A Library of Functional Recombinant Cell-surface and Secreted *P. falciparum* Merozoite Proteins*

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Malaria, an infectious disease caused by parasites of the *Plasmodium* genus, is one of the world's major public health concerns causing up to a million deaths annually, mostly because of *P. falciparum* infections. All of the clinical symptoms are associated with the blood stage of the disease, an obligate part of the parasite life cycle, when a form of the parasite called the merozoite recognizes and invades host erythrocytes. During erythrocyte invasion, merozoites are directly exposed to the host humoral immune system making the blood stage of the parasite a conceptually attractive therapeutic target. Progress in the functional and molecular characterization of *P. falciparum* merozoite proteins, however, has been hampered by the technical challenges associated with expressing these proteins in a biochemically active recombinant form. This challenge is particularly acute for extracellular proteins, which are the likely targets of host antibody responses, because they contain structurally critical post-translational modifications that are not added by some recombinant expression systems. Here, we report the development of a method that uses a mammalian expression system to compile a protein resource containing the entire ectodomains of 42 *P. falciparum* merozoite secreted and cell surface proteins, many of which have not previously been characterized. Importantly, we are able to recapitulate known biochemical activities by showing that recombinant MSP1-MSP7 and P12-P41 directly interact, and that both recombinant EBA175 and EBA140 can bind human erythrocytes in a sialic acid-dependent manner. Finally, we use sera from malaria-exposed immune adults to profile the relative immunoreactivity of the proteins and show that the majority of the antigens contain conformational (heat-labile) epitopes. We envisage that this resource of recombinant proteins will make a valuable contribution toward a molecular understanding of the blood stage of *P. falciparum* infections and facilitate the comparative screening of antigens as blood-stage vaccine candidates. ***Molecular & Cellular Proteomics 12: 10.1074/mcp.O113.028357, 3976–3986, 2013.***

Parasites of the *Plasmodium* genus are the etiological agents responsible for malaria, an infectious disease mostly occurring in developing countries with up to 40% of the world's population described as being at risk of the disease. Among the *Plasmodium* species that can affect humans, *Plasmodium falciparum* is responsible for the highest mortality, causing around one million deaths annually, mostly in children under the age of five (1). The clinical symptoms of malaria occur during the cyclic asexual blood stage of the parasite lifecycle when merozoites, that have invaded and replicated within host erythrocytes, are released into the bloodstream before invading new red blood cells (2). Despite intensive efforts from the research community there is currently no licensed vaccine for malaria. The leading candidate RTS,S/AS01, which targets the pre-erythrocytic stage of the disease and was tested in phase III trials, conferred 30 to 50% protection from clinical malaria, depending on the age group studied (3, 4). This limited efficacy has led to calls for a more effective vaccine and many have suggested that a combinatorial vaccine that additionally targets the blood stage may increase efficacy.

A vaccine targeting the proteins expressed on the surface of the blood stage of the parasite is conceptually attractive because merozoites are repeatedly and directly exposed to the human humoral immune system and naturally acquired antibodies against these proteins have been shown to confer at least partial immunity (5–8). Despite this, only a few antigens discovered before the completion of the parasite genome sequence have been assessed in detail (9) and clinical vaccine trials using antigens that target the blood stage have so far shown limited efficacy, mostly caused by antigenic diversity (10). The sequencing of the parasite genome (11) has identified all possible targets but the systematic screening of these new candidates to assess their potential as a vaccine is hampered by the inability to systematically express recombinant *Plasmodium* proteins in their native conformation (12–

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15). Likely explanations might be the high (~80%) A:T content of the *P. falciparum* genome resulting in low codon usage compatibility in heterologous expression systems, the large size (> 50 kDa) of many proteins, the presence of long stretches of highly repetitive amino acids, and the difficulty in identifying clear structural domains within these proteins using standard prediction computer programs (11). Extracellular proteins, in particular, present an additional challenge because they often have signal peptides and transmembrane regions that can negatively impact expression (16–18) and contain structurally important disulfide bonds. However, unlike most other eukaryotic extracellular proteins, *Plasmodium* cell surface and secreted proteins are not modified by *N*-linked glycans because of the absence of the necessary enzymes (19).

To express *Plasmodium* proteins for basic research and vaccine development, a diverse range of expression systems have been tried (12) ranging from bacteria (17, 18), yeast (13), *Dictyostelium* (20), and plants (21) to mammalian cells (22) and cell-free systems (23–25). To circumvent the problem of codon usage, bacterial (26) and yeast (27) strains with modified tRNA pools have been developed, or sequences of the gene of interest synthesized and codon-optimized to match that of the expression host (28, 29). Although *Escherichia coli* has been the most popular expression system because of its relative simplicity and cost effectiveness, large-scale production of soluble functional *Plasmodium falciparum* recombinant proteins remains challenging with success rates ranging from just 6 to 21% (17, 18) and is often hindered by the need for complex refolding procedures. Similarly, attempts have been made to compile large panels of parasite proteins using *in vitro* translation systems (23, 25, 30, 31). These systems, however, require reducing conditions and are therefore not generally suitable for the systematic expression of extracellular proteins that occupy an oxidizing environment and critically require the formation of disulfide bonds for proper function. As a result, functional analyses of extracellular parasite proteins have often been restricted to smaller subfragments of the proteins that can be expressed in a soluble form rather than the entire extracellular region. Although eukaryotic expression systems are able to add disulfide bonds, they also often inappropriately glycosylate parasite proteins, adding further complication (32). A generic method that would overcome these technical challenges to express, in a systematic way, panels of recombinant *Plasmodium* proteins that have retained their native function and conformation would therefore be a valuable resource for the molecular investigations of erythrocyte invasion and the development of a blood stage vaccine.

To generate a resource of correctly folded recombinant merozoite proteins, we used a mammalian expression system and established the parameters necessary for high-level expression. Using this method, we compiled a panel of 42 proteins that corresponds to the repertoire of abundant cell surface and secreted merozoite proteins of the 3D7 strain of *Plasmodium falciparum*. Biochemical activity of these proteins was demonstrated by recapitulating known protein interactions and by showing conformation-sensitive immunoreactivity of the recombinant proteins using immune sera.

EXPERIMENTAL PROCEDURES

Design and Synthesis of P. falciparum Merozoite Cell Surface and Secreted Expression Plasmids—The regions corresponding to the entire extracellular domains of 51 merozoite cell-surface proteins from the *P. falciparum* 3D7 strain were determined by using transmembrane and GPI-anchor (33, 34), or signal peptide (35) prediction software. Sequences encoding the extracellular domains of these proteins, with the exception of their signal peptide, were made by gene synthesis (GeneartAG) and are presented in Table I. All sequences were codon-optimized for expression in human cells and all potential *N*-linked glycosylation sites (NXS/T) were modified by substituting the serine or threonine residue with an alanine residue to prevent the inappropriate addition of large glycans that are absent in the native *P. falciparum* proteins. The coding sequences were flanked by unique NotI and AscI sites and cloned into a derivative of the pTT3 expression vector (36) between the leader sequence of the mouse variable κ light chain 7–33 (37), and a rat Cd4 domains 3 and 4 tag followed by an enzymatic biotinylation sequence as previously described (38). All expression constructs were cotransfected with the BirA biotinylation enzyme into HEK293E cells and are available from Addgene, a non-profit plasmid repository (www.addgene.org). The soluble biotinylated recombinant proteins were collected from the cell culture supernatant 6 days post-transfection, and dialyzed into HBS before analysis. During gene synthesis, constructs encoding the full-length ectodomain of RH1, RH2b and RH4 proved to be toxic in bacteria, and only subfragments could be produced as presented in Table I.

Enzyme-linked Immunosorbant Assay (ELISA)—The biotinylated ectodomains of the *P. falciparum* library were serially diluted 1:2 up to a final dilution of 1:128 and all dilutions were immobilized on streptavidin-coated plates (NUNC) before being incubated for one hour with 1 μg/ml OX68 antibody (AbD Serotec), which binds the Cd4 tag. The plates were washed in PBS/0.1% Tween20 (PBST) before incubation with an anti-mouse immunoglobulin antibody coupled to alkaline phosphatase (Sigma) for one hour at room temperature. After washes in PBST and PBS, wells were incubated with *p*-nitrophenyl phosphate at 1 mg/ml and optical density measurements taken at 405 nm.

For the immunogenicity study, proteins were either immobilized as above or denatured for 10 min at 80 °C before immobilization on streptavidin-coated plates and incubation with pooled sera from 10 malaria-exposed or malaria-naïve individuals used at a 1:1000 dilution in PBST/2% BSA.

Western Blot—Between 5 and 30 μl of dialyzed transfection medium containing the recombinant proteins was resolved by SDS-PAGE under reducing conditions before blotting onto Hybond-P PVDF membrane (GE Healthcare) overnight at 30 V. Membranes were blocked with 2% BSA in PBST and incubated with 0.02 μg/ml of streptavidin-HRP (Jackson Immunoresearch) diluted in PBST/0.2% BSA and detected with the Supersignal West pico chemiluminescent substrate (Pierce).

AVEXIS Screen—Interactions between the MSP1 and MSP7, and P12 and P41 proteins were identified using the AVEXIS[1] method as

[1] The abbreviations used are: AVEXIS, avidity-based extracellular interaction screen; EBA, erythrocyte binding antigen; HEK, human embryonic kidney; HBS, hepes-buffered saline; MSP, merozoite surface protein; RH, reticulocyte-binding protein homolog.

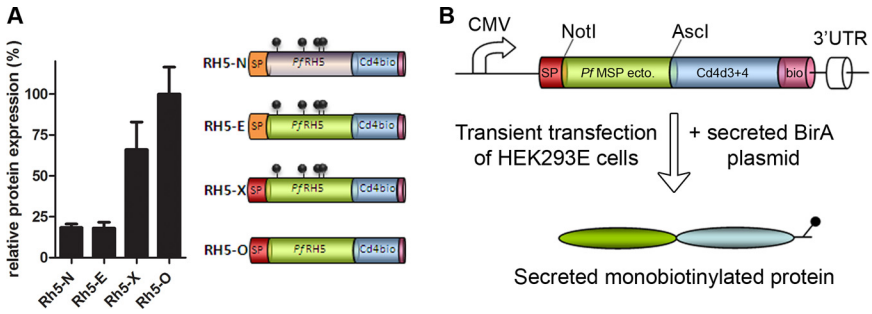

FIG. 1. **Addition of an exogenous signal peptide and mutation of potential *N*-linked glycosylation sites contribute to increased expression of recombinant full-length PfRH5 using a mammalian expression system.** *A*, Four different full-length *P. falciparum* RH5 protein expression constructs were synthesized: RH5-N, ***N***ative sequence; RH5-E, codon-optimized sequence (shaded green) with ***E***ndogenous signal peptide (orange); RH5-X, codon-optimized sequence with an e***X***ogenous high-scoring mouse antibody signal peptide (red); RH5-O, codon-***O***ptimized sequence with exogenous signal peptide and mutation of potential *N*-linked glycosylation sites. The approximate positions of potential *N*-linked glycosylation sites are represented by gray lollipops. The relative levels at which each of these constructs were expressed is shown; bar charts show mean ± S.D.; $n = 3$. *B*, Schematic representation of the merozoite protein constructs and procedure to produce soluble recombinant monobiotinylated Cd4d3 + 4-tagged proteins in mammalian cells.

previously described (38). Briefly, the codon-optimized sequences of the four proteins were subcloned into a prey construct between the leader sequence of the mouse variable κ light chain 7–33 (37), and a rat Cd4 domains 3 and 4 tag followed by the pentamerization domain of rat cartilaginous oligomeric matrix protein and the beta-lactamase coding sequence, as previously described (38). The prey pentamers were screened against the whole biotinylated merozoite library, and positive interactions identified by detecting colorimetric turnover of nitrocefin (Calbiochem, San Diego, CA) at 485 nm.

Flow Cytometry—Biotinylated EBA140-Cd4d3 + 4, EBA175-Cd4d3 + 4 ectodomains or Cd4 domains 3 + 4 alone (negative control) were immobilized around streptavidin-coated Nile Red fluorescent 0.4–0.6 μm microbeads (Spherotech Inc.) by incubation for 45 min at 4 °C and then presented to human erythrocytes. After incubating for 1 h at 4 °C, the cells were analyzed by flow cytometry using an LSR II machine (BD Biosciences). To test for binding specificity, purified human erythrocytes were either treated with 0.1 mU/10^6 cells of *Vibrio cholera* neuraminidase (Sigma) for 1 h at 37 °C and washed twice, or preincubated with the anti-GYPA BRIC 256 monoclonal antibody at a concentration of 0.5 μg/10^6 cells, before incubation with EBA175-coated microbeads.

RESULTS

Optimization of the Parameters for the Expression of Extracellular Merozoite Proteins in Mammalian Cells—To establish which factors are important for the expression of merozoite surface proteins, we selected the *Plasmodium falciparum* RH5 protein as an example. RH5 is a 526 amino acid protein secreted by the rhoptries upon erythrocyte invasion and has features that typify many merozoite surface and secreted proteins: it exhibits a very high (79.8%) A+T content in its coding region, does not possess any obvious structural domains and contains a predicted N-terminal signal peptide that gives very low scores when queried by eukaryotic signal sequence prediction programs such as SignalP3.0 (0.022 using Hidden Markov model, and 0.543 using the neural network model) (35). The protein sequence also contains four *N*-linked glycosylation sequons (NXS/T). Using a mammalian expression system, we have recently shown that an RH5 expression construct containing a codon-optimized sequence, mutated *N*-linked glycosylation sites and an exogenous signal peptide derived from a mouse antibody could produce a biochemically active RH5 protein that is able to bind its receptor, Basigin (39) and to elicit invasion-blocking antibodies (40). In contrast, RH5 expressed in bacterial expression systems required refolding procedures (41).

To determine the relative importance of the factors affecting the expression levels of full-length RH5, we assessed the individual contributions of each modification by comparing four constructs that contained: (1) the native sequence (RH5-N), (2) the codon-optimized coding sequence for expression in human cells in which the endogenous RH5 signal sequence was retained (RH5-E), (3) the codon-optimized coding sequence using an exogenous high-scoring signal peptide (RH5-X), and (4) the codon-optimized coding sequence using an exogenous signal peptide and in which the four threonine residues in the context of potential *N*-linked glycosylation sites were mutated to alanine (RH5-O) (39). Although these four mutations modify the protein coding sequence, we considered they would probably be less disruptive than the inappropriate addition of large glycan moieties, which are likely to sterically hinder protein binding interfaces and antibody epitopes. All four coding sequences were flanked by unique NotI and AscI restriction enzyme sites and subcloned into a plasmid that contained a rat Cd4 domain 3 and 4 (Cd4d3 + 4) tag followed by an enzymatically biotinylatable sequence. The Cd4d3 + 4 tag has been successfully used to express the ectodomains of cell surface proteins from diverse structural families in a soluble form while retaining the native binding properties of the fused protein (42). Using this approach, we were able to detect the secretion of a recombinant RH5 protein expressed at moderate level from the native mRNA sequence by ELISA. Although codon-optimization of the sequence on its own did not significantly improve expression of the RH5 protein, the addition of an exogenous signal sequence improved the expression level by ~3.5-fold (Fig. 1*A*).

This was further enhanced by removal of potential *N*-linked glycosylation sites which increased expression by 5.5-fold. In summary, the addition of an exogenous high-scoring signal peptide and mutation of *N*-linked glycosylation sites led to improved expression of RH5.

A Resource of Recombinant P. falciparum Merozoite Surface Proteins—Having determined the parameters that improved the expression levels of RH5, we used the same systematic approach to compile a resource of recombinant soluble *P. falciparum* merozoite surface proteins in a format that could be used in a wide range of biochemical, immunological and functional studies. From the literature, we selected 51 secreted and membrane-tethered merozoite proteins, which likely represent the repertoire of abundant merozoite surface proteins and consisted of 12 GPI-anchored, 10 peripheral, 9 micronemal, 16 rhoptry, and 4 other proteins (Table I). In the case of membrane-tethered proteins, we truncated the protein just before the predicted transmembrane or GPI-anchor to ensure the protein was soluble while preserving the binding functions of the extracellular region. All coding regions were made by gene synthesis and codon-optimized for expression in human cells, any potential *N*-linked glycosylation sequon was mutated, and the mouse antibody signal sequence was used. For three of the 51 proteins (RH1, RH2b and RH4), cloning of the entire ectodomain concatenated from 1 kbp gene synthesis fragments failed, possibly because of toxicity in bacteria (Geneart AG, personal communication). We therefore used a PCR-based approach to construct smaller subdomains of these proteins from the synthesized fragments (Table I). All proteins were expressed as Cd4d3 + 4-tagged enzymatically monobiotinylated proteins by transiently transfecting HEK293E cells (Fig. 1*B*).

Using this approach, 39 out of 51 proteins (76%) could be detected directly from the spent cell culture supernatant by ELISA, whereas another three (RH1, RH2b and RH4 subdomains) had to be purified first, suggesting extremely low levels of expression. Detectable expression was not observed for nine proteins (EBL1, PTRAMP, RAP1, RAP2, RAP3, CLAG3.2, RhopH2, RON3, and PF3D7_1431400), mostly belonging to the rhoptry and rhoptry neck category. When quantified against a reference, the expression levels of the recombinant soluble proteins varied widely, covering four orders of magnitude, with 10 of them (25%) expressed above 5 μg of protein per ml of cell culture supernatant (Table I). To determine the integrity of the recombinant proteins, the monomeric biotinylated proteins that could be detected by ELISA were normalized, resolved by SDS-PAGE and detected by anti-biotin blotting (Fig. 2). With the exception of RAMA and TLP, which gave smaller fragments than expected, all 37 remaining merozoite proteins were produced at a size compatible with their calculated expected molecular mass. Twelve of the proteins that could be detected from the cell culture supernatant had a molecular mass above 100 kDa, including the entire ectodomain of MSP1, the largest protein in the library. In addition to their full-length ectodomains, smaller fragments were also observed for some of the proteins, suggesting proteolytic processing.

The Recombinant Merozoite Proteins Recapitulate Known Protein–Protein Interactions—One important factor that must be considered when expressing proteins recombinantly is whether they have retained their native biochemical activity. To demonstrate this, we sought to recapitulate well-characterized known protein interactions that were first identified using native proteins. MSP1, one of the most abundant proteins displayed on the surface of merozoites, has been biochemically well characterized and is known to associate in a complex with MSP7 (43–45). To demonstrate that the recombinant MSP1 and MSP7 expressed using our method interacted directly, we used AVEXIS - an assay designed to detect extracellular protein–protein interactions (38). Both MSP1 and MSP7 were expressed as pentameric enzymatically tagged "preys" that were systematically screened for interactions across the entire library of proteins. We observed that both the MSP1 and MSP7 preys were able to interact with the corresponding MSP7 and MSP1 baits and did not interact with any other proteins in the library, including other members of the MSP7 family (46) (Fig. 3*A*). As previously observed by others, no interaction was detected between MSP6 and MSP7, nor between MSP6 and the unprocessed form of MSP1 (45). We used a similar approach to detect the direct interaction between two members of the 6-cys family—P12 and P41—which have recently been shown to form a complex at the merozoite surface (47) (Fig. 3*B*).

To extend this validation, we next focused on two micronemal proteins, EBA175 and EBA140, that are known to interact with host proteins on the erythrocyte surface: Glycophorin-A (GYPA) and Glycophorin-C (GYPC), respectively (22, 48). Both interactions are known to be dependent on sialic acid residues so that pre-treatment of erythrocytes with neuraminidase is sufficient to abolish binding (22, 48). To test whether we could recapitulate these interactions, highly avid arrays of the EBA140 or EBA175 ectodomains were immobilized via their biotin tags around streptavidin-coated fluorescent microbeads and tested to see if they bound human erythrocytes using FACS analysis. Clear binding of both recombinant EBA175 and EBA140 to the erythrocyte surface was observed relative to controls (Fig. 3*C*). Importantly, this binding could be specifically blocked by pre-treatment of erythrocytes with neuraminidase, or, in the case of EBA175-coated microbeads, by pre-incubation of the red blood cells with an anti-GYPA monoclonal antibody. This additionally showed that GYPA is the only major receptor for EBA175 on the erythrocyte surface. Together, these data demonstrate that the recombinant proteins within our library retain their known native extracellular binding properties, suggesting that they are correctly folded and functional.

The Recombinant Merozoite Library Proteins are Immunoreactive and Contain Conformational Epitopes—Recapitulat-

TABLE I

Details of the P. falciparum merozoite surface protein library. Each protein within the library is grouped according to its predicted subcellular localization (Sub-cell. Locn). The region of the protein (typically the full-length ectodomain) that was selected for expression is identified by the N- and C-terminal residues and their positions. The levels at which each protein was expressed was determined by ELISA against a known purified, quantified standard. All transfections were performed transiently using an unoptimized noncommercial transfection reagent, which routinely achieves 40–60% transfection efficiency. Expression levels are given as a guide only given the significant batch-to-batch variability observed using this approach and grouped into "high" (between 5 μg/ml–50 μg/ml:), "medium" (0.5 μg/ml–5 μg/ml) and "low" (0.005 μg/ml–0.5 μg/ml). The expression of nine proteins (PF3D7_1431400, EBL1, PTRAMP, RAP1, RAP2, RAP3, CLAG3.2, RhopH2, and RON3) could not be detected (n.d.). † Pf34 is also GPI-linked Prod: this is a footnote place after detected (n.d)

Sub-cell. Locn	Official nomenclature	Synonym/s	Accession number	Region expressed	Len. (aa)	Exp. level
Surface (GPI-Anchored)	MSP1		PFI1475w	V20-S1701	1682	medium
	MSP2		PFB0300c	I20-N246	227	high
	MSP4		PFB0310c	Y29-S253	225	high
	MSP5		PFB0305c	N22-S251	230	high
	MSP10		PFF0995c	H27-S503	477	medium
	P12	Pf12	PFF0615c	H26-S323	298	medium
	P12p		PFF0620c	Y21-T349	329	low
	P38	Pf38	PFE0395c	Q22-S328	307	medium
	Pf92		PF13_0338	A26-S770	745	low
	Pf113		PF14_0201	Y23-K942	920	medium
	PF3D7_1136200		PF11_0373	L19-G656	638	medium
	PF3D7_1431400		PF14_0293	N25-S968	944	n.d.
Peripheral	MSP3	MSP3.1, SPAM	PF10_0345	K26-H354	328	high
	MSP6	MSP3.2	PF10_0346	Y17-N371	355	low
	H101	MSP3.3	PF10_0347	Q23-N424	402	low
	MSP11	H103, MSP3.7	PF10_0352	K27-Y405	379	medium
	MSP7		PF13_0197	T28-M351	324	high
	MSRP1		PF13_0196	Y22-T379	358	medium
	MSRP2		MAL13P1.174	K24-T280	257	low
	MSRP3		PF13_0193	Q24-S298	275	medium
	P41	Pf41	PFD0240c	K21-S378	358	high
	MSP9	p101, ABRA	PFL1385c	N24-S742	719	low
Micronemal	AMA1	Pf83, RMA1	PF11_0344	Q25-T541	517	high
	EBA140	BAEBL	MAL13P1.60	I26-P1135	1110	low
	EBA175		MAL7P1.176	A21-P1424	1404	low
	EBA181	JESEBL	PFA0125c	I27-S1488	1462	low
	EBL1		PF13_0115	K22-N2584	2563	n.d.
	ASP		PFD0295c	A20-S708	689	low
	MTRAP		PF10_0281	I23-K432	410	medium
	PTRAMP	TRAMP, TSP-3	PFL0870w	N25-S306	282	n.d.
	GAMA	PSOP9	PF08_0008	L22-P710	689	medium
Rhoptry	RH1	RBP1, NBP1	PFD0110w	Q24-T666	643	very low
	RH2b	RBP2b	MAL13P1.176	H25-S1028	1003	very low
	RH4	RBP4	PFD1150c	I27-T1148	1122	very low
	RH5		PFD1145c	F25-Q526	502	low
	RAP1		PF14_0102	I23-D782	760	n.d.
	RAP2		PFE0080c	D22-L398	387	n.d.
	RAP3		PFE0075c	N23-K400	378	n.d.
	CLAG3.2	RhopH1(3.2)	PFC0110w	K21-H1416	1396	n.d.
	RhopH2		PFI1445w	L20-S1378	1359	n.d.
	RhopH3		PFI0265c	K25-L897	873	low
	SPATR		PFB0570w	E22-C250	229	high
	AARP		PFD1105w	K18-P191	174	low
	Pf34†	PV2	PFD0955w	N25-S306	282	high
	RON3		PFL2505c	N22-N249	228	n.d.
	RON6		PFB0680w	F16-T949	934	low
	RAMA		MAL7P1.208	Y17-K838	821	low
Other	TLP	TRAP2	PFF0800w	E24-P1306	1283	low
	PTEX150	Pf112	PF14_0344	A20-N993	974	low
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	PF3D7_0606800		PFF0335c	V23-K299	277	high

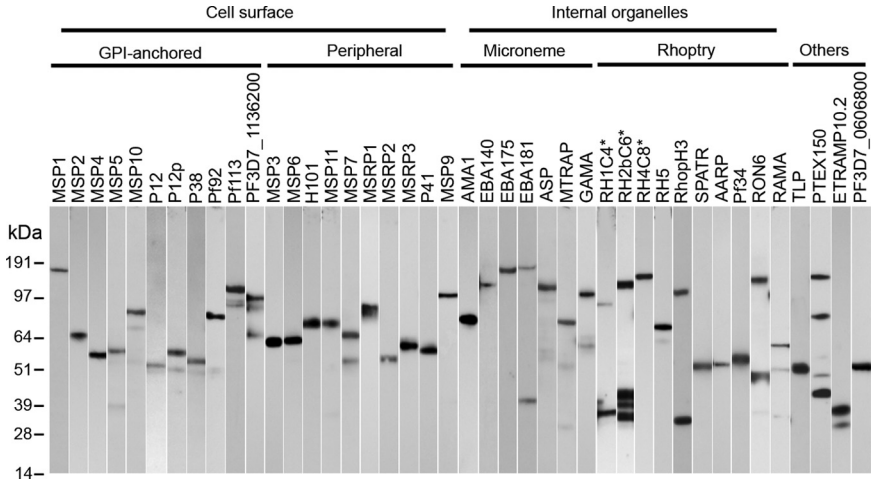

FIG. 2. **A library of recombinant *P. falciparum* merozoite surface and secreted proteins.** A Western blot of the *P. falciparum* merozoite proteins that were normalized, resolved by SDS-PAGE under reducing conditions, blotted, and probed with streptavidin-HRP. Proteins labeled with an asterisk were purified before loading. Each protein additionally contains a C-terminal rat Cd4 tag (~25kDa) and is enzymatically monobiotinylated during expression.

ing known interactions is one way to demonstrate the biochemical activity of the recombinant proteins, however, *P. falciparum* merozoite surface and secreted antigens are relatively poorly characterized and the majority of them have no known interacting partner. Therefore, to systematically demonstrate that the proteins within the panel adopt a native conformation, we tested their immunoreactivity using sera from patients living in malaria-endemic regions with the rationale that protective antibody responses will largely recognize the parasite proteins in their native, folded conformation. Thirty-nine proteins from the library were expressed at sufficient levels, captured in an orientated fashion on a streptavidin-coated microtitre plate and their relative immunoreactivity to pooled sera from Kenyan adults compared with that from malaria-naïve UK individuals (Fig. 4). Most proteins were immunoreactive with strong reactivity observed for most of the GPI-anchored surface proteins, MSP3, MSP7, EBA140, EBA175, AMA1, and RAMA. Seven of them (P12p, MSRP3, ASP, RH5, RON6, TLP, and the very small ETRAMP10.2) showed little serological response when compared with the negative control. Although these proteins may not contain epitopes present in the native protein, we cannot rule out that they are poorly immunogenic. For example, the recombinant RH5 protein, which is biochemically active and can bind the Basigin receptor, is known to be poorly immunogenic in natural infections (49). To show that the antibodies were recognizing conformational epitopes within the protein library, all proteins were denatured by heat-treatment before being captured via their biotin tag. The immunoreactivity of most recombinant proteins was significantly reduced, suggesting that the majority of them contain heat-labile epitopes recognized by conformation-sensitive antibodies (Fig. 4). Only four proteins within the library (MSP3, MSP6, MSP11 and RAMA) exhibited strong immunoreactivity that was insensitive to heat denaturation (Fig. 4); strikingly, all four proteins are distinguished by the presence of repetitive acidic amino acids (50, 51). Three of these four proteins belong to the MSP3 family which are known to be dominated by a heat-stable coiled coil structure and a low complexity glutamic-rich region (52, 53). From these observations, we conclude that most recombinant ectodomains in the library are likely to be correctly folded.

DISCUSSION

One of the enduring technical challenges in malaria research has been the inability to routinely express *Plasmodium* proteins in a recombinant, functionally active form. This challenge has been generally acknowledged as a significant impeding factor for malaria research and the development of an effective vaccine (12). Here, we have used a mammalian expression system and a generic approach to develop a panel of soluble ectodomains representing the repertoire of abundant *P. falciparum* cell surface and secreted merozoite proteins. We have demonstrated their biochemical activity by recapitulating known interactions between parasite proteins and with host receptors.

Previous attempts at expressing *P. falciparum* proteins in heterologous expression systems have primarily relied on empirically identifying tractable fragments yielding soluble proteins in bacterial expression systems, which can be very time-consuming and does not necessarily lead to the production of a functional protein. In both bacterial and cell-free expression systems, proteins are usually produced under reducing conditions, precluding the formation of disulfide bonds that are structurally critical for cell-surface and secreted proteins. These approaches therefore do not produce extracellular proteins in their native fold and subsequently often require complex refolding procedures.

Mammalian expression systems have been previously used to express *Plasmodium* proteins (22, 54–56) but weren't widely adopted, possibly because of low yields. Recent improvements in mammalian expression plasmids and cell lines have led to the development of convenient and high-yielding systems that make this approach more practical (36, 57, 58). We (39, 40, 59) and others (60) have previously reported the successful expression of individual proteins using this system. Yields of the *Plasmodium* proteins did not significantly

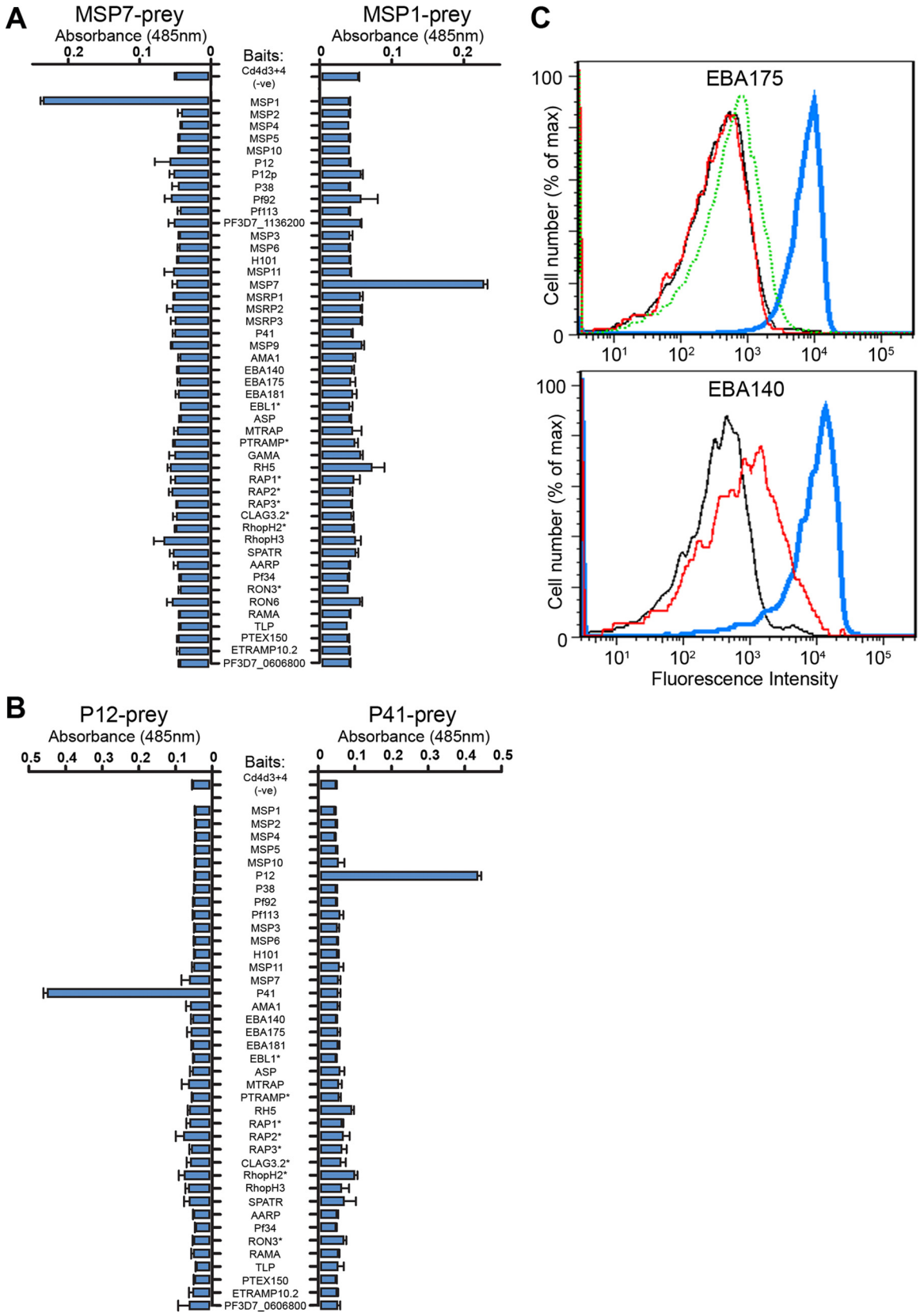

FIG. 3. **Recombinant *P. falciparum* merozoite proteins recapitulate known biochemical activities.** The interactions between recombinant MSP1-MSP7 (*A*), and P12-P41 (*B*) were detected in both bait-prey orientations by screening the *P. falciparum* merozoite library with the

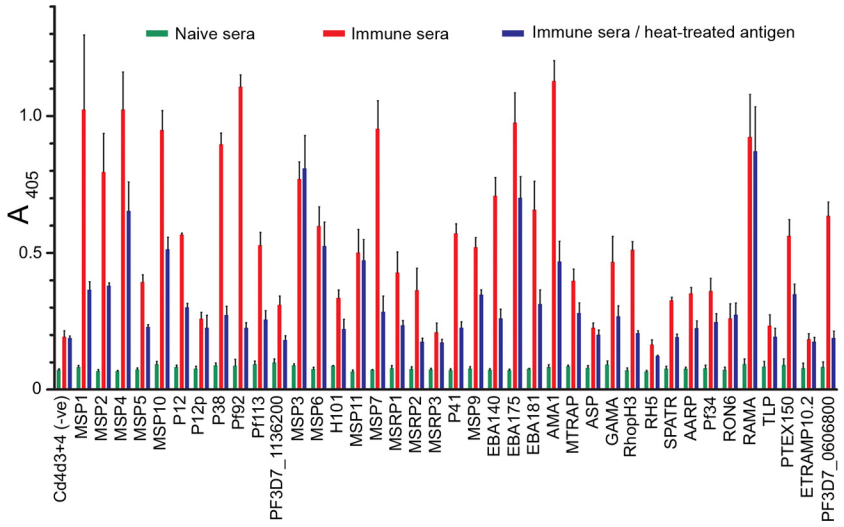

FIG. 4. **Presence of heat-labile conformational epitopes across the *P. falciparum* merozoite protein library by systematic immunoreactivity profiling.** The immunoreactivity of the recombinant *P. falciparum* merozoite proteins was tested using sera pooled from ten malaria-naïve adults from the UK as a control (green bar) or ten immune adults from Kenya (red bar). Reduced response of immune sera to heat-denatured antigens (blue bar) demonstrates the presence of heat-labile (conformational) epitopes. Bar charts show mean ± S.D.; $n = 3$.

differ from other proteins libraries that we have constructed using the same approach; for example, zebrafish (38, 61, 62) and human erythrocyte receptors (39). The proteins were expressed at different levels, spanning several orders of magnitude, with MSP2 and AMA1 giving the highest levels of expression (up to ~50 μg/ml). It should be pointed out that these proteins were expressed using a cost-effective transient transfection approach that typically results in ~40–60% transfection efficiency; yields could therefore be significantly improved by selecting high-expressing stably transfected cell lines. Although the majority of selected merozoite proteins were expressed (Table I), we found that some, particularly those located in the rhoptry, were not expressed at detectable levels, if at all. Attempts to cotransfect multiple members of the same family (RAP1, 2, and 3, for example) with the idea that they may form a complex, did not lead to any further evidence of expression (data not shown). Some proteins trafficked to the rhoptry may require specific chaperones that are not provided by the HEK293 cells.

The inserts encoding each protein within the library are flanked by unique rare-cutting restriction enzymes (NotI and AscI) which enable facile shuttling of the ectodomain regions into a variety of different expression vectors containing useful C-terminal tags for biochemical manipulation. The enzymatically monobiotinylatable tag enables the orientated capture of the proteins on streptavidin-coated microtitre plates or microarray slides (63). Other vectors allow for the production of highly avid pentameric constructs or the purification of recombinant proteins through a C-terminal hexaHis tag, and several of these vectors are already available at plasmid resource providers (64).

We believe that this expression approach and protein library will be a valuable resource to investigate the functional roles of merozoite surface proteins in the biology and pathogenesis of *P. falciparum* malaria but could also be applied to study other aspects of malaria. Indeed, we have been able to express proteins from different stages of the lifecycle including sporozoites and gametes (CC, GJW unpublished) as well as from other *Plasmodium* species, such as *P. vivax* (J. Hostetler, GJW and JR, unpublished) using the same method. The merozoite protein resource will be useful for a wide range of investigations including immunoepidemiology, structural studies, cell-based assays and the identification of novel parasite-parasite and host-parasite interactions. Indeed, individual proteins from the resource have already been used to identify novel host receptors involved in erythrocyte invasion (39, 59). The production of large panels of correctly folded proteins in a systematic way enables the direct and unbiased comparison of proteins relative to each other, rather than the discrete approach using individual proteins that has been frequently used in the past. This will be particularly important for comparative immunoepidemiological studies and a systematic assessment of these proteins might lead to the identification of novel blood stage vaccine candidates.

indicated prey proteins using the AVEXIS assay. Baits labeled with an asterisk were below threshold levels required for the assay. Bar charts show mean ± S.D.; $n = 3$. *C*, Recombinant biotinylated EBA175 (top panel) and EBA140 (bottom panel) immobilized on fluorescent streptavidin-coated beads bound to untreated erythrocytes (blue line). Binding was blocked by pre-treating the erythrocytes with neuraminidase (red line) or, for EBA175, pre-incubating erythrocytes with an anti-GYPA monoclonal antibody (dotted green line). Negative controls were Cd4d3 + 4-coated beads (black line).

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