > ET > R	+	iRBC cytosol/extracellular	+	Immunomodulator
3	PfK22 (PfEK)	202	200	–	LT > S > ET > R	+	iRBC cytosol/extracellular	+	Extracellular kinase
4	PfK139 (PfEP)	25	35	–	LT > S > ET > R	+	iRBC cytosol/extracellular	+	Extracellular phosphatase
5	PfMIF	12	12	–	LT > S > ET > R[a]	–	iRBC cytosol[a]	+	Extracellular cytokine[a]

[a] As studied by Augustijn *et al.* (32).

showed the protein coating the surface of the iRBC and also being released out in the extracellular milieu. PfSEL2 (seen as *dots* coating vesicle-like structures) and PfEK also showed localization close to iRBC plasma membrane while being exported out of the infected red cells (Fig. 4, *b* and *c*). PfEP localized in the extensive membranous network of the parasite from where it seemed to be trafficked to the iRBC cytosol and plasma membrane (Fig. 4*d*). Preimmune sera did not show staining of infected RBCs (not shown). Altogether immunolocalization of the four ES antigens clearly indicated their export beyond the iRBC plasma membrane.

Reactivity of the Parasite ES Antigens with Malarial Patient Sera—Sera collected from *P. falciparum*-infected patients were examined by Western blot to check whether antibodies against ES antigens were elicited during natural infection with *P. falciparum*. Many proteins in the ES culture supernatant, specifically the four recombinant ES antigens, were positively recognized by the patients' sera but not by sera from control subjects (Fig. 5). Incidentally the same patient sera samples also recognized PfMIF. A previous study has also reported reactivity of PfMIF with Kenyan patient samples (33). These results thus showed that many of the malaria parasite extracellular/secreted antigens elicit an immune response during natural infection.

Functional Characterization of the Selected ES Antigens—To get an insight into the functions of parasite 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* SEL1 is a secreted or membrane-associated protein and a negative regulator of LIN-12 and GLP-1 receptors of the Notch signaling pathway (26, 27). As PfSEL1 and PfSEL2 are homologs of *C. elegans* SEL1, we studied their possible role in Notch pathway modulation. The main aim of the microarray experiment was to investigate the intriguing possibility that PfSEL1 and PfSEL2 proteins might have immunomodulatory effects *in vivo* (in the mouse model). This was done by analyzing changes in expression level of genes involved in the Notch signaling pathway in immunized mice. Four groups of mice (three mice/group) were immunized: first with PfSEL1 in PBS/CFA, second with PfSEL2 in PBS/CFA, third with PfMSP1 (a well characterized malarial antigen that is known not to be involved in the Notch signaling pathway) in PBS/CFA, and fourth with PBS/CFA only (as a control). Spleen cells were then isolated (*ex vivo*) from these immunized mice and restimulated with corresponding recall antigens/control immunogen so that these splenocytes from primed mice were able to produce a strong and detectable immune response. RNA extracted from stimulated and control splenocytes was used as a probe to blot against Notch pathway arrays. Differences in expression of genes corresponding to Notch signaling pathways were measured for PfSEL1/PfSEL2-stimulated cells *versus* unstimulated/PfMSP1-stimulated cells (supplemental Fig. 4b). Change was measured as -fold ratios be-

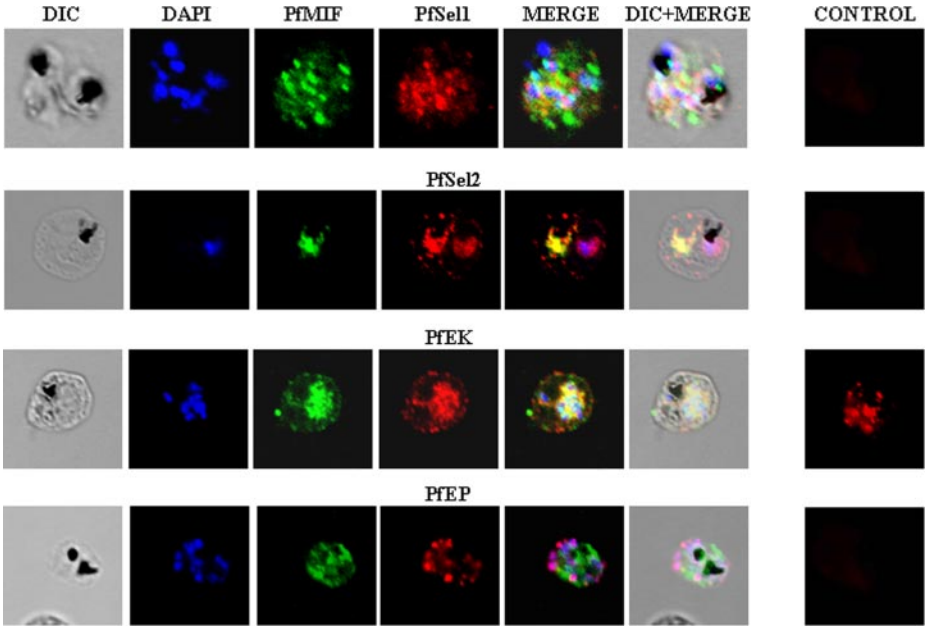

FIG. 3. **Indirect immunofluorescence assay and confocal microscopy to localize the selected ES antigens by co-immunostaining of *P. falciparum*-infected erythrocytes.** Air-dried infected erythrocytes were incubated with mouse antibodies specific to the selected proteins at 1:100 dilution (*column 4*; *red*). Rabbit αPfMIF antibody was used as a co-localization marker (*column 3*; *green*). The parasite nuclei were stained with 4′,6-diamidino-2-phenylindole (*DAPI*) (*column 2*; *blue*). *Columns 1*, *5*, and *6* show a differential interference contrast (*DIC*) image, merged image, and differential interference contrast with merged image, respectively. Negative controls were performed using antibodies preincubated with the respective recombinant proteins before incubating with the fixed infected RBCs (*column 7*).

tween individual spots of SEL1/SEL2-stimulated arrays *versus* control/MSP1-stimulated arrays by densitometric scan. For better visualization of gene expression changes, log values of these -fold ratios were tabulated (Fig. 6). Immunization of mice with MSP1 (control immunogen) did not cause significant change in the expression of genes of the Notch pathway (supplemental Table 1). It was also observed that splenocytes from naïve mice/adjuvant control mice did not respond to *in vitro* stimulation with either SEL1 or SEL2, and there was no change in the pathway-specific gene expression profile compared with that of splenocytes from control mice. Hence the observed changes in the pathway-specific gene expression profile were indeed due to *in vivo* priming of mice with specifically either SEL1 or SEL2. Based on this pathway-specific microarray gene expression profiling system, we observed that Notch-binding genes (*Delta1/3/4* and *Jag1/2*), some of the Notch receptor processing genes (*Adam10*, *Aph1a*, and *Nctsn*), and some genes regulating cell differentiation (*Notch1/3/4* and others) were prominently down-regulated, whereas *Notch2*, *Psen2*, and *Psenen* were found to be up-regulated compared with control (Fig. 6). These array results suggest that the extracellular PfSEL1/PfSEL2 proteins modulate Notch signaling in host cells by altering the expression of many genes involved in the Notch pathway, which eventually regulates the peripheral immune system through its ability to influence cell survival and growth.

We also studied whether two other ES antigens, PfEK and PfEP were functionally active. As shown in Fig. 7*a*, recombinant PfEK was found to be active as it was able to phosphorylate both myelin basic protein and Histone2A (Fig. 7*a*). Interestingly recombinant PfEK, when added to *in vitro P. falciparum* culture in the presence of cell-impermeable [γ-^{32}P]ATP, phosphorylated a ~70-kDa protein in the extracellular culture supernatant over and above basal phosphorylation (by either host erythrocytic kinases or other kinases exported by the intracellular parasite beyond the iRBC membrane in culture). However, in similar conditions, PfEK did not phosphorylate any protein in the ES culture supernatant of uninfected RBCs, suggesting that the substrate is parasite-specific (Fig. 7*b*). This clearly showed that the parasite exports active kinases to the outer surface of or beyond the iRBC plasma membrane that phosphorylate other proteins in the extracellular milieu. Recombinant PfEP was also found to be catalytically active as it dephosphorylated protein kinase C-phosphorylated myelin basic protein and also Src-phosphorylated myelin basic protein substrates. Heat inactivation of PfEP abolished its dephosphorylation potential (Fig. 7*c*). Some proteins of the ES culture supernatant, prelabeled using protein kinase C, were dephosphorylated by the recombinant PfEP (Fig. 7*d*), thus indicating a potential role of the parasitic phosphatase in the extracellular culture medium.

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.*

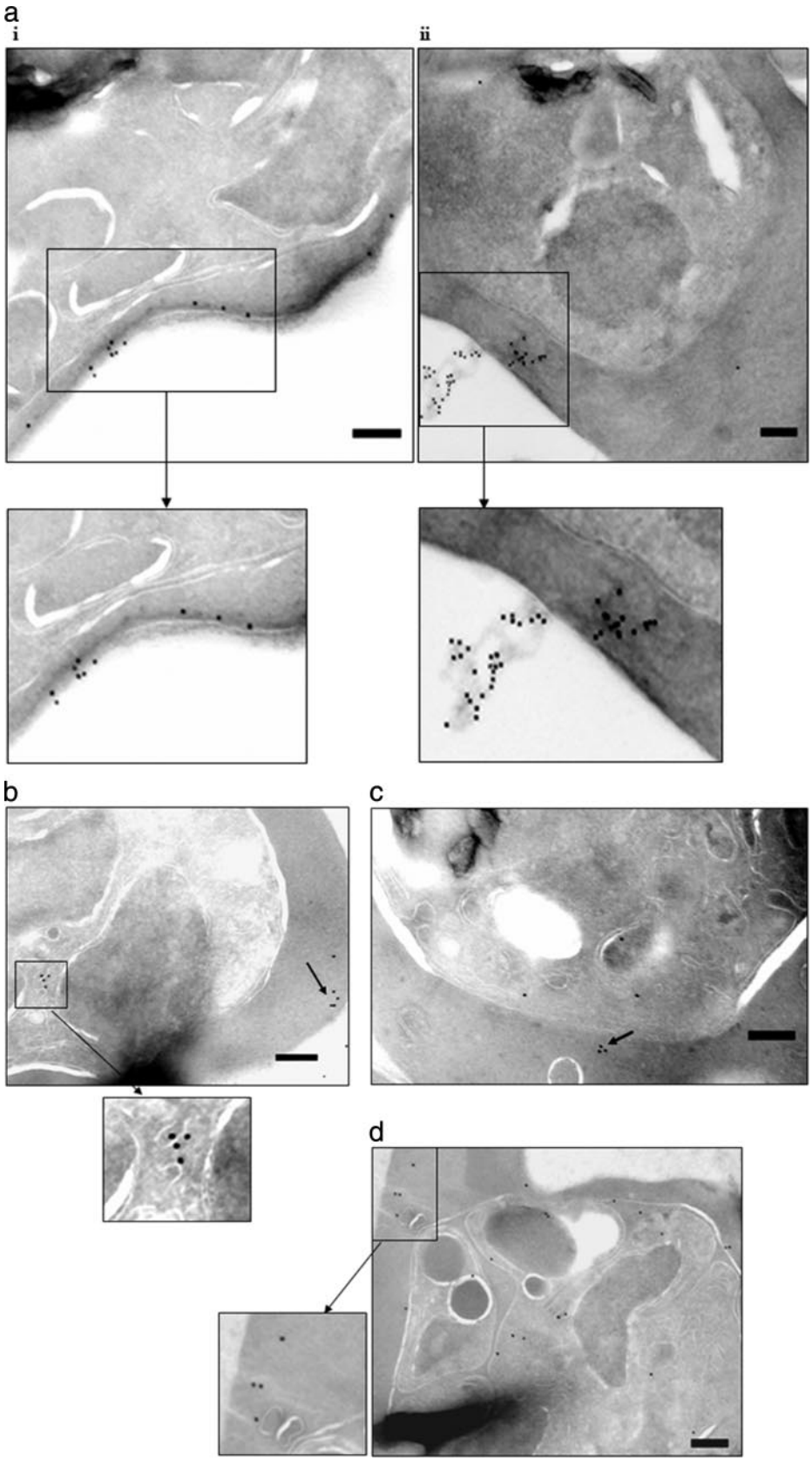

FIG. 4. ***a–d*, localization of the selected ES antigens by immunoelectron microscopy.** Ultrathin sections of *P. falciparum*-infected erythrocytes (at late trophozoite/schizont stages) were labeled with specific sera (against the four selected proteins) and gold-conjugated secondary antibody. Localization 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.

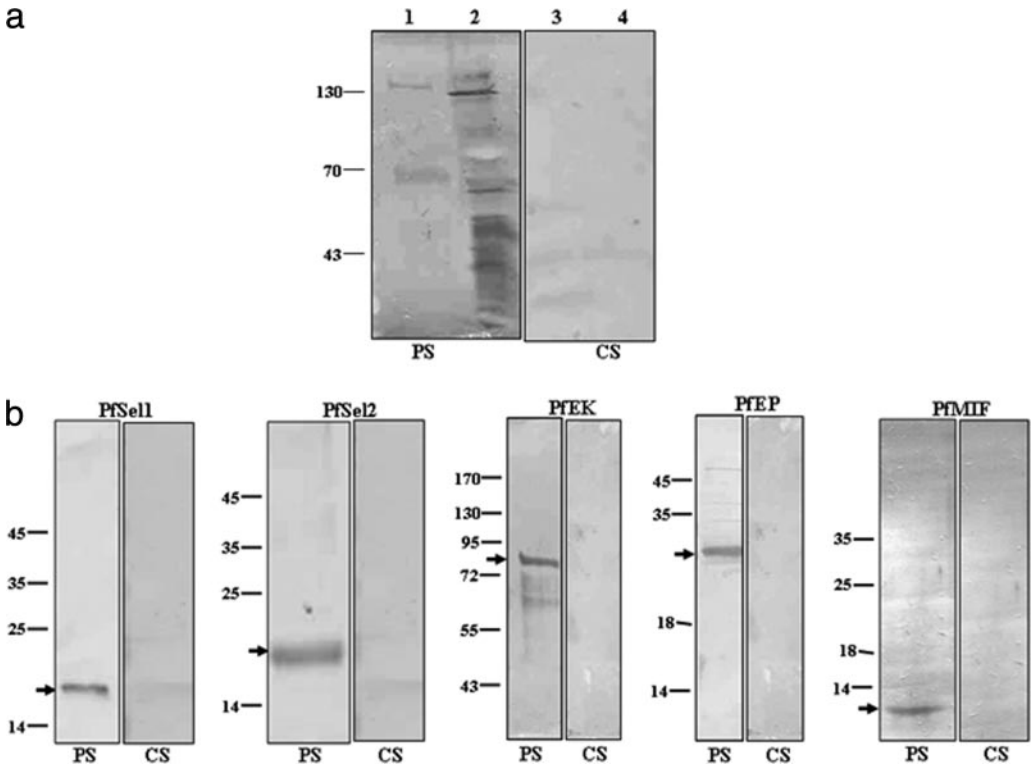

FIG. 5. ***a* and *b*, reactivity of parasite ES antigens with malarial patient sera.** Western blot analyses were performed using sera collected from *P. falciparum*-infected patients (*PS*) and from control subjects (*CS*) at 1:50 dilution. *a*, *lanes 1* and *3*, extracellular culture supernatant 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 PfSEL1, PfSEL2, PfEK, PfEP, and PfMIF (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).

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 +HIS 3D7 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 ^{35}S-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 by 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

Down-regulated genes with Log2(Fold Ratio)< -6

Gene name	Description	Position of Spot	Log2(Fold Ratio Sel1)	Log2(Fold Ratio Sel2)
Adam17	*A disintegrin and metallopeptidase domain 17*	*A3*	*-6.89*	*-6.74*
Catnb1	*Catenin (cadherin associated protein), beta 1*	*A7*	*-6.73*	*-8.33*
Cd44	*CD44 antigen*	*B3*	*-6.79*	*-8.96*
Chuk	*Conserved helix-loop-helix ubiquitous kinase*	*B8*	*-7.96*	*-7.38*
Dtx2	*Deltex 2 homolog (Drosophila)*	*C5*	*-2.13*	*-9.96*
Fgf	*C-fos induced growth factor*	*D1*	*-2.13*	*-6.50*
Fos	*FBJ osteosarcoma oncogene*	*D2*	*-7.15*	*-7.64*
Fzd10	*Frizzled homolog 10 (Drosophila)*	*D5*	*-6.50*	*-4.75*
Fzd4	*Frizzled homolog 4 (Drosophila)*	*D8*	*-6.64*	*-4.60*
Fzd5	*Frizzled homolog 5 (Drosophila)*	*E1*	*-6.26*	*-5.21*
Fzd6	*Frizzled homolog 6 (Drosophila)*	*E2*	*-4.64*	*-7.64*
Hes6	*Hairy and enhancer of split 6 (Drosophila)*	*F5*	*-8.96*	*-7.96*
Heyl	*Hairy/enhancer-of-split related with YRPW motif-like*	*F8*	*-4.64*	*-6.15*
hr	*Hairless*	*G2*	*-8.38*	*-5.44*
Jag1	*Jagged 1*	*G8*	*-3.73*	*-7.38*
Lmo2	*LIM domain only 2*	*H7*	*-8.96*	*-6.96*
Maml1	*Mastermind like 1 (Drosophila)*	*I3*	*-6.23*	*-5.00*
Ncor2	*Nuclear receptor co-repressor 2*	*J1*	*-8.89*	*-4.64*
Up-regulated genes with Log2(Fold Ratio)>1				
Aes	**Amino-terminal enhancer of split**	**A4**	**1.02**	**1.01**
Fosl1	**Fos-like antigen 1**	**D3**	**1.03**	**1.00**
Gsk3b	**Glycogen synthase kinase 3 beta**	**F2**	**1.01**	**1.00**
Hes1	**Hairy and enhancer of split 1 (Drosophila)**	**F3**	**1.14**	**1.03**
Hes5	**Hairy and enhancer of split 5 (Drosophila)**	**F4**	**1.09**	**1.02**
Lrp5	**Low density lipoprotein receptor-related protein 5**	**I2**	**1.09**	**1.08**
Notch2	**Notch gene homolog 2 (Drosophila)**	**J7**	**1.59**	**2.00**
Nr4a2	**Nuclear receptor subfamily 4, group A, member 2**	**K2**	**1.02**	**1.00**
Numbl	**Numb-like**	**K5**	**-0.04**	**1.73**
Pparg	**Peroxisome proliferator activated receptor gamma**	**L2**	**1.58**	**2.01**
Psen2	**Presenilin 2**	**L4**	**0.95**	**1.04**
Psenen	**Presenilin enhancer 2 homolog (C. elegans)**	**L5**	**1.02**	**1.00**

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 PfSEL1, PfSEL2, and medium only (control) for 24 h. 1 μg 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 $\log_2$ (-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-Jinkins.

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-

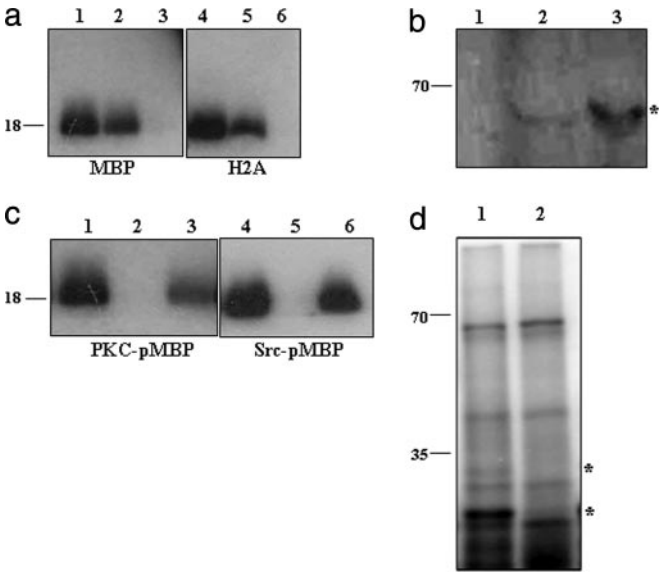

FIG. 7. ***a–d*, catalytic activity of PfEK and PfEP.** Kinase assays were performed in 20-μl reactions in buffer containing 100 mM Tris (pH 8.0), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 10 mM $CaCl_2$, [γ-^{32}P]ATP, and 0.1 mM orthovanadate. *a*, 2 μg of substrate myelin basic protein (*MBP*) (*lane 1*, with 0.1 μg of PfEK; *lane 2*, with protein kinase C; *lane 3*, with 0.1 μg of heat-inactivated PfEK), and 2 μg of substrate Histone2A (*H2A*) (*lane 4*, with 0.1 μg of PfEK; *lane 5*, with protein kinase C; *lane 6*, with 0.1 μg of heat-inactivated PfEK). *b*, recombinant PfEK was added to *in vitro P. falciparum* culture in the presence of cell-impermeable [γ-^{32}P]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 extracellular kinases of the parasite. Activity of purified PfEP 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 PfEP; *lane 3*, with heat-inactivated PfEP; *lane 4*, Src-phosphorylated myelin basic protein substrate (0.5 μg), *lane 5*, substrate with PfEP; *lane 6*, with heat-inactivated PfEP. *d*, *lane 1*, protein kinase C-phosphorylated concentrated culture supernatant from *P. falciparum* culture; *lane 2*, protein kinase C-phosphorylated concentrated culture supernatant incubated with PfEP. Molecular mass markers are indicated (kDa). *Asterisks* show phosphorylated proteins in *panel B* whereas dephosphorylated proteins in *panel D*.

cytes were detected; the latter were either released or fragmented and accumulated in the extracellular fraction during sample preparation; for example, the malarial adhesion protein PfEMP1; an acidic phosphoprotein precursor, PCEMA1; and others. A few merozoite surface proteins (Pf12, PFSUB2, GBP130, and PfRh2) were also detected that could have been released because of inadvertent parasite lysis, which happens even in the best of sample preparations. The presence of protein PFG377, a *P. falciparum* female gametocyte-specific antigen, can be explained by earlier studies that have suggested that the parasite commitment to sexual differentiation occurs prior to schizont maturation (31). Hence the parasite might have already started the buildup of its repertoire of proteins required for the sexual stages. Alternatively studies have reported that although sexual development is a fundamental developmental switch gametocytes and asexual trophozoites share the same features in their *early* G_1 phase of growth with their protein expression patterns being quite similar, thus reflecting conserved mechanisms of parasite development in different stages. For example, 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 proteases like PfSUB1 and serine repeat antigen protein that are released in the extracellular milieu just as the schizont-infected RBCs rupture (38). DegP-like serine protease, the homolog of a bacterial ectoprotease, was an interesting exception (25). These observations strengthen the validity of this proteomics study.

Validation of Extracellular Nature of P. falciparum ES Antigens—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 punctate vesicle-mediated export of PfSEL1, PfSEL2, 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 PfSEL1, a large number 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 trafficking of these proteins in the infected erythrocyte as they lack the host cell targeting/PEXEL motif shown to target proteins to the host erythrocyte. In addition to immunolocalization studies, we also observed that all four ESAs were strongly recognized by sera from *P. falciparum*-infected patients. Together these results provided strong support for the extracellular nature of the identified *P. falciparum* proteins.

Potential Roles of P. falciparum ES Antigens—The excretory/secretory proteins produced by parasites are key players in host-parasite interactions. Many parasites use genetic variability to defeat host immunity and drug treatments. For example, 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-

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 $\gamma\delta^+$ lymphocytes (44). The LCCL family of Apicomplexa-specific proteins (PfCCP1 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 $\alpha\beta$ 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 $\gamma\delta$ 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 PSEL1/PSEL2 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 mice 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 erythro-

[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 (PfEK and PfEP 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. PfEK and PfEP 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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