

One-step design of a stable variant of the malaria invasion protein RH5 for use as a vaccine immunogen

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Many promising vaccine candidates from pathogenic viruses, bacteria, and parasites are unstable and cannot be produced cheaply for clinical use. For instance, *Plasmodium falciparum* reticulocyte-binding protein homolog 5 (PfRH5) is essential for erythrocyte invasion, is highly conserved among field isolates, and elicits antibodies that neutralize *in vitro* and protect in an animal model, making it a leading malaria vaccine candidate. However, functional RH5 is only expressible in eukaryotic systems and exhibits moderate temperature tolerance, limiting its usefulness in hot and low-income countries where malaria prevails. Current approaches to immunogen stabilization involve iterative application of rational or semirational design, random mutagenesis, and biochemical characterization. Typically, each round of optimization yields minor improvement in stability, and multiple rounds are required. In contrast, we developed a one-step design strategy using phylogenetic analysis and Rosetta atomistic calculations to design PfRH5 variants with improved packing and surface polarity. To demonstrate the robustness of this approach, we tested three PfRH5 designs, all of which showed improved stability relative to wild type. The best, bearing 18 mutations relative to PfRH5, expressed in a folded form in bacteria at >1 mg of protein per L of culture, and had 10–15 °C higher thermal tolerance than wild type, while also retaining ligand binding and immunogenic properties indistinguishable from wild type, proving its value as an immunogen for a future generation of vaccines against the malaria blood stage. We envision that this efficient computational stability design methodology will also be used to enhance the biophysical properties of other recalcitrant vaccine candidates from emerging pathogens.

malaria | immunogen design | Rosetta | PROSS

Malaria places the gravest public-health burden of all parasitic diseases, leading to ~215 million human clinical cases and ~440,000 deaths annually (1). The most virulent parasite species, *Plasmodium falciparum*, is endemic in large parts of sub-Saharan Africa and Southeast Asia. It causes the majority of malaria deaths and is a major target for vaccine development. There are currently no licensed malaria vaccines on the market, and the leading anti-sporozoite subunit vaccine, RTS,S/AS01, has achieved only modest efficacy of 30–50% in phase II/III clinical trials (2, 3). There is therefore an urgent need to develop additional vaccines, including those that target various stages of the parasite life cycle (4).

One of the leading malaria vaccine candidates is *Plasmodium falciparum* reticulocyte-binding protein homolog 5 (PfRH5), a protein required for the establishment of blood stage infection. PfRH5 is released onto the surface of infective *Plasmodium falciparum* merozoites, binding to human basigin in an interaction that is essential for erythrocyte invasion (4–7). Compared with other *Plasmodium* surface antigens, it is remarkably conserved across field isolates (7–11) and antibodies that bind either PfRH5 or basigin show robust growth-inhibitory effects *in vitro* against all tested strains of *Plasmodium falciparum* (5, 7–9, 11–13). Additionally, in a challenge trial, immunization with PfRH5-based vaccines protected *Aotus* monkeys against heterologous challenge with a virulent *Plasmodium falciparum* strain (12). PfRH5 is therefore the best-performing antigen against the blood stage of the parasite, and

clinical trials are already underway to test its safety, immunogenicity, and efficacy in immunized human volunteers (4).

Despite this promise, PfRH5 suffers from two significant shortcomings as a subunit vaccine candidate. First, the protein has limited stability at high temperatures, and second, despite extensive protein engineering (11), correctly folded, soluble, and functional PfRH5 has not been produced in microbial expression hosts. Instead, production has relied on more expensive eukaryotic expression systems, such as transiently transfected HEK293 cells (7) or stable insect cell lines (11, 14). Because the most likely use for PfRH5-based vaccines would require infant immunization in hot and underdeveloped regions, where a cold chain for transporting vaccine formulations is very challenging, a stabilized and lyophilized variant that can be cheaply produced in microbial cells, and that will retain efficacy when stored at elevated temperatures, is highly desirable. We therefore aimed to design versions of PfRH5 with improved expression levels and thermal stability, without compromising their effectiveness as immunogens.

Many potential vaccine immunogens are only marginally stable. To address this problem, approaches for immunogen stabilization or grafting of immunogenic epitopes onto stable scaffolds have been implemented (15–21). However, key vaccine immunogens frequently have complex folds with significant flexibility and low stability. Together with the strict requirement to maintain neutralizing immunological responses, this means that current efforts for

Significance

Malaria is one of the world's most devastating infectious diseases, affecting hundreds of millions of people and resulting in nearly half a million deaths each year. The parasites that cause malaria must invade the red blood cells of an infected patient, while blocking this process prevents the disease. The PfRH5 protein is an exciting vaccine candidate required for red blood cell invasion by *Plasmodium falciparum*, the most deadly malaria parasite. Here, we describe our use of a streamlined computational methodology to design variants of PfRH5 that can be produced more simply and cheaply and that show greater thermal stability. This method has broad potential to help the design of vaccines that target many of the world's most deadly diseases.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 5M10).

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immunogen stabilization often require time-consuming and labor-intensive cycles. For instance, in the design of superior HIV and respiratory syncytial virus immunogen variants, multiple rounds of rational design, random mutagenesis, and biochemical, immunological, and structural characterization were applied (15–21). Although effective, such iterative strategies limit the ability to respond quickly to emerging pathogens.

We recently described a stability-design algorithm, called PROSS (22), and demonstrated its effectiveness in designing variants of challenging human enzymes with much improved thermal stability and increased bacterial expression levels, without affecting protein function. Guided by the recent structures of PfrH5, alone and in complex with its human receptor basigin and with neutralizing antibodies (11, 23), we embarked on a one-step, structure-based computational design process to generate PfrH5 variants, aiming to improve expression levels and thermal stability while retaining structure, ligand binding, and immunogenic properties.

Results

Extending Sequence Analysis Below the “Twilight Zone.” The PROSS workflow comprises three stages. It first analyzes homologs of the target protein to identify, at each amino acid position, mutations that are most likely to occur through natural diversity of the protein family. Second, starting from a molecular structure of the target protein, Rosetta atomistic design simulations suggest a subset of these mutations, which are individually predicted to stabilize the wild-type protein. At the last step, Rosetta combinatorial sequence optimization is used to design several optimized variants, typically comprising >10 mutations each, with improved native-state energy. In all steps, amino acid conformations in the active site are held fixed to preserve function. In this workflow, high natural sequence diversity provides an essential source of information on tolerated variation, and we have so far applied PROSS only to targets with dozens and even hundreds of unique homologs (22).

PfrH5 presented an unusual challenge for the phylogenetic sequence analysis implemented in PROSS. As of May 2015, sequences of PfrH5 from *Plasmodium falciparum* field isolates showed 99% sequence identity to one another, and only one ortholog, from *Plasmodium reichenowi* (with 66% sequence identity to PfrH5) was available. This extremely low sequence diversity made application of the PROSS methodology “as is” impractical. To increase diversity, we extended the search below the twilight zone of sequence identity (<25%) and took special measures to validate the sequence alignment. Specifically, context-specific iterated BLAST (CSI-BLAST) identified 72 unique homologs at 15–25% sequence identity to PfrH5, more than 90% of which showed only 15–18% sequence identity to PfrH5. From this set, we excluded sequences from genera other than *Plasmodium*, and any sequences that showed more than 1% gaps in the aligned segment. Because the RH5 fold is considered unique and highly conserved in *Plasmodium* invasion proteins (11), these restrictions increase the likelihood that the sequences in the alignment belong to the same fold. As a further safety measure, we generated two alignments, a “permissive” alignment containing all the homologs described above and a “strict” one at >18% sequence identity. These alignments had 14 and 8 sequences, respectively (Datasets S1 and S2). As a template structure, we used a version of RH5 with both the flexible N terminus (residues 1–140) and a disordered loop (residues 248–296) removed, as this PfrH5ΔNL variant contains the structured region of RH5 and retains the capacity to bind to basigin and induce production of inhibitory antibodies (11). The two alignments and the PfrH5ΔNL structure were then provided to the PROSS algorithm in two independent runs. To preserve the function and immunological efficacy of the designed PfrH5 variants, we did not allow mutations at amino acid positions within 5 Å of the contact sites of either basigin or two anti-PfrH5 inhibitory antibodies, 9AD4 and QA1 (11, 13) (Table S1). We then visually inspected the designs and selected three for experimental testing,

one based on the strict alignment bearing 18 mutations relative to PfrH5 (PfrH5ΔNL_{HS1}) and two using the permissive alignment bearing 25 and 15 mutations (PfrH5ΔNL_{HS2} and PfrH5ΔNL_{HS3}, respectively) (Fig. 1A, Table S1, and Datasets S1 and S2).

Generation of a Stabilized, Functional, and Bacterially Expressed PfrH5.

Genes encoding each of these three variants were designed and tested in *Escherichia coli* expression strain Rosetta-gami under different growth conditions. Although no detectable soluble expression was observed for PfrH5ΔNL, each of the stabilized variants

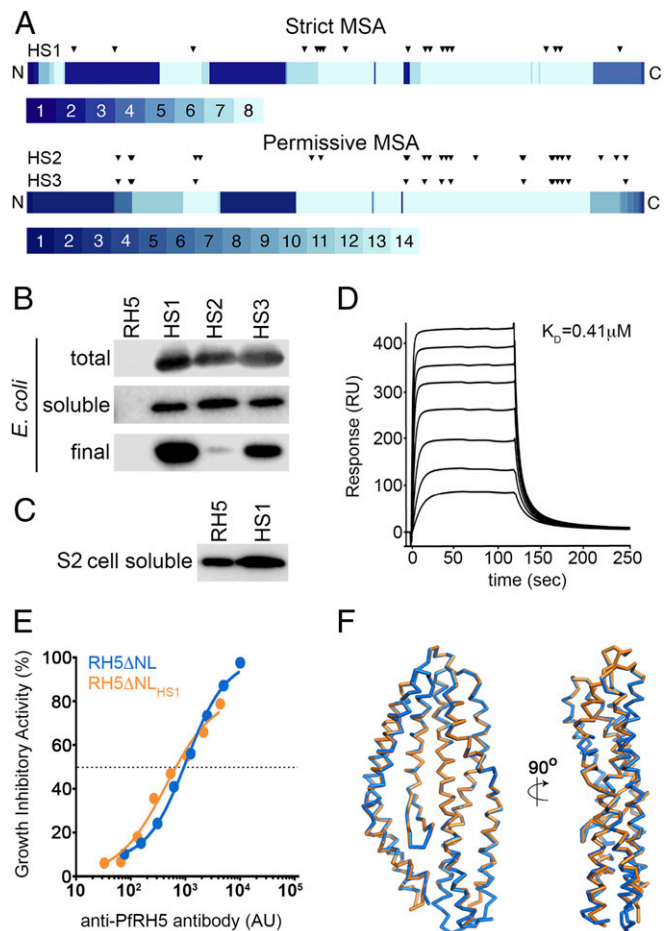


Fig. 1. Design of an *E. coli*-expressible PfrH5 variant. (A) Eighty percent of mutations in all three designed variants are located in the C-terminal half of PfrH5, where most of the aligned sequences contribute information on sequence diversity. The PfrH5 sequence is schematically shown from N to C terminus, and each position is colored according to the number of unique sequences contributing to the strict and permissive alignments, ranging from 1 to 8 and from 1 to 14, respectively. The locations of the mutations in each designed variant relative to wild-type PfrH5ΔNL are indicated by triangles. (B) Expression levels of PfrH5ΔNL (RH5), PfrH5ΔNL_{HS1} (HS1), PfrH5ΔNL_{HS2} (HS2), and PfrH5ΔNL_{HS3} (HS3) from *E. coli*. “Total” is whole lysed cells, “soluble” is material after cell lysis and clarification, and “final” is after immobilized metal ion affinity chromatography and size exclusion chromatography. (C) Expression levels of PfrH5ΔNL (RH5) and PfrH5ΔNL_{HS1} (HS1) secreted in the cell supernatants from a stable *Drosophila melanogaster* Schneider 2 (S2) cell line. (D) Surface plasmon resonance analysis of the binding of PfrH5ΔNL_{HS1} to basigin, with twofold dilutions of PfrH5ΔNL_{HS1} from a maximal concentration of 8 μM. (E) In vitro GIA of purified IgG against 3D7 clone parasites from mice immunized with either PfrH5ΔNL or PfrH5ΔNL_{HS1}. The anti-PfrH5 antibody response was measured for each sample of purified IgG and is plotted against the measured level of GIA. The dashed line indicates 50% GIA, and each GIA data point represents the mean of each sample tested in triplicate. (F) The structure of PfrH5ΔNL_{HS1}:9AD4 (orange) overlaid on the structure of PfrH5ΔNL:9AD4 (blue).

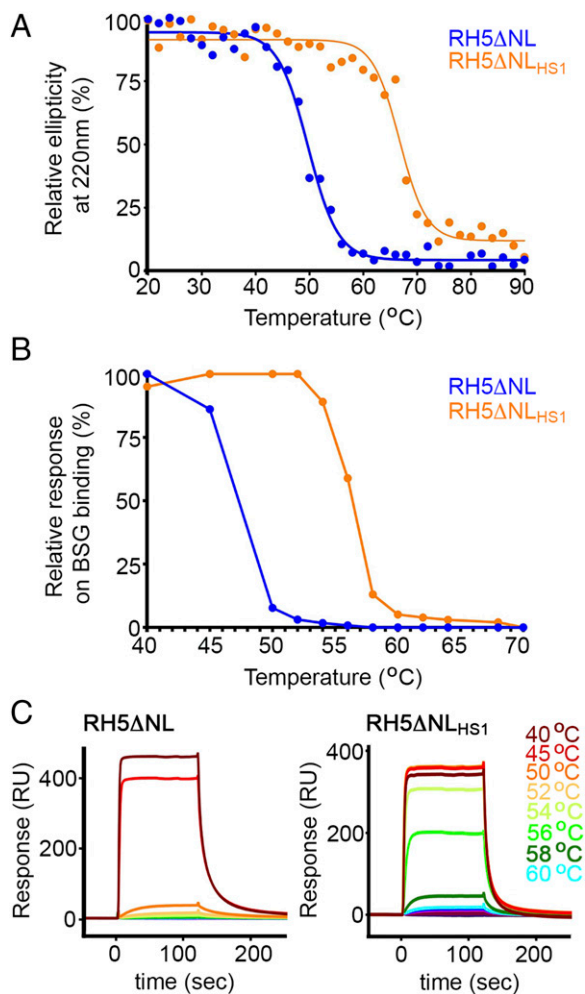


Fig. 2. Increased thermal stability of variant PfrH5ΔNL_{HS1}. (A) Determination of the effect of temperature on the ellipticity of PfrH5ΔNL and PfrH5ΔNL_{HS1} at a wavelength of 220 nm as measured by circular dichroism. (B and C) Determination of the effect of temperature on the binding of PfrH5ΔNL and PfrH5ΔNL_{HS1} to basigin (BSG), measured by surface plasmon resonance. Protein, at 16 μM, was incubated for 60 min at the specified temperature before analysis at 8 μM.

expressed at similar levels (Fig. 1B). Protein from each variant was purified by immobilized metal ion chromatography, followed by size exclusion chromatography (Fig. S1). This showed PfrH5ΔNL_{HS1} to have the best production properties, yielding ~50-fold more protein than PfrH5ΔNL_{HS2} and approximately fourfold more than PfrH5ΔNL_{HS3}. PfrH5ΔNL_{HS1} was produced with a final yield of ~1.3 mg from each liter of *E. coli* culture. To determine whether these improvements in expression properties are limited to prokaryotic expression systems, we also generated a stable *Drosophila melanogaster* Schneider 2 (S2) cell line that expresses PfrH5ΔNL_{HS1}, allowing us to assess its expression levels in what is currently the leading system for PfrH5 expression (11, 14). Here too, we observed an increased yield of protein, with PfrH5ΔNL_{HS1} expressed at levels threefold to fourfold higher than PfrH5ΔNL (Fig. 1C). Therefore, stability design resulted in the first versions of PfrH5 that express in a stable, soluble, and folded form in *E. coli* and were also shown to have the potential to increase the level of expression of PfrH5 in insect cell systems.

We next assessed the structural integrity and functionality of PfrH5ΔNL_{HS1} purified from bacteria. We used surface plasmon resonance to show that this protein bound to basigin with an affinity of 0.41 μM (Fig. 1D), comparable to the 0.29 μM observed for PfrH5ΔNL (Fig. S2). In addition, basigin binding with an

affinity of ~0.3 μM was retained after lyophilization and resuspension of both PfrH5ΔNL and PfrH5ΔNL_{HS1}, increasing the options for vaccine preparation (Fig. S2). To ensure that PfrH5ΔNL_{HS1} contains the epitopes required to elicit an inhibitory immune response, we raised mouse polyclonal IgG and tested their ability to neutralize parasites in an in vitro assay of growth inhibition activity (GIA) (Fig. 1E). IgG raised against PfrH5ΔNL_{HS1} showed a strong inhibitory effect, similar to that for IgG raised against PfrH5ΔNL. Indeed, these polyclonal IgG were qualitatively comparable, requiring very similar amounts of PfrH5-specific IgG to neutralize 50% of parasites. Finally, we determined the crystal structure of PfrH5ΔNL_{HS1} in complex with the Fab fragment from 9AD4, an inhibitory monoclonal antibody raised previously against PfrH5ΔNL (13), to 2.35-Å resolution (Fig. 1F, Fig. S3, and Table S2). Composite omit maps showed clear electron density for mutated residues, largely in their designed positions (Fig. S3). However, there were no other significant changes in the PfrH5 structure with r.m.s.d. values of 0.7 Å for the backbone Cα positions and 1.0 Å for the complex, when comparing PfrH5ΔNL_{HS1} and PfrH5ΔNL. The stabilized and bacterially expressed PfrH5ΔNL_{HS1} variant therefore retains the structure, ligand binding, and immunogenic properties of native PfrH5.

To assess the thermal stability of PfrH5ΔNL_{HS1} (defined as the temperature at which one-half of the protein is inactive), we first conducted circular dichroism with a thermal melt, following ellipticity at 220 nm as a measure of α-helicity (Fig. 24). Although ellipticity for PfrH5ΔNL was reduced by 50% at 48 °C, the stabilized variant, PfrH5ΔNL_{HS1}, showed the same loss of ellipticity at 66 °C, showing a ~20 °C improvement in thermal stability. To assess whether this loss of ellipticity is reversible, we heated PfrH5ΔNL_{HS1} to 75 °C and cooled to 20 °C before repeating circular dichroism measurements, and observed no recovery of secondary structure, showing that thermal denaturation is irreversible (Fig. S4). As circular dichroism measures secondary-structure content rather than protein functionality, we also conducted an experiment in which we incubated the PfrH5 variants at different temperatures for an hour before returning them to room temperature and testing basigin

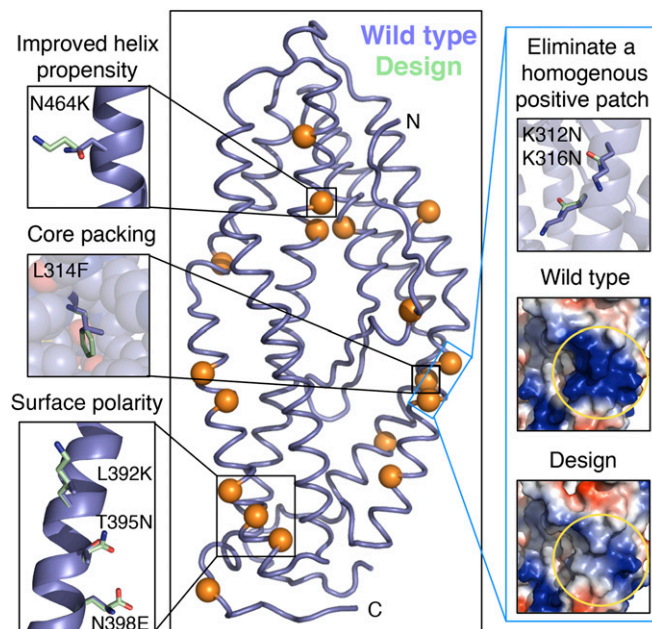


Fig. 3. Structural insights into thermal stability of PfrH5ΔNL_{HS1}. Structure of wild-type PfrH5 is shown in blue, and the 18 mutated positions, throughout PfrH5ΔNL_{HS1}, are indicated by orange spheres in the central panel. Surrounding thumbnails highlight stabilizing effects of selected mutations.

binding by surface plasmon resonance (Fig. 2*B* and *C*). P_fRH5ΔNL lost 50% of its basigin-binding capacity at ~47 °C, whereas P_fRH5ΔNL_{HS1} showed a similar loss at ~57 °C, showing a ~10 °C improvement in thermal stability. We have therefore designed a variant of P_fRH5 that retains both the structure and immunogenicity of the native protein, while allowing expression in *E. coli* and increased thermal stability.

To assess the reasons for its higher bacterial expression levels and improved thermal stability, we compared the structure of P_fRH5ΔNL_{HS1} with that of P_fRH5ΔNL. The comparison showed several of the hallmarks expected for stabilizing mutations (Fig. 3 and Table S3). Of the 18 mutations, 15 occurred on the surface, of which 8 improved surface polarity, and an additional 2 (Lys312Asn and Lys316Asn) eliminated a homogenous positively charged patch. Indeed, such patches have been associated with aggregation and poor stability (24, 25). Furthermore, P_fRH5 comprises a mostly helical backbone (75% of the sequence), and seven mutations increased helix-forming propensity relative to P_fRH5. Last, the design contains three mutations that improve hydrophobic core packing (Asp183Glu, Leu314Phe, and Ser467Ala). It is by combining a large set of mutations in a single variant that significant gains in expression levels and thermal stability can be achieved.

Discussion

In conclusion, we have used a computational method to design improved expression properties and thermal stability into a leading malaria vaccine candidate, P_fRH5. This generated an improved immunogen that could be scaled economically for production in *E. coli* and is more thermally stable for storage and delivery. This immunogen will be valuable in future generations of malaria vaccines. The approach used here is also highly applicable for the development of vaccine immunogens against other stages of the life cycle of *Plasmodium falciparum* or other pathogens. Indeed, a major challenge in applying PROSS to P_fRH5 was the lack of sequence variation in this protein across different parasite strains, requiring a modified strategy for selecting distant sequence homologs. Although this will be an issue for some other vaccine candidates, such as highly conserved transmission-blocking targets, the majority of pathogen surface proteins, from viral surface proteins to the diverse *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family of *Plasmodium* surface proteins (26), are highly variable as they evolve under diversifying selection pressure to avoid detection by the acquired immune system. With judicious selection of natural variants on which to base the design process, the methods outlined here will be applicable to many of these cases, whether highly conserved or highly diverse.

Current methods for vaccine immunogen stabilization rely on laborious cycles of design and experimental testing. By contrast, the design strategy implemented here allows for rapid and significant enhancement of stability and expression levels in one test cycle. We therefore expect this method to contribute to vaccine immunogen production across a wide range of the most deadly diseases affecting humanity.

Materials and Methods

Collection and Filtering of Homologous Sequences. The P_fRH5 structure was downloaded from the Protein Data Bank (PDB) [ID code 4WAT (23)], and homologous sequences were collected using CSI-BLAST (27, 28) to search the nonredundant (nr) database in May 2015, with *e* value of <10⁻⁴, three iterations, a maximum of 500 sequences, and default values on all other parameters. Hits were clustered using cd-hit (29) at 98% threshold and default parameters. Hits from genera other than *Plasmodium* were excluded. Hits were also

excluded if their sequence identity to the query was lower than 15% or if they showed more than 1% gaps in the aligned segment. Of the remaining sequences, two sets of hits were defined: a strict set containing only hits sharing 19% sequence identity to the query or more (P_fRH5ΔNL_{HS1}), and a permissive set containing all remaining hits (8 and 14 hits, respectively, including the query sequence) (P_fRH5ΔNL_{HS2} and P_fRH5ΔNL_{HS3}). The strict alignment contained the following UniProt entries: Q8IFM5 (PDB ID code 4WAT), A0A078K5N4, B4X6H6, K6VIX0, A0A060RXZ9, W7J6M4, A0A024WYW5, and Q7YWE8; the permissive alignment contained the entries in the strict alignment and the following additional entries: I6QQT7, C1IW27, I6RGY9, A5K940, A5K696, and A0A060S1Z4. MUSCLE (30) was used with default parameters to derive a multiple sequence alignment from each set of hits.

PROSS Stability Design. To preserve the P_fRH5 binding interface with its natural target basigin [PDB ID code 4U0Q (11)] and with two neutralizing antibodies, 9AD4 and QA1 [PDB ID codes 4U0R and 4U1G (11), respectively], 63 residues within 5 Å of all three interfaces were held fixed throughout all Rosetta simulations (Table S1).

Two independent runs of the PROSS algorithm (22) were carried out based on the strict and permissive alignments. As a template structure, we used a version of P_fRH5 with both the flexible N terminus (residues 1–140) and a disordered loop (residues 248–296) removed (P_fRH5ΔNL) (11). Because P_fRH5 is large (>400 aa), we decided to experimentally test designs with 15 mutations or more. Using the strict alignment, only one design had more than 15 mutations (P_fRH5ΔNL_{HS1}), and using the permissive alignment, two designs were selected for experimental testing based on visual inspection (P_fRH5ΔNL_{HS2} and P_fRH5ΔNL_{HS3}) (Table S1). Datasets S1 and S2 contain the strict and permissive alignments, respectively, as well as the three design variants.

Design Model Analysis. Sequence and structural features of P_fRH5ΔNL_{HS1} were compared with the sequence and structure of wild-type P_fRH5ΔNL. Mutations were defined as improving helical propensity if they were in helical regions and if Rosetta energy calculations showed $\Delta\Delta G_{calc}$ of less than -0.15 Rosetta energy units for the energy term that accounts for the compatibility of the amino acid identity with the local backbone ϕ and ψ dihedral angles (*p_aa_pp*). Positions were defined as buried if they had >21 and >75 neighboring nonhydrogen atoms within 10 and 12 Å, respectively, according to the Rosetta Features Reporter (31, 32).

Expression, Purification, and Characterization of Thermally Stabilized RH5 Variants. A gene for *Plasmodium falciparum* RH5 spanning from K141 to Q526 with both the flexible N terminus (residues 1–140) and a disordered loop (residues 248–296) removed (P_fRH5ΔNL) was available from a previous study (11). Synthetic genes were designed to express the equivalent regions from the thermally stabilized variants with codon use optimized for *Drosophila melanogaster* expression. They were expressed in the Rosetta-gami strain of *E. coli* and were purified using immobilized affinity and size exclusion chromatography. P_fRH5ΔNL and basigin were expressed as described (11). Determination of the structure of the P_fRH5ΔNL_{HS1}:9AD4 complex and its biophysical and immunological characterization were characterized using protocols similar to those described (11) and are detailed in *SI Materials and Methods*. All procedures on mice were performed in accordance with the terms of the UK Animals (Scientific Procedures) Act Project License and were approved by the University of Oxford Animal Welfare and Ethical Review Body.

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