Technical Note

A new method for the capture of surface proteins in *Plasmodium falciparum* parasitized erythrocyte

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Abstract
Introduction: We propose a new method for the selective labeling, isolation and electrophoretic analysis of the *Plasmodium falciparum* protein exposed on the erythrocyte cell surface. Historically, membrane surface proteins have been isolated using a surface biotinylation followed by capture of biotin-conjugated protein via an avidin/streptavidin-coated solid support. The major drawback of the standard methods has been the labeling of internal proteins due to fast internalization of biotin.
Methodology: To solve this problem, we used a biotin label that does not permeate through the membrane. As a further precaution to avoid the purification of non surface exposed proteins, we directly challenged whole labeled cells with avidin coated beads and then solubilized them using non ionic detergents.
Results: A marked enrichment of most of the RBC membrane proteins known to face the external surface of the membrane validated the specificity of the method; furthermore, only small amounts of haemoglobin and cytoskeletal proteins were detected. A wide range of *P. falciparum* proteins were additionally described to be exposed on the erythrocyte surface. Some of them have been previously observed and used as vaccine candidates while a number of newly described antigens have been presently identified. Those antigens require further characterization and validation with additional methods.
Conclusion: Surface proteins preparations were very reproducible and identification of proteins by mass spectrometry has been demonstrated to be feasible and effective.

Key words: Plasmodium *falciparum*; erythrocyte; malaria; surface protein

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Introduction

The erythrocyte membrane undergoes profound changes during the intracellular development of malaria parasites. *Plasmodium falciparum* induces a vast array of modifications from the early phases of its growth with the insertion of ring-infected erythrocyte surface antigen (RESA) and additional proteins occurring immediately after invasion [1-4], and with an increasing number of proteins inserted at the trophozoite and schizont stages [5,6]. Additionally, several modifications also involve red cell membrane proteins and cause overall reorganization of the parasitized erythrocyte membrane [7-10]. The reorganization of the host cell membrane has a functional role during the later stages of parasite growth, ending in erythrocyte membrane disruption and parasite egress [11-13]. The development of a powerful immune response to the parasitized erythrocyte membrane components is the counterpart of such modifications. Deeper knowledge of surface modifications of malaria parasitized erythrocytes is required for the design of new vaccines [14-16]. Unfortunately, the impressive genetic variability of the major malaria antigens such as P*f*EMP1 partially defuse the effectiveness of the immune response, which apparently takes years to adapt to the antigenic complexity of the parasite and to acquire proficiency [17-19]. Lack of adequate immune response is considered to be one of the major causes of severe complications in young children. For this reason the development of vaccines aimed at speeding up an effective immune response in children remains one of the major goals of malaria research. Analysis and monitoring of the immune response to malaria would require expensive and complicated methodological approaches such as protein microarrays containing hundreds of recombinant antigen domains. The assessment of the functional meaning of such results would also require further information about the extracellular accessibility of the epitopes.

In this report we present a relatively simple and very reproducible method to isolate surface exposed proteins in parasitized erythrocytes based on biotin labeling and capture through surface-

surface interactions. The results have been validated through immunological and proteomic methodologies demonstrating that this technique may represent a consistent approach for the analysis and monitoring of effective surface directed immunological responses to malaria.

Methodology

Cultivation of P. falciparum-*RBC membranes*

Freshly drawn blood (Rh+) from healthy adults of both sexes was used, after being anticoagulated with heparin and kept in citrate-phosphate-dextrose with adenine (CPDA-1). Red blood cells (RBC) were separated from plasma and leukocytes by three washings in wash medium (RPMI 1640 medium containing 2 mM glutamine, 24 mM $NaHCO_3$, 25 mM HEPES, 20 mM glucose, and 32 mg of gentamicin per liter [pH 6.80]). P *falciparum* strain Palo Alto (mycoplasma free) was cultivated at a 0.5% hematocrit and synchronized as described previously [20]. *P. falciparum* RBCs with different maturation stages at 14 hours to 18 hours (rings) or 34 hours to 38 hours (trophozoites), were studied. The parasitemia proportions were usually 20%. To assess total parasitemia and the relative contributions of rings and trophozoites, slides were prepared from cultures at the indicated times and stained with Diff-Quik kit stain (Fisher Scientific, Pittsburgh, USA) and 400 to 1,000 cells were examined microscopically.

Isolation of normal and parasitized erythrocyte surface proteins

Briefly, 1 ml of control and parasitized (Trophozoite stage) packed erythrocytes were incubated for 10 minutes with 0.5 mg/ml of Sulfo-NHS-Biotin (Pierce) at 4°C under gentle mixing. The reaction was stopped by three washes with phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 8.1mM K_2HPO_4, 1.5mM KH_2PO_4, pH 7.4) in the presence of glucose 5 mM at pH 7.4 (PBS-glucose) and pelleted at 1000 x g for 4 minutes in a refrigerated centrifuge at 4°C. Biotinylated surface proteins from control and parasitized packed erythrocytes were incubated with avidin-agarose beads (500µl/ml of cells) for 30 minutes at 4°C. Samples were pelleted at 1500 x g in a refrigerated centrifuge at 4°C. After removal of the supernatant, 15 volumes of cold hemolysis buffer (1%Triton X-100, 5mM DDT, 5mM EDTA) in the presence of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) were added to the samples. Unbound components were removed by three washes at 15.000 x g using a cold hemolysis buffer. Proteins captured on the surface of the beads were eluted with Laemmli buffer at a volume ratio of 1:1, heated for 5 minutes at 100°C, and then separated electrophoretically in 6%-15% gradient polyacrylamide gel, 20 x 20cm. Protein content was quantified using the DC Protein Assay (Biorad, Hercules, CA, USA). Next 50 µg of control and 150 µg of parasitized erythrocyte proteins were loaded for each lane. Electrophoresis was performed for 15 minutes at 50V and then continued at 150V. Proteins from SDS-PAGE were stained with colloidal Coomassie Blue. All SDS/PAGE experiments were performed at least in triplicate.

Identification by MALDI-TOF MS

Isolated surface proteins were identified by mass spectrometry. Bands were excised from SDS/PAGE and proteins were digested with trypsin. Bands were destained by way of several washes in 5 mM NH_4HCO_3/ACN (acetonitrile) (50/50 v/v) and successively dried with pure ACN. The gel slices were rehydrated for 45 minutes at 4°C in 20 µl of 5 mM NH_4HCO_3 digestion buffer containing 10 ng/µL of trypsin. Excess protease solution was removed and the volume adjusted with 5 mM NH_4HCO_3 to cover the gel slices. Digestion was allowed to proceed overnight at 37°C.

Samples were loaded onto a MALDI target using 1 µL of the tryptic digests mixed 1:1 with a solution of CHCA (alpha-Cyano-4-hydroxycinnamic acid) (10 mg/ml in ACN/TFA 0.1%, 40/60). MS analysis of peptides was performed as previously describe [7].

Immunoblot analysis

Following the mapping of the P *falciparum* surface proteome, malaria antigens were stained by SDS/PAGE western blotting using specific antibodies and immune sera kindly provided by the Pasteur Institute (Paris).

Membranes were probed with anti-G8 and anti-tR96 (both developed in rabbit); with anti-band 3, anti-HrpI, anti-Heat shock protein 70 and 90, anti-RhopH, anti-Hrp II (developed in mouse); with a healthy human serum; and with a pool of malaria hyper-immune sera. All the antibodies were diluted 1:50 and incubated for 1 hour. After three washes with PBS/ tween 01%, the membranes were incubated with specific secondary antibody conjugated with alkaline phosphatase.

Results

Isolation of surface proteins and their proteomic characterization

We isolated surface membrane proteins from trophozoite parasitized erythrocytes. To minimize the labeling of internal proteins we used a non

permeable cross-linker (Sulfo-NHS biotin) for a short period at 4°C. Cells were then incubated with avidin bound agarose beads to allow the binding of biotinylated / surface exposed proteins to the beads. After extensive washing of non adherent cells, we added a nonionic detergent to wash out all cellular components that were not directly bound to the beads surface. Proteins bound to the avidin coated beads were then eluted using SDS and separated by SDS/PAGE. Figure 1 shows the results obtained with control and parasitized erythrocytes after Coomassie Blue staining (lanes 2, 4). Table 1 shows the list of all proteins identified by mass spectrometry of the bands visualized in the gels. Relatively few proteins were separated from control erythrocyte surfaces while a much higher number of proteins were isolated from parasitized erythrocytes. A total of 18 out of 29 protein bands belonged to the parasites, while the rest belonged to the host cell. Performing replicate experiments we observed a good reproducibility of the results.

It is important to note that some of the identified proteins, such as spectrins, actin and protein 4.1, are part of the cytoskeletal complex. Their presence in extracted surface membrane proteins could be due to their tight interaction with transmembrane proteins such as band 3 [21,22] and P*f*EMP1[23].

Immunological characterization of the surface proteins

To test the antigenicity of the isolated surface proteins using the same surface isolated proteins, we then performed a western blot with a pool of hyper-immune sera (Figure 1, lanes 1, 3). Approximately 12 protein bands reacted with the sera indicating their antigenicity. Notably, no reactivity was observed with non-parasitized human erythrocytes. Some proteins such as RhopH 1 and Rhoptry 2 (bands 11, 12) and elongation factor 2 (band 15) appear to correspond to known malaria antigens. All other antigens require further validation using additional methods for their unequivocal identification.

Validation of the methodology to isolate surface exposed proteins

To validate the method we tested our isolated surface proteins using high affinity antibodies directed to proteins known to be not surface expressed, such as HSP 70 and 90, and to proteins known to be surface exposed, such as tR96, G8, and band 3 (Table 2). Figure 2 shows that western blots were positive only with antibodies directed to known surface exposed proteins while no reactivity was observed with antibodies for internal antigens. Those data therefore demonstrate that the hereby developed methodology is able to effectively enrich surface exposed proteins.

Discussion

The characterization of the surface antigenic modifications induced by the intracellular growth of *P. falciparum* still represents a major challenge for the development of malaria vaccines and for the understanding of the wide variability of clinical responses to malaria. So far, many genetic and proteomic approaches have been used [24-27] that have provided valuable information about *P. falciparum* antigens and their use for vaccine development; however, direct evidence about the actual surface expression of the antigens would be of great value in selecting new targets.

The present report demonstrates that it is possible to isolate surface proteins from *P. falciparum* parasitized erythrocyte cultures using a simple method that is both labor and cost effective. The protein patterns obtained with replicate experiments reveal significant reproducibility and a consistent enrichment of surface proteins. Western blot analysis of the cell surface isolated proteins using hyper-immune sera demonstrated that several proteins are highly immunogenic, and therefore represent potential candidates for vaccine development. While some of these proteins such as Rhoptry are known to be malaria antigens and have been easily identified, most of our results require additional validation using different methodologies.

Proteomic analysis of surface isolated proteins revealed the presence of membrane proteins with little contamination by proteins known to be not surface expressed. The method could be easily parallelized to increase its throughput and further decrease cost and labor efficiency. The present methodology could be therefore applied to fresh isolates for the study of the variability of parasite strains and to analyze the individual immune responses. This methodology also could be readily used to study the age dependent development of an immune response directed to surface determinants, the interference of human protective mutations, and the effect of antimalarial therapy.

Table 1. Surface membrane proteins identified by MALDI-Tof analysis.

N	Protein	Species	MW	Score	Coverage
1	Spectin alpha	Hum	279,7	140	22
2	Spectrin beta	Hum	246	183	36
3	Band 3	Hum	102	70	13
4	Band 3	Hum	102	91	16
5	Band 4.2	Hum	77	93	17
6	Actin	Hum	41,7	82	9
7	Tropomodulin	Hum	34	53	13
8	Spectin alpha	Hum	279,7	44	27
9	Spectrin beta	Hum	246	106	27
10	Spectrin beta	Hum	246	91	15
11	RhopH 1	P*f*	168,7	70	11
12	Rhoptry 2	P*f*	168,7	88	21
13	Glicophorin binding protein	P*f*	95,8	66	10
14	Band 3	Hum	102	117	15
15	Band 3	Hum	102	54	7
	Elongation factor 2	P*f*	93,4	71	11
16	Band 3	Hum	102	81	14
	HSP 86	P*f*	86	71	10
17	Band 3	Hum	102	47	12
18	Band 4.1	Hum	97	87	18
	Band 3	Hum	102	49	13
19	Band 4.2	Hum	77	99	16
20	Band 4.2	Hum	77	109	16
21	Band 3	Hum	102	81	14
22	Band 3	Hum	42,5	95	8
	Catalase	Hum	59,6	53	6
23	EMP1	P*f*	14,3	55	5
24	Beta tubulin	P*f*	49,7	65	7
	Pyruvate kinase	P*f*	55,6	117	10
25	Elongation factor 1	P*f*	49	94	10
26	Actin	Hum	41,7	110	11
27	Actin	Hum	41,7	129	14
28	Phosphoribosyl-pyrophosphate synthetase	P*f*	49,3	79	8
29	Endoplasmatic retic-rest calcium binding protein	P*f*	39,3	42	6
30	Elongation factor 1B	P*f*	32	54	6
31	L. lactate dehydrogenase	P*f*	34	92	10
32	L. lactate dehydrogenase	P*f*	34	63	7
33	Hemoglobin	Hum	15,9	68	6
34	Hemoglobin	Hum	15,9	101	8
35	Hemoglobin	Hum	15,9	52	7
36	Hemoglobin	Hum	15,9	70	6
37	2-Cys peroxiredoxin	P*f*	21,8	60	5
38	2-Cys peroxiredoxin	P*f*	21,8	65	6
	EMP	P*f*	14,3	57	5
39	Hemoglobin	Hum	15,9	69	11
	96 tR antigen	P*f*	17,6	107	9
40	Hemoglobin	Hum	15,9	89	9

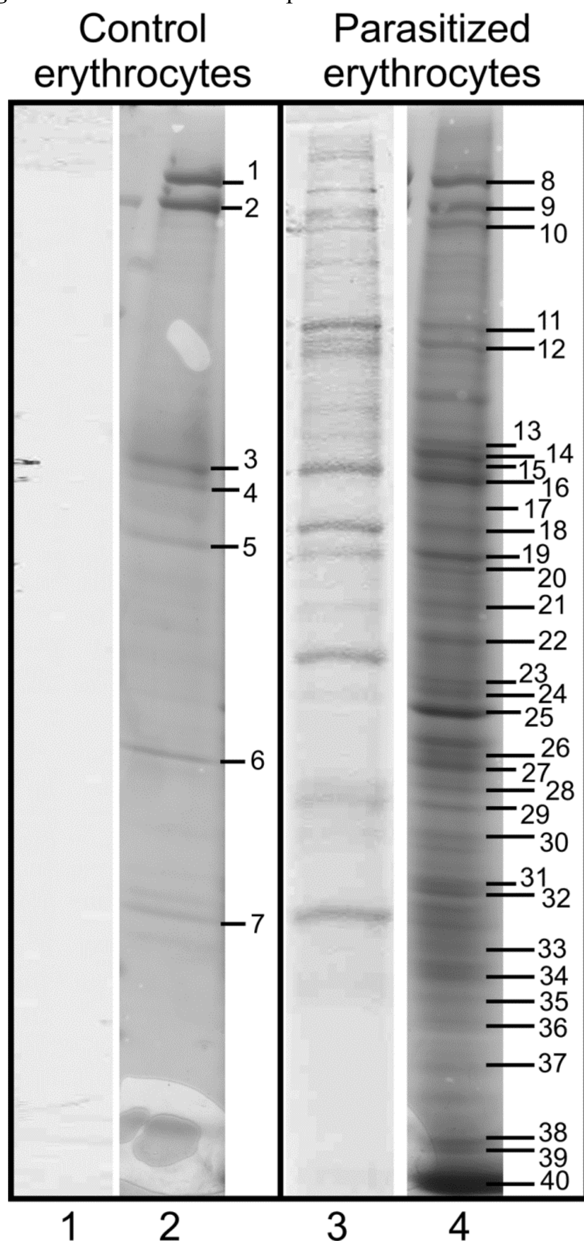
Numbers correspond with the band numbered in figure 1.

Table 2. Antibodies used to validate surface membrane protein isolation method.

Lane	Antibody	Site	Reactivity
1	Anti-G8	Esternal	Positive
2	Anti-Band 3	Trans-membrane	Positive
3	Hrp I	Internal	Negative
4	HSP 70	Internal	Negative
5	HSP 90	Internal	Negative
6	Anti-RhopH	Esternal	Positive
7	Hrp II	Internal	Negative
8	tR 96	Esternal	Positive
9	Healthy human serum	Esternal	Negative
10	Pool of malaria hyper-immune sera	Esternal	Positive

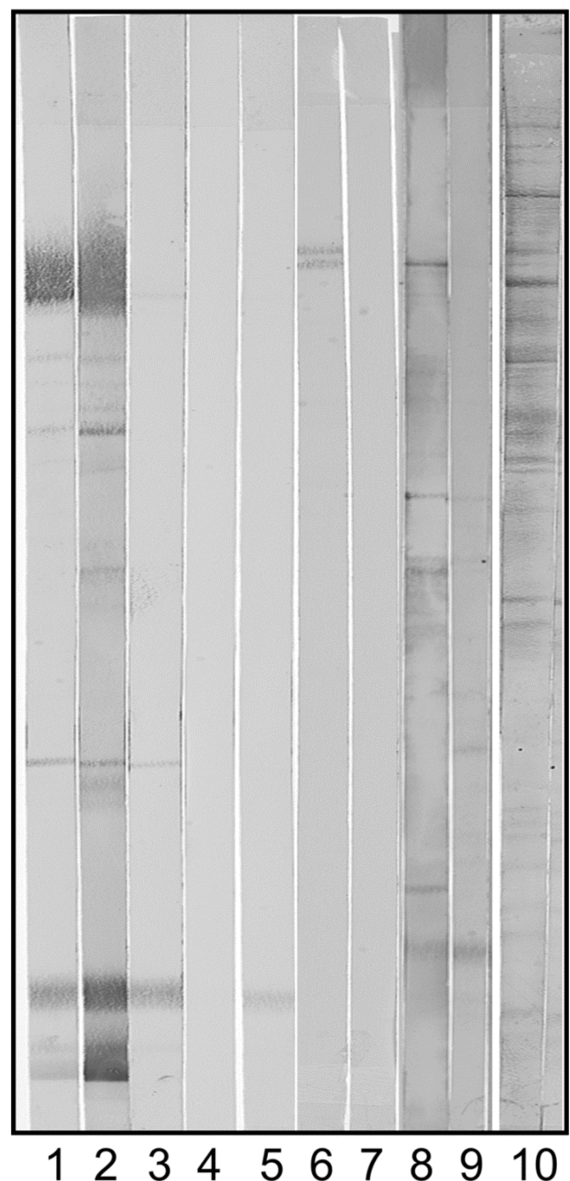
Numbers of listed antibodies correspond with lane numbers in the figure 2.

Figure 1. Surface membrane proteins identification



Isolated surface proteins from control (lanes 1, 2) and parasitized erythrocytes (lanes 3, 4) were separated by 10% SDS-PAGE and blotted on nitrocellulose membrane stained with a pool of malaria hyper-immune sera (lanes 1, 3) or stained by Blue colloidal Coomassie (lanes 2, 4). Proteins numbered in lanes 2 and 4 were identified by mass spectrometry and listed in table 1.

Figure 2. Validation of the surface membrane protein isolation methodology



Extracted surface protein from parasitized erythrocytes were separated by 10% SDS-PAGE and blotted on nitrocellulose membrane stained with specific antibodies listed in table 2.

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