

Improving the production of the denatured recombinant N-terminal domain of rho-try-associated protein 2 from a *Plasmodium falciparum* target in the pathology of anemia in falciparum malaria

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Rho-try-associated protein 2 (RAP2) is known to be discharged from rho-try onto the membrane surface of infected and uninfected erythrocytes (UEs) ex vivo and in vitro and this information provides new insights into the understanding of the pathology of severe anemia in falciparum malaria. In this study, a hexahistidine-tagged recombinant protein corresponding to residues 5-190 of the N-terminal of Plasmodium falciparum RAP2 (rN-RAP2) was produced using a new method of solubilization and purification. Expression was induced with D-lactose, a less expensive alternative inducer to the more common isopropyl-β-D-thio-galactopyranosidase. The recombinant protein was purified using two types of commercially-available affinity columns, iminodiacetic and nitrilotriacetic. rN-RAP2 had immunogenic potential, since it induced high titers of anti-RAP2 antibodies in mice. These antibodies recognized full-length RAP2 prepared from Triton X-100 extracts from two strains of P. falciparum. In fact, the antibody recognized a 29-kDa product of RAP2 cleavage as well as 82 and 70-kDa products of RAP1 cleavage. These results indicate that the two antigens share sequence epitopes. Our expressed protein fragment was shown to contain a functional epitope that is also present in rho-try-derived ring surface protein 2 which attaches to the surface of both infected and UEs and erythroid precursor cells in the bone marrow of malaria patients. Serum from malaria patients who developed anemia during infection recognized rN-RAP2, suggesting that this protein fragment may be important for epidemiological studies investigating whether immune responses to RAP2 exacerbate hemolysis in falciparum malaria patients.

Key words: *Plasmodium falciparum* - rho-try-associated protein 2 - 6xHis-tagged protein - purification - Western blotting

Rho-try-associated protein 2 (RAP2) is a 398-amino acid protein encoded by a single exon located on chromosome 5 of *Plasmodium falciparum* (Perez-Leal et al. 2005). It is discharged from rho-try, an apical organelle of the merozoite, onto the surface of the erythrocyte membrane during the blood stage of malaria infections. This discharge leads to the formation of a parasitophorous vacuole (PV) membrane. RAP2 is considered to be an important candidate for vaccine development, since monkeys immunized with RAP2 were partially protected against parasite challenge (Perrin et al. 1985, Ridley et al. 1990).

Douki et al. (2003) and Layez et al. (2005) provided new insights into the rho-try antigen, especially RAP2, by discharging this antigen onto the surface of uninfected erythrocytes (UEs) ex vivo and in vitro. These authors suggested that the anti-RAP2 specific immune

response plays a role in the pathology of severe anemia by inducing lysis of UEs immediately after discharge of this antigen onto the surface membrane.

Saul et al. (1992) cloned the entire *RAP2* gene and described its expression and purification using a hexahistidine system. Their methods yielded a low level of expression; further, the purification of RAP2 presented a range of difficulties due to the protein's lipophilicity. Expression in their study was induced using isopropyl-β-D-thio-galactopyranosidase (IPTG), which substantially raised the cost of protein production due to the many liters of culture necessary to obtain a reasonable amount of protein. The authors described that RAP2 precipitates even at very low concentrations in the absence of a strong detergent; 6 M guanidine was needed to solubilize inclusion bodies. Furthermore, Saul et al. (1992) reported that recombinant RAP2 could be eluted effectively only with a denaturation buffer containing 0.5 M imidazole (iDB) and not with a pH gradient.

In the present study, a hexahistidine-tagged RAP2 fragment containing the N-terminal (N-RAP2) residues 5-190 of *P. falciparum* RAP2 was produced. We achieved efficient induction of expression in *Escheria coli* using D-lactose as a less expensive alternative to IPTG. Furthermore, we compared the purifying power of two types of commercially-available affinity columns, iminodiacetic (Ni-IDA) and nitrilotriacetic (Ni-

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NTA). Our expression system is relevant for future studies aiming to understand the molecular mechanisms of host-parasite interactions in falciparum malaria.

MATERIALS AND METHODS

Cloning of N-RAP2 - Bioinformatic analyses were performed using DNASTAR software (DNASTAR Inc). The following primers were used in this study: 5' - ggatcctTTTATGTATTAGTTTT-3' (sense) and 5' - gaattcGGTGGTGGTGGTGGATCCTACTATAAAG-3' (anti-sense). These primers were designed based on the GenBank sequence XM-001351539. *P. falciparum* 3D7 parasites were cultured in vitro using routine methods and parasite extracts were prepared as previously described (Ljungström et al. 2004a, b). For amplification of a 585 bp fragment containing the N-terminal residues 5-190 of *P. falciparum* RAP2 (N-RAP2), PCR was performed with the following parameters: 94°C for 5 min, 35 cycles of 94°C for 1.5 min, 60°C for 1 min, 72°C for 1.5 min and a final 10 min incubation at 72°C. After agarose gel purification, the fragment was cloned into the pGEM T easy vector (Promega) and transformed into *E. coli* DH5a. White transformant colonies were screened using PCR.

The sequence of the cloned N-RAP2 DNA was confirmed using the Thermo sequence primer cycle sequencing kit. After digestion with *Bam* HI and *Eco* RI, the N-RAP2 fragment was sub-cloned into the pRSET vector (Invitrogen) and transformed into *E. coli* TG1. Colonies were screened by PCR using a primer for the T7 promoter (sense) and a RAP2 primer (anti-sense).

Expression and purification of recombinant N-RAP2 - Bacterial extract was prepared based on the methods of Petty (1996) with some modifications developed by our group. After transforming *E. coli* BL21 cells with the *RAP2-pRSET* construct, a single transformant colony was used to grow a 1 L culture in M9 synthetic medium supplemented with 100 µg/mL ampicillin at 30°C. When an optical density of 0.8 was reached, 10 g/L of D-lactose was added and the culture was incubated for another 16 h.

The culture was harvested by centrifugation (4000 rpm/15 min/4°C) and resuspended in MCAC buffer (20 mM Tris-HCl, pH 7.9; 0.5 M NaCl; 10% glycerol; 0.15 mM PMSF) containing 33 µL of a protease inhibitory cocktail (0.1 µg/mL leupeptin and 0.1 µg/mL pepstatin). The cells were resuspended in the ratio of 2.5-5.0 mL of buffer per 50 mL of culture. Triton X-100 was added and three cycles of freezing in liquid nitrogen and thawing at RT were performed. Deoxyribonuclease I was added at the concentration recommended by the supplier and the induced extract was harvested by centrifugation (15,000 rpm/15 min/4°C). Pellets were resuspended in denaturation buffer (DB) (8 M urea; 10 mM 2-mercaptoethanol; 20 mM Tris-HCl, pH 7.9; 0.5 M NaCl; 10% glycerol; 0.15 mM PMSF) and incubated at RT for 1-2 h on a horizontal shaker. The extract was then harvested by centrifugation (15,000 rpm/15 min/rt) and the denatured supernatant was stored at -80°C. A 5-L culture was also induced using 10 g/L of D-lactate and the denatured supernatant was prepared as described above.

Prior to purification, Ni-IDA and Ni-NTA affinity columns were equilibrated with MCAC buffer. The pH of the denatured supernatant was readjusted with 5N NaOH to 9.0 and the supernatant was incubated in batch with the affinity resin for 1 h at RT with rotation. In the Ni-NTA column, the resin was washed with 30 mL of DB at pH 6.3 adjusted with HCl 1M. It was gradually washed as follows with denaturation buffer (DB) containing increasing concentrations of imidazole (iDB): 1st, 10 mL of DB supplemented with 5 mM iDB; 2nd, 10 mL of 10 mM iDB; 3rd, 10 mL each of 15 mM iDB and 20 mM of iDB; finally, 10 mL of DB pH 5.9. The bound protein was eluted with DB at pH 4.5. Ni-IDA resin was similarly washed with 120 mL of DB in a 0-100 mM iDB gradient, and all elutions were performed with 1M iDB. For each resin, the purity of the final eluate was analyzed by SDS-PAGE and Coomassie blue staining.

Preparation of anti-rN-RAP2 serum from mice - rN-RAP2 purified from the Ni-NTA column was quantified and 4 µg of protein was homogenized in complete Alum adjuvant. Three female BALB/c mice (6-8 weeks old) were immunized by intraperitoneal injection. After 15 days, a booster was performed under the same conditions. Sera were collected from the tail vein 15 days after the booster.

Preparation of total extract from Plasmodium 3D7 for Western blotting - A culture of *P. falciparum*, clone FCR3, was provided as a gift by Fabio Costa (Instituto de Biologia – Universidade de Campinas). Ring stage-rich parasites were subjected to osmotic lysis by diluting them 150 fold with ultrapure water containing protease inhibitors (0.1 µg/mL leupeptin, 0.1 µg/mL pepstatin, 0.15 mM PMSF and 5 mM EDTA). After 16 h of incubation at 4°C, the cellular lysate was harvested by centrifugation (15,000 rpm/4°C/2 h) and washed twice with ultrapure water containing protease inhibitors in order to eliminate any trace of hemoglobin. The pellet was resuspended in 100 µL of 1% Triton X-100 and incubated for 30 min on ice. Subsequently, the protein was harvested by centrifugation and the supernatant subjected to immunoblotting.

Immunoblotting to detect reactivity against rN-RAP2 - SDS-PAGE and Western blots were performed following routine methods. First, to confirm the expression of rN-RAP2, a lysate from *E. coli* BL21 pLysS transformed with the *RAP2-pRSET* plasmid was blotted onto a nitrocellulose membrane and probed with a commercially-available monoclonal antibody (mAb) (46-1008, Invitrogen, Carlsbad, CA, USA) against the histidine tag. Second, conformational and linear epitopes of rN-RAP2 were compared with those of native RAP2 obtained from extracts of *P. falciparum* 3D7 and FCR3 parasites. *E. coli* lysates and falciparum extracts were blotted onto nitrocellulose membranes and probed using murine anti-rN-RAP2 IgG antibodies raised as described above. Third, we determined whether rN-RAP2 contains a functional RSP2 epitope using an RSP2 mAb generously provided by Dr. Jurg Gysin from the Mediterranean University (Marseille, France). In all three blots, goat anti-mouse peroxidase was used as the secondary antibody.

In order to determine whether natural antibodies recognize the recombinant protein, purified rN-RAP2 was blotted onto a nitrocellulose membrane and incubated with human sera from malaria-infected individuals (a kind gift from Dr. Marcus Lacerda of Fundação de Medicina Tropical, state of Amazonas, Manaus, Brazil). Two black spots were drawn with a pencil to allow precise placement of the protein onto the nitrocellulose membrane. Goat anti-human peroxidase was used as the secondary antibody. Tetramethylbenzidine was used as the substrate in all reactions and the reactions were stopped after 30 min by washing with distilled water.

RESULTS

Sequencing and expression of recombinant N-terminal RAP2 - The 585 bp fragment containing the N-terminal residues 5-190 of *P. falciparum* RAP2 was sequenced and confirmed with 97 score the identity of the RAP2 fragment with RAP2 sequence XM_001351539 (Fig. 1). The rN-RAP2 was expressed in *E. coli* BL21 pLysS using D-lactose as an inducer. An antibody against the His tag was used to confirm the presence of hexahistidine in the ~27-29 kDa protein (Fig. 2).

Purification of rN-RAP2 - The rN-RAP2 was purified using two commercially-available affinity resins, Ni-IDA and Ni-NTA. Much purer protein was obtained on the Ni-NTA resin than Ni-IDA resin (Fig. 3). Despite extensive washing, the final preparation of protein from the Ni-IDA resin contained impurities visible by Coomassie blue staining (Fig. 3A). In contrast, no impurities could be detected using Coomassie blue staining on the

Ni-NTA resin (Fig. 3B). A test purification from 5 L of culture demonstrated that production could be scaled up for larger quantities (Fig. 4).

Recognition of rN-RAP2 by antibodies raised in mice - Mice immunized with rN-RAP2 in the presence of alum adjuvant developed high titers of anti-RAP2 antibodies, demonstrating the immunogenicity of this protein fragment (data not shown).

To characterize the conformational and sequence epitopes in rN-RAP2, anti-rN-RAP2 antibodies from immunized mice were analyzed for their ability to recognize native RAP2 prepared from the Triton X-100 protein extract from both erythrocyte membranes and the insoluble fraction containing parasites. The extracts were blotted onto nitrocellulose membranes and incubated with anti-rN-RAP2 sera from several mice (Fig. 5). Sera pooled from mice stimulated with alum adjuvant alone served as a negative control. In the soluble fraction, the pooled anti-rN-RAP2 sera recognized one protein of ~29 kDa. Two products of 70 kDa and 82 kDa were recognized in the insoluble fraction.

These results are similar to those of Stowers et al. (1996), who found that an anti-RAP1 mAb recognized the 65 kDa and 70 kDa products of RAP1 cleavage. Together with other fragments of 29 and 42 kDa, these products correspond to uncleaved and cleaved forms of RAP2 in Triton X-100 extracts from parasites. These products are formed prior to merozoite invasion (Howard et al. 1998). We chose to focus on rN-RAP2 rather than full-length RAP2 in our work because the former can be produced in much higher yields and is sufficient to induce

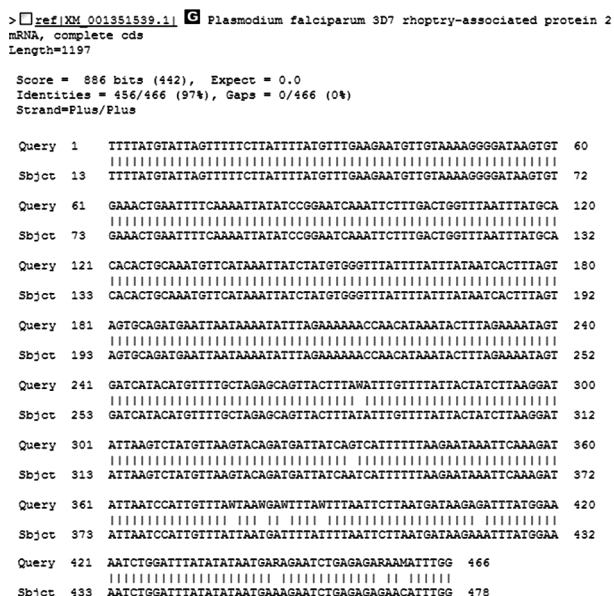


Fig. 1: confirmation of the identity of the 585 bp fragment containing the N-terminal residues 5-190 of *Plasmodium falciparum* rhooptry-associated protein 2 (RAP2). The sequence of the cloned N-terminal of *P. falciparum* (N-RAP2) DNA was confirmed using the Thermo sequenase primer cycle sequencing kit.

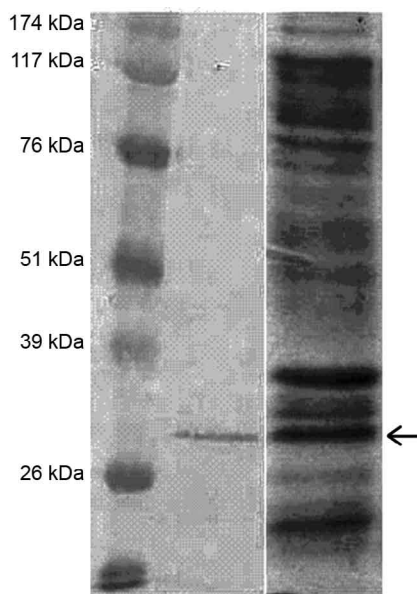


Fig. 2: confirmation of the presence of the hexahistidine tag in the N-terminal of *Plasmodium falciparum* (rN-RAP2) protein. Western blots showing binding of the protein by a commercially available monoclonal anti-histidine antibody. Antibody binding was revealed using an anti-mouse IgG antibody conjugated to peroxidase. The blot shows the fusion protein to have a mass of 27-29 kDa, consistent with the prediction.

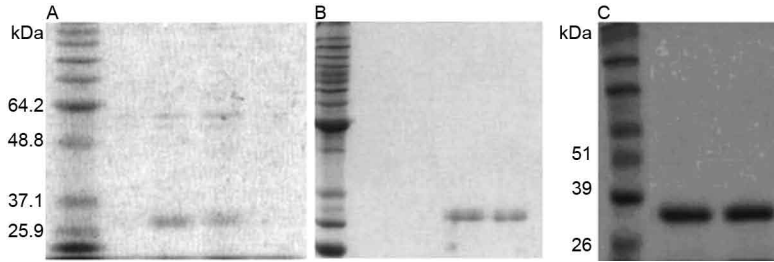


Fig. 3: a comparison of N-terminal of *Plasmodium falciparum* (rN-RAP2) purification using two different nickel affinity columns. SDS-PAGE analysis of rN-RAP2 purification using two commercially available columns. A: two eluates from a iminodiacetic (Ni-IDA) column contained impurities visible following by Coomassie blue staining; B: two eluates from a nitrilotriacetic (Ni-NTA) column showed no detectable impurities following Coomassie blue staining; C: improvement of rN-RAP2 purification. Higher yields of protein were achieved by increasing the culture volume and using the inexpensive inducer D-lactose. The protein elutes from a Ni-NTA column in two fractions, as shown by SDS-PAGE.

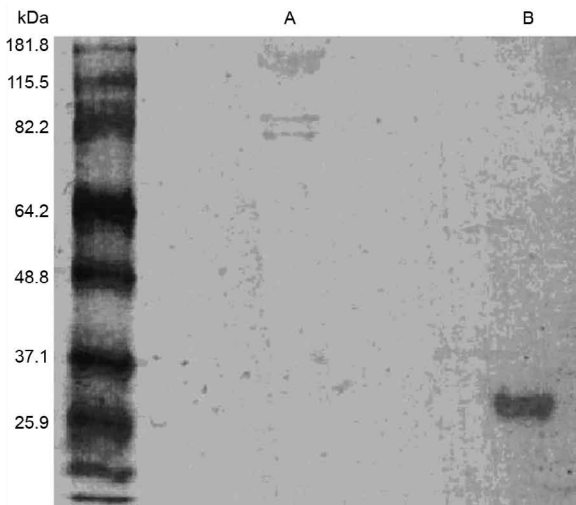


Fig. 4: characterization of cross-reactivity epitopes in N-terminal of *Plasmodium falciparum* (rN-RAP2). Western blot using anti-rNRAP2 mice IgG against extracts from two *P. falciparum* strains. Lanes A: recognition of proteins of ~70 and 82 kDa from insoluble Triton X-100 extracts. These bands correspond to two products of RAP1 cleavage; B: a 29-kDa band corresponding to a cleavage product of RAP2.

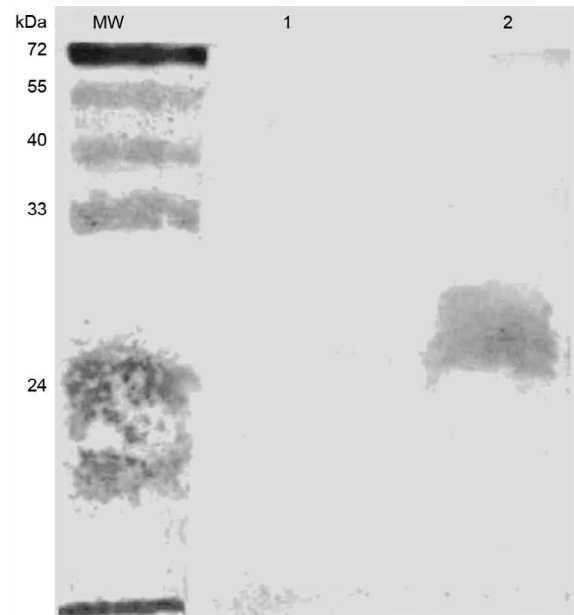


Fig. 5: detection of a functional epitope associated with host-parasite interaction in falciparum malaria. Western blot of two extracts of *Escherichia coli* BL21 pLysS containing rhostry-associated protein 2 (RAP2-pRSET) construct under (1) non-inducing and (2) inducing conditions. An anti-ring surface protein 2 (RSP2) monoclonal antibody was used as the primary antibody and goat anti-mouse IgG peroxidase was used as the secondary antibody. The protein (~29 kDa) present in the induced extract was recognized by an antibody against RSP2, demonstrating the presence of a target epitope on the expressed N-terminal of *Plasmodium falciparum* (rN-RAP2). This suggests that the N-terminal fragment contains an epitope involved in the host-parasite interactions in falciparum malaria recently described by Douki et al. (2003) and Layez et al. (2005).

the production of specific polyclonal antibodies in mice that recognize RAP2 from two strains of *P. falciparum*. Furthermore, the fact that anti-rN-RAP2 antibodies recognize a cleaved form of RAP2 as well as two products of RAP1 processing indicates that (i) both antigens share sequence epitopes and (ii) these epitopes reside within the first 180 residues of RAP2.

Epitopes associated with host-parasite interaction in falciparum malaria - Recent studies have shown that the RSP2, a protein derived of rhostry encoded by a *RAP2* gene, is discharged onto the surface of both infected and UEs and erythroid precursor cells in the bone marrow of malaria patients (Douki et al. 2003, Layez et al. 2005). A mAb against RSP2 was able to detect rN-RAP2 (Fig. 6, Lane 2). This result shows that the recombinant N-terminal fragment of RAP2 protein expressed in *E. coli* possesses epitopes similar to those implicated in the molecular mechanism of host-parasite interaction in falciparum anemia (Douki et al. 2003, Layez et al. 2005).

Additionally, rN-RAP2 was recognized by serum from a malaria patient who developed anemia during infection (Fig. 6), demonstrating that this patient had antibodies against sequence epitopes found in our protein fragment. The successful purification of rN-RAP2 may permit epidemiologic studies aimed at determining the prevalence of anti-RAP2 antibodies in malaria patients with or without severe anemia and understanding the role of this target in the pathology of anemia in patients with falciparum malaria.

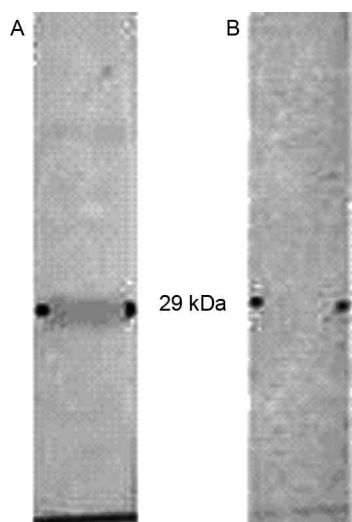


Fig. 6: recognition of N-terminal of *Plasmodium falciparum* (rN-RAP2) by sera of patients who developed anemia during malaria. Western blotting shows recognition of rN-RAP2 by human sera. A: serum from a human exposed to malaria. Two black spots were made with pencil to allow precise placement of the protein on the nitrocellulose. Goat anti-human peroxidase was used as the secondary antibody; B: serum from an individual with no past history of malaria served as the negative control.

DISCUSSION

The rhoptry organelles in the invasive merozoite form of *Plasmodium* species are thought to play a central role in the invasion of erythrocytes (Howard & Reese 1990). More specifically, they have been implicated directly in the sequence of events leading to host cell invasion, PV membrane formation and maintenance of the blood stage of the parasite cycle.

Here, we present a cost-effective method of RAP2 production developed by modifying the solubilization and purification methods of Stowers et al. (1995). In contrast to these researchers who used the full-length protein, we worked with the recombinant N-terminal fragment of RAP2 and achieved purification in denaturing conditions using 8 M urea. We eluted the recombinant protein using a pH gradient. Our results further indicate that the N-terminus portion has epitopes that are common to RAP1 and recognized by the immune system of patients exposed to malaria.

Initially, we obtained only very low levels of rN-RAP2. The fragment was undetectable by SDS-PAGE and Coomassie blue, but it could be observed in Western blots using a commercially-available mAb against hexahistidine. Saul et al. (1992) reported similar results when expressing this protein in *E. coli* clone SG13009 using the pDS56/RBSII hexahistidine vector. Many factors (e.g., the fact that RAP2 is a lipophilic protein) could contribute to this difficulty. The full-length protein contains five cysteines and codons rarely used in *E. coli*. Our N-terminal fragment has three of these cysteines and three rare codons. According to expression protocols, the oxidation of these cysteines may also make expres-

sion and solubilization difficult for inhibit growth of the host cells and rare codons can lead to a high level of frame shifting (QIAexpressionist 2003).

We have observed that D-lactose is as efficient as IPTG for inducing the expression of recombinant protein (data not shown). However, we did not compare expression yields in side-by-side experiments with D-lactose and IPTG as done by Woyski & Cupp-Vickery (2001), who performed such a comparison when inducing the expression of cytochrome P450s with D-lactose. According to these authors, IPTG is the most expensive component of expression experiments and its use at a concentration of 1 mM results in a cost of ~\$6.00/L of culture. In large-scale fermentation, the cost of IPTG could become significant; in our studies, for example, the IPTG for a 5 L production run would cost \$30. Instead, we used D-lactose at 10 g/L. This compound has a total cost of \$0.30 (\$0.06/L), which is 100 fold less expensive than IPTG. In this way, we removed the inducing agent as the most expensive part of expression.

Purification of rN-RAP2 on Ni-IDA resin was less efficient than that on Ni-NTA resin. Despite extensive washing using a gradient of 5-90 mM iDB, the eluate from the Ni-IDA contained impurities visible by Coomassie blue staining (Fig. 3A). However, using the Ni-NTA resin and performing the wash step with denaturation buffer at pH 6.3 and a quick gradient of 5, 10, 15 and 20 mM iDB was sufficient to eliminate Coomassie-visible impurities in the eluate (Fig. 3B). This difference in purification power may be due to the fact that Ni-IDA has only three metal-chelating sites and is unable to bind metal ions tightly. This weak binding leads to ion leaching when the resin is loaded with strongly chelating proteins and peptides or during wash steps. This results in low yields, impure products and metal-ion contamination of isolated proteins. In contrast, the Ni-NTA resin is a tetradentate chelating agent that binds nickel ions at four of the six ligand binding sites, leaving two sites free to interact with the His affinity tag. This means that Ni-NTA can bind metal ions much more tightly than Ni-IDA and it can retain them even under stringent wash conditions (QIAexpressionist 2003).

The data shown here indicate that rN-RAP2 produced by a convenient and inexpensive protein expression system in prokaryotes can elicit an IgG response in mice and that the antisera produced can recognize native antigens from two strains of *P. falciparum*. Our results show that rN-RAP2 shares at least one cross-reactive epitope with RAP1. The same data were found by Stowers et al. (1996), who detected 65 and 70 kDa products of RAP1 processing during merozoite invasion as well as other bands of 29 and 42 kDa in Triton X-100 parasite extracts. All of these bands correspond to uncleaved and cleaved forms of RAP2 (Howard & Reese 1998).

Stowers et al. (1996) demonstrated that anti-RAP2 antibodies cross-react with several linear epitopes of RAP1, such as C₃LTEFSKLY₁₂ in RAP2 and L₂₀₂TPLEELY₂₀₉ in RAP1. The authors noted that the cross-reactivity of anti-RAP2 antibodies with RAP1 occurs because the epitopes in the two proteins are identical in three of the four flanking amino acids (underlined). This region

of RAP1 has been shown to inhibit parasites in vitro and confer partial protection in monkeys (Ridley et al. 1990). RAP1 and RAP2, together with a poorly understood third protein RAP3, form a low molecular weight complex within the rhoptries of *P. falciparum*. Experiments in which the *RAP1* gene was disrupted have shown that RAP1 is required to localize RAP2 to rhoptries. RAP1 normally exists in complexes with RAP2 and RAP3, and some of these complexes are stabilized through merozoite invasion and the development of young ring-stage parasites (Balbi et al. 2002). Moreover, anti-RAP1 antibodies immunoprecipitate both RAP1 and RAP2 (Stowers et al. 1996). However, truncation mutants of RAP1 cause mislocalization of RAP2 but do not affect the efficiency of in vitro blood-stage growth. These results have suggested that the low molecular weight complex is not required for *P. falciparum* invasion and growth in human erythrocytes (Baldi et al. 2000), supporting the idea anti-RAP2 antibodies may not be involved in protection against the parasite.

The fact that rN-RAP2 could be detected by the same mAb that recognized the RSP2 attached on the surface of infected and UEs and by serum from a malaria patient who developed anemia indicates that the recombinant protein can still be recognized by the immune system. We have thus achieved the recombinant expression of rN-RAP2 containing a “ligand” sequence involved in the host-parasite interaction for attaching onto surface of infected and UEs.

Future studies with rN-RAP2 may permit better understanding of this target in the pathology of anemia in patients with falciparum malaria. Our expression system may therefore prove valuable for generating an immunogenic protein fragment for epidemiological studies designed to evaluate the effects of the anti-RAP2 immune response in anemic malaria patients.

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