

Production of non-glycosylated recombinant proteins in *Nicotiana benthamiana* plants by co-expressing bacterial PNGase F

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Received 30 November 2011;

revised 28 February 2012;

accepted 29 February 2012.

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Summary

Application of tools of molecular biology and genomics is increasingly leading towards the development of recombinant protein-based biologics. As such, it is leading to an increased diversity of targets that have important health applications and require more flexible approaches for expression because of complex post-translational modifications. For example, *Plasmodium* parasites may have complex post-translationally modified proteins such as Pfs48/45 that do not carry *N*-linked glycans (*Exp. Parasitol.* 1998; **90**, 165.) but contain potential *N*-linked glycosylation sites that can be aberrantly glycosylated during expression in mammalian and plant systems. Therefore, it is important to develop strategies for producing non-glycosylated forms of these targets to preserve biological activity and native conformation. In this study, we are describing *in vivo* deglycosylation of recombinant *N*-glycosylated proteins as a result of their transient co-expression with bacterial PNGase F (Peptide: *N*-glycosidase F). In addition, we show that the recognition of an *in vivo* deglycosylated plant-produced malaria vaccine candidate, Pfs48F1, by monoclonal antibodies I, III and V raised against various epitopes (I, III and V) of native Pfs48/45 of *Plasmodium falciparum*, was significantly stronger compared to that of the glycosylated form of plant-produced Pfs48F1. To our knowledge, neither *in vivo* enzymatic protein deglycosylation has been previously achieved in any eukaryotic system, including plants, nor has bacterial PNGase F been expressed in the plant system. Thus, here, we report for the first time the expression in plants of an active bacterial enzyme PNGase F and the production of recombinant proteins of interest in a non-glycosylated form.

Keywords: *N*-linked glycosylation, PNGase F, deglycosylation, plant transient expression, recombinant proteins, malaria vaccine candidate Pfs48/45.

Introduction

N-linked glycosylation is a post-translational modification which is critical for correct folding, stability and biological activity of many proteins including recombinant subunit vaccines and therapeutic proteins produced in heterologous expression systems (Gomord *et al.*, 2010; Wujek *et al.*, 2004). Some eukaryotic (as well as bacterial) proteins may not contain *N*-glycans in the native host, but their proteins may contain multiple potential glycosylation sites that are aberrantly glycosylated when these proteins are expressed in heterologous eukaryotic expression systems, potentially leading to impaired functional activity. Indeed, the attachment of carbohydrates strongly affects the physico-chemical properties of a protein, therefore can alter its essential biological properties such as specific activity, ligand–receptor interactions and immunogenicity and may pose a safety risk when used *in vivo*. For example, aberrant *N*-linked glycosylation of cell surface receptors such as integrins and cadherins has been shown to stimulate carcinoma progression and metastasizing (Guo *et al.*, 2002; Partridge *et al.*, 2004), indicating significant changes in these proteins' behaviour. When a malaria vaccine candidate, Pfs48/45, containing seven putative *N*-glycosylation sites, was expressed in a yeast system (*Pichia pastoris*), it did not have a transmission-blocking (TB) activity

and did not induce TB antibodies in mice (Milek *et al.*, 2000). Pfs48/45 was also expressed in plants (*Nicotiana benthamiana*) at Fraunhofer USA Center for Molecular Biotechnology (FhCMB). FhCMB has engineered Pfs48/45 as a poly-histidine (6xHis)-tagged protein, which was purified using immobilized metal ion affinity chromatography (IMAC), but the TB activity of this plant-derived vaccine candidate was low (data not shown). The low TB activity of Pfs48/45 may be associated with an incorrect/altered folding or masking of important epitopes of the protein because of glycosylation. Although according to a recent report (Bushkin *et al.*, 2010) *Plasmodium* has the potential to transmit GlcNAc proteins, it was shown that the native Pfs48/45 protein does not contain *N*-linked glycans (Milek *et al.*, 1998). Because the Pfs48/45 protein has seven putative *N*-linked glycosylation sites, these sites can be aberrantly glycosylated when the protein is expressed in any of the available eukaryotic hosts. Indeed, the C-terminal fragment (containing 10 cysteine residues) of the Pfs48/45 protein of *Plasmodium falciparum* expressed in *Escherichia coli* as a maltose-binding protein fusion has been shown to be correctly folded (when co-expressed with four *E. coli* chaperones) and elicits functional TB antibodies in mice (Outchkourov *et al.*, 2008). However, the microbial expression system has drawbacks: (i) the full-length form of Pfs48/45 (M-Pfs16C, 16 cysteine residues and seven

putative glycosylation sites) did not properly fold and had poor solubility and much weaker epitope recognition; (ii) the Pfs48/45 fragments expressed in *E. coli* are rapidly and significantly degraded by host cell proteases in the *E. coli* periplasm; (iii) the expression level is low; and (iv) purified vaccine candidates may not be free from bacterial endotoxin contamination.

Plants, on the other hand, offer several advantages compared to other recombinant protein expression systems; these include the possession of eukaryotic post-translational modification machinery, simple low-cost scale up for manufacturing, and safety of use of plant-derived products in humans or animals because of the lack of any harboured microbial or mammalian pathogens (Mett *et al.*, 2008; Yusibov and Mamedov, 2010). Plant viral vector-based transient expression in *N. benthamiana* plants has been successfully used for a time- and cost-efficient production of recombinant vaccine candidates against various infectious diseases (Farrance *et al.*, 2011; Mett *et al.*, 2011). The biological activity of many therapeutic proteins is dependent on their glycosylation status, and mammalian glycoproteins are efficiently glycosylated when they are expressed in plants. However, plants can also efficiently produce *N*-linked glycosylated recombinant proteins that do not require *N*-linked glycosylation. Thus, for improved functional activity and immunogenicity of certain vaccine candidates and therapeutic proteins including antibodies, it is important to develop a strategy to produce non-glycosylated forms of proteins using transient expression in *N. benthamiana* plants.

Here, we describe a method for producing deglycosylated proteins in plant cells, by transiently expressing bacterial PNGase F (Peptide: *N*-glycosidase F) in combination with a target protein of interest. PNGase F is a 34.8-kDa enzyme secreted by a Gram-negative bacterium *Flavobacterium meningosepticum* (Plummer *et al.*, 1984; Tarentino *et al.*, 1990). It cleaves a bond between the innermost GlcNAc and asparagine residues of high-mannose, hybrid and complex oligosaccharides in *N*-linked glycoproteins, except when the α (1–3) core is fucosylated. In this study, we transiently co-expressed several recombinant proteins including the malaria vaccine candidate Pfs48F1, protective antigen (PA) of *Bacillus anthracis* and an antibody against PA of *B. anthracis* with bacterial PNGase F in *N. benthamiana* plants. We have demonstrated that bacterial PNGase F is fully active *in vivo* and successfully cleaves *N*-linked oligosaccharides in target glycoproteins, resulting in the uniformity of the expressed proteins.

Results

Expression, purification and characterization of bacterial PNGase F produced in *N. benthamiana* plants

The bacterial PNGase F gene sequence encompassing 314 amino acids (the full length of the catalytically active protein without a signal sequence) was optimized for the expression in *N. benthamiana* plants, cloned into the pGRD4 vector (Shoji *et al.*, 2009) and expressed in *N. benthamiana* plants as described in Experimental procedures. All constructs generated and used in this study are schematically shown in Figure 1. The average expression level of PNGase F was approximately 150 mg/kg of fresh leaf biomass. The expression of PNGase F was confirmed by the Western blot analysis using an anti-FLAG monoclonal antibody (mAb) (Figure 2a).

The recombinant enzyme was purified from *N. benthamiana* leaves using an anti-FLAG agarose column as described in Experimental procedures. As shown by SDS–PAGE and Coomassie staining, the purified PNGase F protein was highly homogeneous (Figure 2b).

To evaluate the deglycosylating activity of recombinant PNGase F *in vitro*, the purified plant-produced enzyme was incubated with a glycosylated malaria antigen, the purified Pfs48F1 protein of *P. falciparum*, which was also expressed in *N. benthamiana* plants. The results of the Western blot analysis indicated that purified plant-derived PNGase F was able to deglycosylate Pfs48F1 *in vitro* and that the degree of deglycosylation was proportional to the amount of purified PNGase F used in the incubation reaction (Figure 2c). Similar results were obtained when Pfs48F1 was incubated with a crude extract from PNGase F-expressing plants or commercial PNGase F (New England Biolabs, Ipswich, MA) (Figure 2c).

In addition, *N. benthamiana* plants expressing PNGase F remained healthy at 7, 8 and 9 days post-infiltration (dpi) (data not shown) with no visible symptom development or change in growth when co-expression of target reached the highest level.

In vivo deglycosylation of recombinant Pfs48F, *B. anthracis* PA and an anti-PA antibody in *N. benthamiana* plants by co-expressing PNGase F

To evaluate *in vivo* cleavage of *N*-linked oligosaccharides decorating Pfs48F1, bacterial PNGase F and Pfs48F1 were transiently co-expressed in *N. benthamiana* plants via co-agroinfiltration

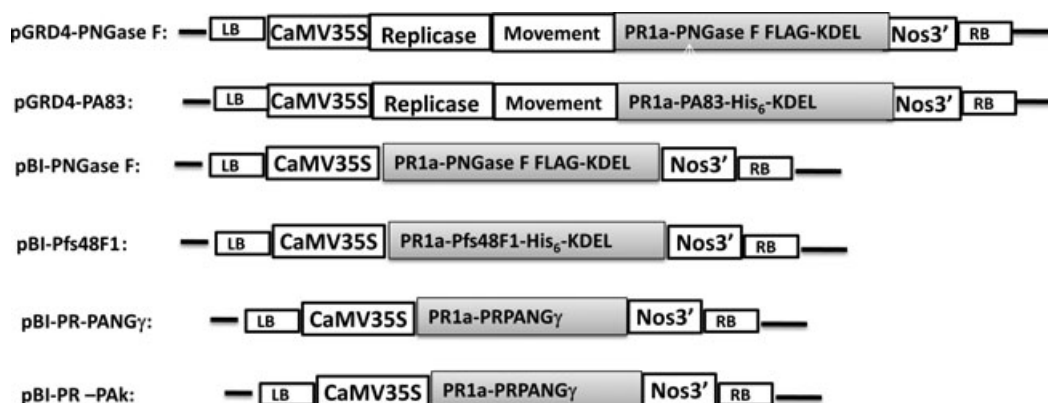


Figure 1 Schematic representation of different plant expression cassettes based on the pGRD4 and binary vectors generated in this study. CaMV35S, Cauliflower mosaic virus promoter; LB, left border; RB, right border; nos, nopaline synthase gene terminator.

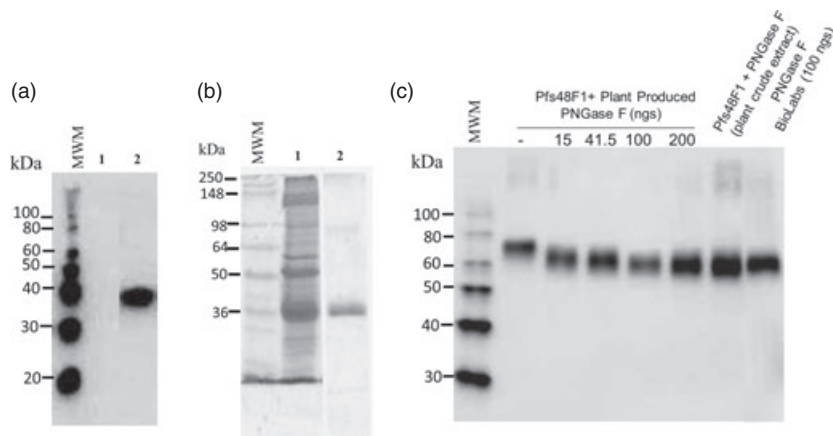


Figure 2 (a) Western blot analysis of bacterial PNGase F produced in *Nicotiana benthamiana* plants. Samples were prepared as described in Experimental procedures. Briefly, leaf samples were taken at 7 dpi and homogenized in three volumes of extraction buffer. After centrifugation at 13 000 *g* for 20 min, 10 μ L of 10-fold diluted samples were run on SDS-PAGE prior to Western blotting. PNGase F was detected using the anti-FLAG polyclonal antibody (Sigma). Lanes: 1—crude extract prepared from control plant; 2—crude extract prepared from plant infiltrated with bacterial PNGase F (pGRD4-PNGase F). (b) SDS-PAGE of purified PNGase F. PNGase F was purified from *N. benthamiana* plants using an anti-FLAG agarose column as described in Experimental procedures, and the purified protein was separated by SDS-PAGE and stained with Coomassie. Lanes: 1—TSP; 2—purified PNGase F. (c) Western blot analysis of *in vitro* and *in vivo* Pfs48F1 deglycosylation by plant-produced PNGase F. Purified Pfs48F1 was incubated with either PNGase F expressed in and purified from plants, or with the commercial enzyme (New England Biolabs). The 0.75 μ g amounts of the deglycosylated and intact Pfs48F1 protein preparations were run on SDS-PAGE followed by Western blotting. Pfs48F1 was detected using the anti-4xHis tag mAb. MWM refers to molecular weight markers: (a, c) SeeBlue® Plus2 Pre-Stained Standard, Cat. No. LC5925, Invitrogen, Carlsbad, CA; (b) Magic-Mark™ XP Western Protein Standard, Cat. No. LC5602, Invitrogen.

with both pGRD4-PNGase F and pBI121-Pfs48F1 constructs. As shown by SDS-PAGE and the Western blot analysis, co-expression with PNGase F led to the accumulation of Pfs48F1 that was similar in size to that of the *in vitro* deglycosylated molecule (Figure 3a), suggesting that Pfs48F1 was enzymatically deglycosylated (Figure 3a). The expression level of deglycosylated Pfs48F1 co-expressed with PNGase F was about 50 mg/kg of fresh leaf biomass, and solubility was about 95%. This degly-

cosylated form of Pfs48F1 had significantly enhanced rates of recognition by mAbs raised against various epitopes (I, IIb, III and V) of the Pfs48/45 protein (Outchkourov *et al.*, 2007) when measured using the enzyme-linked immunosorbent assay (ELISA, Figure 4). As shown in Figure 4, epitope-specific mAbs I, III and V recognize the deglycosylated form of Pfs48F1 2–6-fold better than the glycosylated form of the same protein. It should also be noted that mAbs I and III are conformation-specific. This

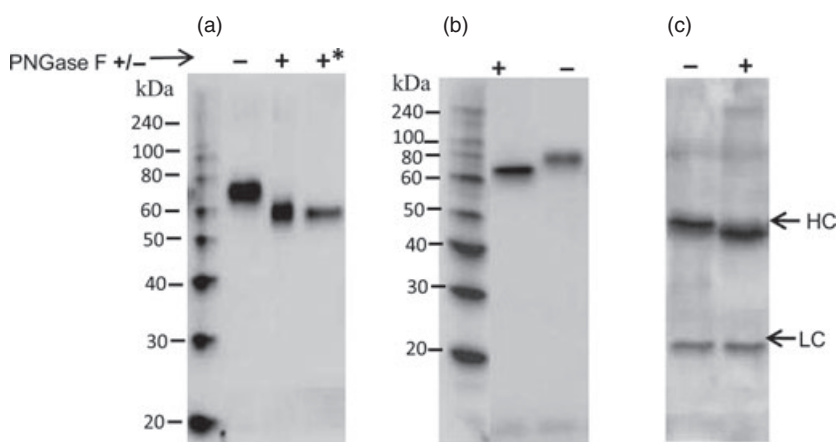


Figure 3 Western blot analysis of co-expression of Pfs48F1 (a), *Bacillus anthracis* PA (b) or an antibody against *B. anthracis* PA (c) with bacterial PNGase F in *N. benthamiana* plants. *N. benthamiana* plants were infiltrated with combinations of the pGRD4-PNGase F/pBI-Pfs48F1, pGRD4-PA83-1/pBI-PNGase F or pBI-PA/pGRD4-PNGase F constructs, for the production of Pfs48F1, PA and the anti-PA antibody, respectively. Leaf samples were taken at 7 (for Pfs48F1), 5 (for PA) and 6 (for the anti-PA antibody) dpi and were homogenized in three volumes of extraction buffer. After centrifugation at 13 000 *g*, samples were diluted 10-fold in the SDS sample buffer. Ten microlitre of samples were run on SDS-PAGE followed by Western blotting. Pfs48F1 bands were probed using the anti-4xHis tag mAb (a). PA bands were probed with the anti-4xHis tag mAb (b), while the anti-PA antibody bands were probed with a rabbit anti-human IgG polyclonal antibody (c). Pfs48F1, PA and the anti-PA antibody were expressed alone (–) and with PNGase F (+). Purified Pfs48F1 was incubated with the commercial PNGase F enzyme (+*, Cat. No. P0704S, New England Biolabs).

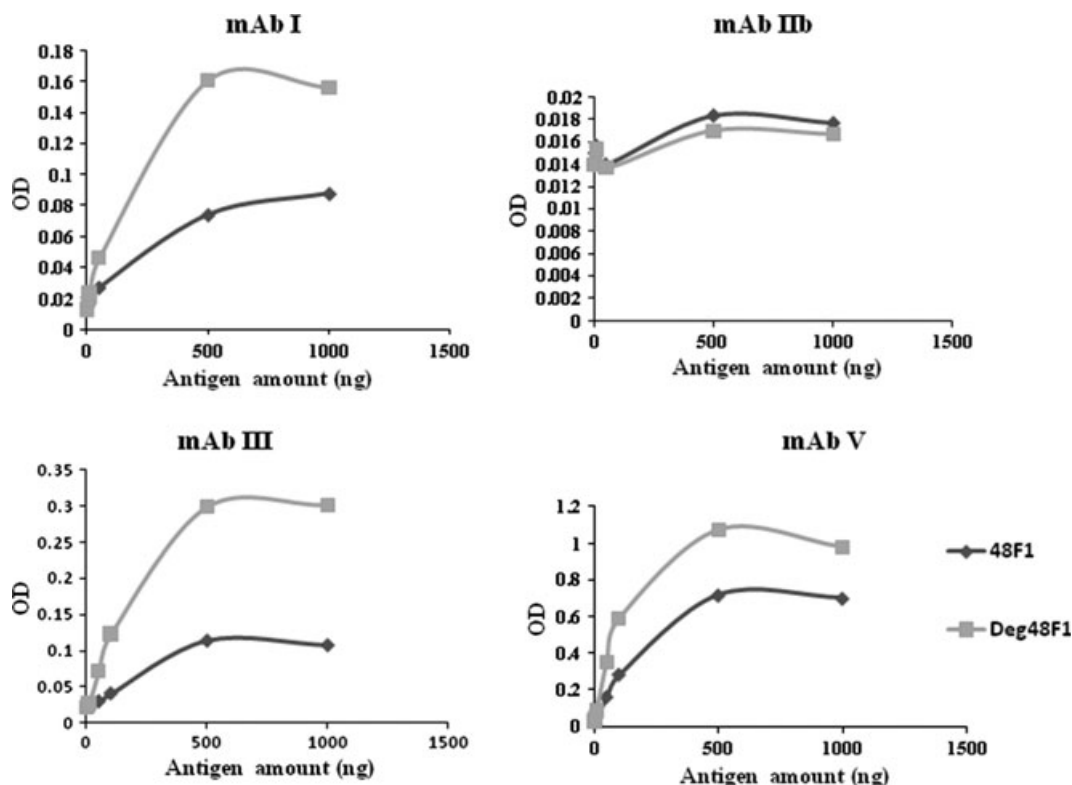


Figure 4 Comparative ELISA analysis of the glycosylated (Pfs48F1, ◇) and deglycosylated forms of Pfs48F1 (■). Recognition of the glycosylated and deglycosylated forms of Pfs48F1 by various rat mAbs that detect epitopes I, IIb, III and V of Pfs48/45 was measured by ELISA as described in Experimental procedures. ELISA plates were coated overnight with the anti-4xHis tag mAb followed by incubation with 1–1000 ng of the glycosylated or deglycosylated forms of the Pfs48F1 antigen for 2 h. Bound antibodies were detected by a HRP-conjugated goat anti-rat polyclonal antibody and visualized using OPD as a substrate. Values shown represent the optical density at the wavelength of 490 nm.

indirectly suggests that, perhaps, deglycosylated Pfs48F1 has native-like folding.

This approach was further tested by co-expressing the enzyme with PA of *B. anthracis* or with an antibody against PA. It should be noted that PA of *B. anthracis* is not a glycoprotein, but it is glycosylated when expressed in *N. benthamiana* plants. The Western blot analysis performed at 5 dpi demonstrated a shift in the mobility of PA co-expressed with PNGase F (Figure 3b), indicating protein deglycosylation. When PNGase F was co-expressed with an anti-PA antibody, the protein mobility shift on SDS-PAGE was observed with the heavy chain (HC) that has one glycosylation site, but not with the light chain (LC) that lacks glycosylation sites (Figure 3c). These results, along with the observation of *in vivo* deglycosylation of Pfs48F1 following co-expression with PNGase F, demonstrate that PNGase F successfully cleaved *N*-linked glycans from all tested glycoproteins and suggest that the PNGase F co-expression strategy can be used to produce therapeutic proteins in a non-glycosylated form in the *N. benthamiana*-based transient expression system.

Mass spectrometry/peptide mapping analysis of glycosylated and deglycosylated Pfs48F1 variants

To further demonstrate *in vivo* deglycosylation of Pfs48F1 co-expressed with PNGase F, we performed the mass spectrometry/peptide mapping analysis of glycosylated and deglycosylated Pfs48F1 variants as described in Experimental procedures. Pfs48F1 contains seven predicted *N*-linked glycosylation sites: N23, N104, N163, N177, N227, N272 and N276.

Mass spectrometry data confirmed that all sites in Pfs48F1, except for N104, have some level of glycosylation. The site N104 showed no increase in detection either before or after treatment with PNGase F. The mass spectrometry data for Pfs48F1 co-expressed with PNGase F *in vivo* showed a high level of deamidated Asp (change of Asn to Asp during deglycosylation) similar to that in Pfs48F1 treated *in vitro* with PNGase F. *In vitro* treatment of Pfs48F1 co-expressed with PNGase F (*in vivo* deglycosylated Pfs48F1) showed no enhancement of Asp residues which were deamidated, implying that co-expression with PNGase F offers the same level of deglycosylation as *in vitro* treatment.

Glycan detection analysis of glycosylated and deglycosylated Pfs48F1 variants

To demonstrate the *in vivo* deglycosylation of Pfs48F1, we also performed the glycan detection analysis of glycosylated and deglycosylated Pfs48F1 variants as described in Experimental procedures. As shown in Figure 5a, the glycan was only detected in plant-produced glycosylated Pfs48F1 (lane 1, Figure 5a). Figure 5b shows the Western blot analysis of the same samples (1/10 diluted) probed with an anti-4xHis tag mAb.

Affinity analysis of mAb V binding to glycosylated and PNGase F-deglycosylated Pfs48F1

Solution binding affinity measurements were performed using mAb V and plant-produced glycosylated, *in vitro* deglycosylated (treated with PNGase F) and *in vivo* deglycosylated

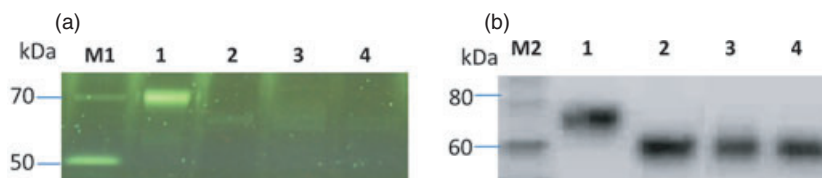


Figure 5 Glycan detection and Western blot analysis of glycosylated and deglycosylated Pfs48F1 variants. (a) 0.25 µg of the plant-produced Pfs48F1 protein from each sample was run on 10% SDS-PAGE followed by in-gel glycan detection using the Pro-Q Emerald 300 glycoprotein stain kit (Cat. No. P33378, Molecular Probes, Grand Island, NY). Stained proteins were visualized using UV illumination. Lanes: 1—glycosylated Pfs48F1, 2—glycosylated Pfs48F1 treated with commercial PNGase F, 3—*in vivo* deglycosylated Pfs48F1, 4—*in vivo* deglycosylated Pfs48F1 treated with commercial PNGase F *in vitro*. (b) Western blot analysis of the same sample using the anti-4xHis tag antibody. PNGase F treatment and Western blot analysis were performed as described above. M1, CandyCane™ glycoprotein molecular weight standards (Molecular Probes), 250 ng of each protein per lane; M2, MagicMark™ XP Western Protein Standard (Cat. No. LC5602; Invitrogen).

(co-expressed with PNGase F) Pfs48F1 as described in Experimental procedures. mAb V was equilibrated at three different concentrations of each antigen. The resulting affinities for glycosylated, and *in vitro* and *in vivo* deglycosylated Pfs48F1 were 9.7 nM (17.5–4.5 nM), 3.8 nM (11.7–1.1 nM) and 2.6 nM (6.3–1.0 nM), respectively (Figure 6). These findings indicate that the affinity of mAb V binding to the deglycosylated form of Pfs48F1 is higher than to the glycosylated form of Pfs48F1, but only a minimal difference exists between the forms of Pfs48F1 deglycosylated *in vitro* and *in vivo*.

The active concentration of mAb V was determined in experiments with glycosylated Pfs48F1 (Figure 6a). From this activity, a ligand concentration multiplier (LCM) was assigned to the two deglycosylated Pfs48F1 versions (Figure 6b,c). This concentration assignment was made because of an unknown nature of the *in vivo* deglycosylated Pfs48F1 concentration. It also normalizes the affinity values based on the activity of the antibody. Without this, any errors in the antigen concentrations would directly impact the calculated dissociation constant (K_d). Because the antigen concentrations are determined based on the antibody activity, exact concentrations of the antigens are not required for analysis. The LCM for *in vitro* deglycosylated Pfs48F1 was determined to be 0.97 or 97% the nominal value. The LCM for *in vivo* deglycosylated Pfs48F1 was 58% the nominal value, which is not unexpected based on the method of the concentration estimation.

Inhibition of mAb III with glycosylated and PNGase F-deglycosylated Pfs48F1

Experiments to compare mAb III binding to each of the Pfs48F1 variants were also performed using the Kinetic Exclusion Assay (KinExA) 3200 instrument (Sapidyne, Boise, ID). Initial tests of the mAb III signal indicated that the binding of mAb III to Pfs48F1 was much weaker compared with mAb V (data not shown). The limited availability of reagents reduced the extent of mAb III titration that could be performed; therefore, we used a single-point inhibition assay to assess differences in the affinity of mAb III to the three forms of Pfs48F1 (glycosylated, *in vivo* deglycosylated and *in vitro* deglycosylated with PNGase F). Solutions containing 140 nM of mAb III (nominal binding site concentration) with 250 nM of one of the three Pfs48F1 forms or without Pfs48F1 were tested using the KinExA 3200 instrument on a single-point inhibition screen. Concentration of Pfs48F1 was made based on the LCM values determined in the experiments with mAb V. Figure 7 shows the results of the mAb III signal inhibition observed when mAb III was incubated

with each of the three Pfs48F1 forms. The strongest binding to and the maximum mAb III signal inhibition were observed with *in vivo* PNGase F-deglycosylated Pfs48F1, while the *in vitro* deglycosylated and glycosylated forms of Pfs48F1 were equivalent in their ability to inhibit the mAb III signal. Although these results are qualitative in nature, they suggest that mAb III has the higher affinity to *in vivo* deglycosylated Pfs48F1 compared to both the *in vitro* deglycosylated and glycosylated forms of Pfs48F1.

Discussion

Recently, an increasing interest in using plants for recombinant protein production, including vaccine antigens, antibodies and therapeutic proteins, has been observed. Plants offer several advantages as a production platform, including eukaryotic post-translational modifications that are required for the biological activity of recombinant therapeutic proteins. Plants also represent an excellent production system because of safety, reduced costs and the eukaryotic-type expression of pharmaceuticals that do not require *N*-glycosylation. von Horsten *et al.* (2010) have reported the production of non-fucosylated antibodies by co-expression of the prokaryotic enzyme GDP-6-deoxy-D-lyxo-4-hexulose reductase. Some eukaryotic proteins (as well as bacterial proteins, for example, PA of *B. anthracis*) contain no *N*-glycans in the native host, but because of the presence of multiple sites for potential glycosylation, these proteins may become aberrantly glycosylated when expressed in plants. Therefore, it is very important to develop a strategy to express these types of recombinant proteins in plants in the non-glycosylated form. It has been previously demonstrated that blocking of *N*-glycosylation by tunicamycin, a specific inhibitor of the enzyme that transfers acetylglucosaminephosphate (GlcNAc-1-P) onto dolichol phosphate (Dol-P), results in a non-uniform expression of proteins in plants (Frank *et al.*, 2008; Hori and Elbein, 1981). However, this strategy may not be useful for the production of recombinant proteins in the non-glycosylated form for a number of reasons: (i) tunicamycin is very toxic; (ii) even short-term treatment of plants with tunicamycin significantly affects protein folding (D'Amico *et al.*, 1992; Sparvoli *et al.*, 2000) and (iii) inhibits extracellular secretion of proteins (Faye and Chrispeels, 1989); and (iv) moreover, a long-term treatment with tunicamycin has a lethal effect on plants (Gomord *et al.*, 2010).

In this study, we have demonstrated for the first time the expression of a deglycosylating bacterial enzyme PNGase F in plants using FhCMB's plant virus-based transient expression

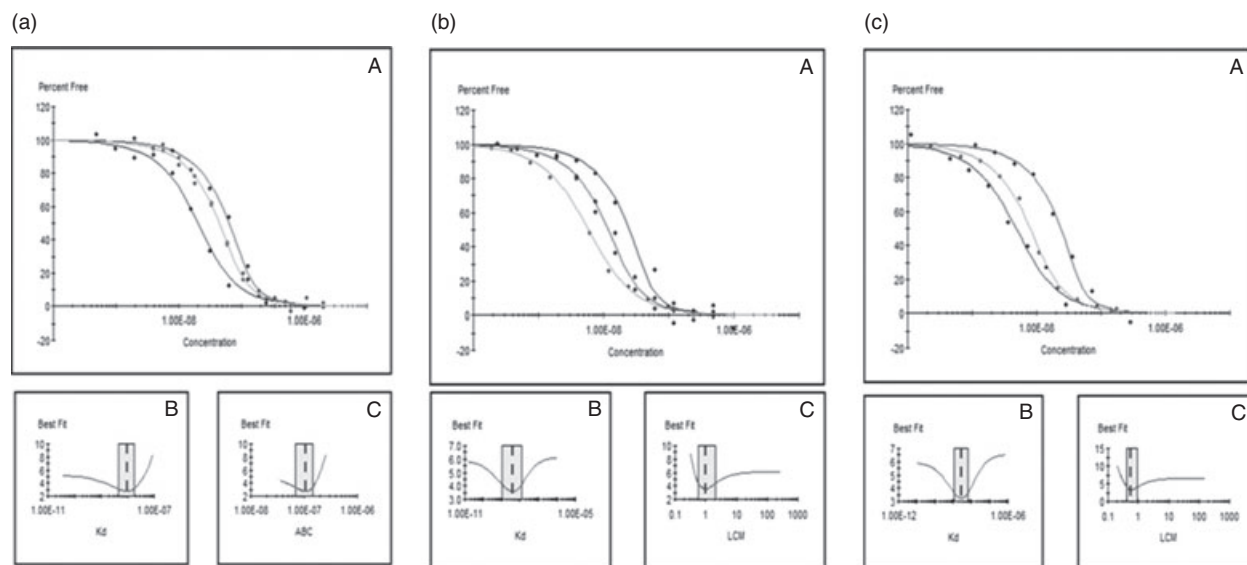


Figure 6 Affinity of mAb V binding to plant-produced Pfs48F1. (a) Affinity of mAb V binding to plant-produced glycosylated Pfs48F1. A. Three-curve binding titration fit for mAb V and glycosylated Pfs48F1. mAb V binding site concentrations were held constant at 107 nM, 69 nM and 22 nM in titrations of Pfs48F1. B. K_d determined for mAb V and glycosylated Pfs48F1 was 9.7 nM (17.5–4.5 nM). C. The active binding site concentration, reported in A, was determined from the three-curve fit to be 107 nM (144–69 nM) for the initial curve. (b) Affinity of mAb V binding to *in vitro* deglycosylated Pfs48F1. A. Three-curve binding titration fit for mAb V and *in vitro* deglycosylated Pfs48F1. mAb V binding site concentrations were held constant at 42.4 nM, 16.2 nM and 4.2 nM in titrations of Pfs48F1. B. K_d determined for mAb V and *in vitro* deglycosylated Pfs48F1 was 3.8 nM (11.7–1.1 nM). C. LCM is 97%, consistent with the nominal concentration after PNGase F treatment. (c) Affinity of mAb V binding to *in vivo* deglycosylated Pfs48F1. A. Three-curve binding titration fit for mAb V and *in vivo* deglycosylated Pfs48F1. mAb V binding site concentrations were held constant at 42.4 nM, 10.6 nM and 4.2 nM in titrations of Pfs48F1. B. K_d determined for mAb V and *in vivo* deglycosylated Pfs48F1 was 2.6 nM (6.3–1.0 nM). C. LCM indicates that the concentration is 54% of that expected from the Western blot analysis.

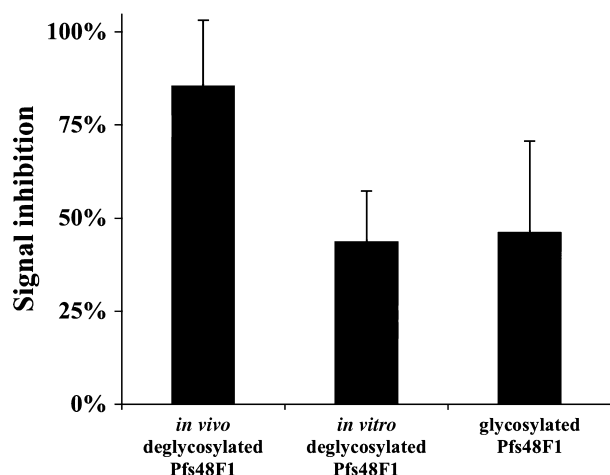


Figure 7 Inhibition of the mAb III signal with glycosylated and PNGase F-deglycosylated Pfs48F1. Pfs48F1 was measured using a single-point inhibition test as described in Experimental procedures. The error bars indicate the range of values from duplicate samples.

system and utilized the strategy of *in vivo* enzymatic deglycosylation to produce glycan-free target proteins. We found that the recombinant plant-produced PNGase F is fully active *in vivo* and successfully cleaved *N*-linked glycans from all tested glycoproteins, including the malaria vaccine candidate Pfs48F1, PA of *B. anthracis* and an antibody against PA of *B. anthracis*.

The efficiency of *in vivo* deglycosylation of the Pfs48F1 protein was demonstrated by the glycan detection and mass

spectrometry analyses. The mass spectrometry analysis of Pfs48F1 co-expressed with PNGase F *in vivo* showed a high level of Asn deamidation (Asn23, 163, 177, 227, 272 and 276 of *in vivo* deglycosylated Pfs48F1 were deamidated into Asp) similarly to that in Pfs48F1 treated *in vitro* with PNGase F, indicating the same activity of PNGase F *in vivo* and *in vitro*. In addition, when the deglycosylated and glycosylated forms of Pfs48F1 were compared using ELISA, the deglycosylated form was recognized by mAbs I, III and V at a greater level than the glycosylated form. The binding affinity of mAb V for glycosylated Pfs48F1 and *in vitro* and *in vivo* deglycosylated Pfs48F1 determined using the KinExA 3200 instrument was 9.7 nM, 3.8 nM and 2.7 nM, respectively, suggesting that aberrant glycosylation might have led to masking of important epitopes or caused incorrect/alterd folding of the Pfs48F1 protein. In addition, qualitative results of the signal inhibition analysis suggest that mAb III had the highest affinity to *in vivo* deglycosylated Pfs48F1 compared to both *in vitro* deglycosylated and glycosylated Pfs48F1. Based on all these results, we anticipate that the PNGase F deglycosylation strategy would allow for production of a malaria vaccine candidate Pfs48F1 which can provide a high TB activity. In an ongoing animal study, mice were immunized with either a glycosylated or a deglycosylated form of Pfs48F1, and TB activities of their sera are being evaluated.

In plants, most proteins of the extracellular compartment and the endomembrane system are glycosylated, and *N*-linked glycosylation of proteins has a great impact on their biological functions (Rayon *et al.*, 1998). Therefore, we have tested the deglycosylating effect of plant-expressed PNGase F on endogenous total soluble proteins (TSP) of *N. benthamiana* plants during the short transient expression period. No significant

difference in SDS-PAGE mobility patterns was observed for TSP from control (non-transformed) plants and plants transformed with bacterial PNGase F (Figure 8). Importantly, *N. benthamiana* plants transformed with PNGase F remained healthy when the target expression reached the maximum level. Taken together, these data suggest that because of the transient nature of expression and brief time span, the effect of PNGase F on the endogenous protein folding and extracellular secretion is not significant. Therefore, the enzymatic *in vivo* deglycosylation strategy can be used to produce therapeutic proteins and antibodies in the non-glycosylated form in *N. benthamiana* plants using the transient expression system. As mentioned above, this is especially important for those proteins that are not glycosylated in their native hosts but become aberrantly glycosylated when expressed in other eukaryotic hosts, potentially leading to reduced functionality and immunogenicity because of incorrect/alterd folding and/or masking of epitopes. Our findings can be also important for producing fucose- and xylose-free recombinant proteins, which would reduce a potential risk of immunologic and allergic reactions to these epitopes in humans. We suggest that co-expression with a deglycosylating enzyme such as PNGase F can be applied to various targets including antibodies and vaccine candidates that can be post-translationally modified in the endoplasmic reticulum (ER) of *N. benthamiana* plants. Finally, because the recombinant enzyme PNGase F has not been previously expressed in plants, our results on PNGase F expression support the utility of plants as an expression system for the production of an active, endotoxin-free PNGase F at reduced costs.

Experimental procedures

Cloning, expression and purification of recombinant PNGase F produced in *N. benthamiana* plants, and evaluation of its deglycosylating activity *in vitro* and *in vivo*

The PNGase F gene was optimized for expression in *N. benthamiana* plants (for codon optimization, mRNA stability, etc.) and

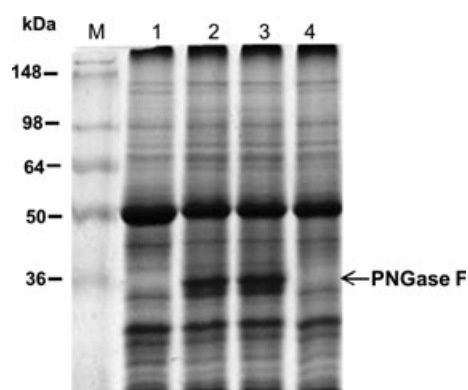


Figure 8 SDS-PAGE of soluble proteins from control (non-transformed) *Nicotiana benthamiana* plants and *N. benthamiana* plants transformed with bacterial PNGase F. Samples were prepared as described in Experimental procedures. Briefly, leaf samples from either control (non-transformed, lane 1) *N. benthamiana* plants or plants transformed with pGRD4-PNGase F (lane 2), pGRD4-PNGase F plus p19 (lane 3) or p19 (lane 3) were taken at 7 dpi and ground in three volumes of extraction buffer. Ten microlitre of the 10-fold diluted samples were run on SDS-PAGE followed by staining with Coomassie.

synthesized by GENEART AG (Regensburg, Germany) with flanking *PacI* (5' terminus) and *XhoI* (3' terminus, after stop codon) restriction enzyme sites. To transiently express PNGase F in *N. benthamiana* plants, the signal peptide (amino acids 1–40) was removed from the PNGase F sequence, and *Nicotiana tabacum* PR-1a signal peptide (MGFVLFSQLPSFLLVSTLLFLVISHSCRA) was added to the N-terminus. In addition, the KDEL sequence (the ER retention signal) and the FLAG epitope (the affinity purification tag) were added to the C-terminus. The resulting sequence was inserted into the launch vector pGRD4 (Shoji *et al.*, 2009) or the binary expression vector pBI121 (Chen *et al.*, 2003) using *PacI/XhoI* sites to obtain pGRD4-PNGase F and pBI-PNGase F, respectively. pGRD4-PNGase F and pBI-PNGase F (together with pSoup for pGRD4-PNGase F, which provides a replication function *in trans* [Hellens *et al.*, 2000]) were then introduced into the *Agrobacterium tumefaciens* strain GV3101. The resulting bacterial strain was grown in BBL medium (10 g/L soy hydrolysate, 5 g/L yeast extract, 5 g/L NaCl, 50 mg/L kanamycin) overnight at 28 °C. The bacteria were introduced by manual infiltration into 6-week-old *N. benthamiana* plants grown in soil. At 5, 6 and 7 dpi, leaf tissues were harvested and homogenized using a bullet blender (Zymo Research, Irvine, CA). Extracts were clarified by centrifugation (13 000 *g* for 30 min) and analysed using Western blotting.

PNGase F was purified from *N. benthamiana* leaves using an anti-FLAG antibody column chromatography. For purification of PNGase F, 8 g of frozen leaves was ground in 24 mL of TBS buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) using a mortar and a pestle. Plant debris was removed by filtration through Miracloth (Calbiochem, San Diego, CA) followed by centrifugation at 13 000 *g* for 10 min and then filtered through a 0.22- μ m syringe filter (ACROS Organics, Morris Plains, NJ). An anti-FLAG M2 affinity gel column (Anti-FLAG M2 Affinity gel, Cat. No. A2220; Sigma, St. Louis, MO) was prepared according to the manufacturer's instructions (Sigma). Twenty four millilitre of a clear supernatant were mixed with the gel and rotated at 4 °C for 1 h, after which the whole mixture was returned to the column and the column was washed with 20 volumes of TBS buffer. Bound proteins were eluted using 0.1 M glycine-HCl buffer, pH 3.5 into tubes containing 1 M Tris solution to neutralize glycine buffer.

To assess the deglycosylating activity of the recombinant plant-produced PNGase F *in vitro*, different amounts (10–200 ng) of the purified enzyme in phosphate buffered saline (PBS) were incubated at 37 °C for 1 h with Pfs48F1 expressed in *N. benthamiana* plants and purified by IMAC, and 0.75 μ g amounts of glycosylated and *in vitro* PNGase F-treated Pfs48F1 were subjected to SDS-PAGE and the Western blot analysis. Similarly, *in vivo* deglycosylated Pfs48F1 (co-expressed with PNGase F in *N. benthamiana*) was analysed by SDS-PAGE and Western blotting.

Potential deglycosylation of *N. benthamiana* endogenous proteins by plant-produced PNGase F was evaluated by comparing TSP from control (non-transformed) *N. benthamiana* plants and *N. benthamiana* plants expressing bacterial PNGase F, PNGase F plus p19 (plant suppressor of RNA silencing [Voinnet *et al.*, 2003]) or p19 alone on SDS-PAGE. Leaf punches from each sample were homogenized using a bullet blender (Zymo Research) at 4 °C for 2 min, homogenate was centrifuged twice at 13 000 *g* for 10 min, samples were boiled with 1 $\times$ SDS sample buffer and 10 μ L of each sample was loaded on SDS-PAGE.

Co-expression of PNGase F with Pfs48F1, *B. anthracis* PA and an anti-PA antibody

To co-express PNGase F with Pfs48F1, *B. anthracis* PA or an antibody against PA, pBI-Pfs48F1/pGRD4-PNGase F, pGRD4-PA/pBI-PNGase F or pBI-PA/pGRD4-PNGase F plasmids were constructed and used for infiltration of *N. benthamiana* plants. The sequences of Pfs48F1 (amino acids 28–401, GenBank accession number EU366251), *B. anthracis* PA (amino acids 30–764, GenBank accession number AAA22637) and HC and LC of the anti-PA antibody (Mett et al., 2011) were inserted into the launch vector pGRD4 or the binary expression vector pBI using *PacI/XhoI* sites. All genes described above were codon-optimized for expression in *N. benthamiana* plants. The Pfs48F1 gene optimized for expression in *N. benthamiana* plants encoded the wild-type Pfs48/45 protein (Kocken et al., 1993). All targets were expressed with a signal sequence of *N. tabacum* PR-1a protein at their N-termini. PA and Pfs48F1 were expressed with the 6xHis tag followed by the ER retention signal KDEL at their C-termini. *Agrobacterium* transformation, plant infiltration and leaf protein extraction were performed as described above.

Purification of Pfs48F1 from *N. benthamiana* plants

For purification of the Pfs48F1 recombinant protein from *N. benthamiana* plants, 750 g of plant material was homogenized in 2.25 L of extraction buffer (50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 20 mM Imidazole, 20% glycerol, 1 mM DTT) and incubated with 0.5% Triton (final concentration) for 20 min at 4 °C with stirring. After incubation, the lysate was centrifuged at 48 000 *g* for 40 min, and crude extract was filtered through Miracloth and loaded onto column with 70 mL Chelating Sepharose Big Beads charged with Ni. Column was washed with 15 volumes of 50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 20 mM Imidazole, 20% glycerol. Proteins were eluted with 50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 300 mM Imidazole, 20% glycerol. Eluted protein fraction was dialysed first against 10 mM sodium phosphate buffer, pH 6.5, 50 mM NaCl, 10 mM EDTA, 10% glycerol and then into 10 mM sodium phosphate buffer, pH 6.5, 10% glycerol. After spin down, dialysed sample was loaded onto a 5-mL Capto Q column equilibrated with 10 mM sodium phosphate buffer, pH 6.5, 10% glycerol. Proteins were eluted with 10 mM sodium phosphate buffer, pH 6.5, 10% glycerol, 600 mM NaCl. Eluted fraction was concentrated up to 2.6 mg/mL and dialysed against PBS, pH 7.5. The purity of Pfs48F1 estimated by Coomassie-stained SDS–PAGE was 72%.

Purification of *in vivo* deglycosylated Pfs48F1 from *N. benthamiana* plants

For purification of *in vivo* deglycosylated recombinant Pfs48F1 protein from *N. benthamiana* plants, 50 g of plant material infiltrated with pBI-Pfs48F1 and pGRD4-PNGase F was homogenized in 150 mL of extraction buffer (50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 20 mM Imidazole, 1 mM DTT) and incubated with 0.5% Triton (final concentration) for 20 min at 4 °C with stirring. After incubation, the lysate was centrifuged at 48 000 *g* for 40 min, and crude extract was filtered through Miracloth and loaded onto a 5-mL HisTrap FF column (Cat. No. 17-5255-01; GE Healthcare, Waukesha, WI) followed by washing with 15 volumes of 50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 20 mM Imidazole. Proteins were eluted with

50 mM sodium phosphate buffer, pH 8.0, 0.5 M NaCl, 100 mM Imidazole. Eluted fraction was concentrated and dialysed against PBS, pH 7.5.

Mass spectrometry/peptide mapping analysis

Plant-produced, partially purified Pfs48F1 samples (Pfs48F1 glycosylated, Pfs48F1 glycosylated treated with commercial PNGase F, *in vivo* deglycosylated Pfs48F1 and *in vivo* deglycosylated Pfs48F1 treated with commercial PNGase F) were electrophoresed on a 10% SDS–PAGE, and the target bands were excised and sent for in-gel enzymatic protein digestion followed by the liquid chromatography–tandem mass spectrometry analysis of the resulting peptide map (MS Bioworks, Ann Arbor, MI). Peptide mapping utilized three different peptidases, trypsin, chymotrypsin and elastase, in an attempt to maximize sequence coverage. The peptide maps were assessed for the presence of *N*-glycan-containing peptides by the shift of deamidated Asn residues to Asp. The gel slices that were digested with trypsin were washed with 25 mM ammonium bicarbonate followed by an acetonitrile wash. Samples were then reduced with 10 mM dithiothreitol at 60 °C followed by alkylation with 50 mM iodoacetamide at room temperature. Trypsin was added, and digestion was performed for 4 h at 37 °C. The reaction was quenched with formic acid, and the supernatant was analysed directly without further processing. Chymotrypsin and elastase digestion was performed in a similar manner, except digestion was performed at 37 °C overnight. Quenched supernatants were analysed using a Waters nanoACQUITY high-performance liquid chromatography system (Waters Corporation, Milford, MA) with peaks detected with a Thermo Scientific Orbitrap Velos Pro (Thermo Fisher Scientific, Waltham, MA). Resulting data were searched using a local copy of Mascot (Matrix Science, Boston, MA) in the UniProt *Nicotiana* and *A. tumefaciens* databases against the Pfs48F1 sequence. The Mascot Database (DAT) files were parsed into the Scaffold software (Proteome Software, Portland, OR) for validation, filtering and creation of a non-redundant list per sample.

Glycan detection analysis

The presence of glycans in plant-produced, partially purified Pfs48F1 samples (Pfs48F1 glycosylated, Pfs48F1 glycosylated treated with commercial PNGase F, *in vivo* deglycosylated Pfs48F1, and *in vivo* deglycosylated Pfs48F1 treated with commercial PNGase F) was detected by Pro-Q Emerald 300 glycoprotein staining. For this, 0.25 µg of the plant-produced Pfs48F1 protein from each sample was run on 10% SDS–PAGE followed by the detection of glycans in the gel using the Pro-Q Emerald Glycoprotein Stain Kit according to the manufacturer's protocol (Molecular Probes). Stained proteins were visualized using UV illumination.

Western blot analysis

Samples from infiltrated *N. benthamiana* leaves were separated on 10% SDS–PAGE, transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA) and blocked with 0.5% I-block (Applied Biosystems, Carlsbad, CA). The membrane was then incubated with a primary anti-4xHis tag mAb (for His-tagged proteins; Roche Applied Science, Indianapolis, IN) followed by a secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse polyclonal antibody. For detection of PNGase F, a rabbit anti-FLAG mAb (Cat. No. F2555; Sigma) was used as

a primary antibody and a HRP-conjugated anti-rabbit IgG was used as a secondary antibody. Proteins reacting with the anti-4xHis tag and the anti-FLAG mAbs were visualized using Super-Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The images were taken using the GeneSnap software on a GeneGnome and quantified using the Gene Tools software (Syngene Bioimaging, Frederick, MD).

Comparative ELISA analysis

Recognition of the deglycosylated and glycosylated forms of Pfs48F1 by rat mAbs raised against various epitopes (I, IIb, III and V) of the *P. falciparum* surface protein Pfs48/45 (Outchkourov *et al.*, 2007; Roeffen *et al.*, 2001) was assessed using ELISA. ELISA plates (96-well MaxiSorp plates [NUNC, Rochester, NY]) were coated with the anti-4xHis tag mAb (Cat. No. 34670; Qiagen, Valencia, CA) in PBS at 50 µL/well (5 µg/mL) overnight at 4 °C. After blocking with 0.5% I-block in PBS, desired amounts (1–1000 ng) of the deglycosylated and glycosylated forms of Pfs48F1 were added and incubated for 2 h. After washing plates, 50 µL (2 µg/mL in I-block) of various mAbs raised against Pfs48/45 was added and incubated for 2 h. Bound antibodies were detected using a HRP-conjugated goat anti-rat polyclonal antibody (1 : 25 000 in I-block) and visualized using o-phenylenediamine (OPD) as a substrate, at the wavelength of 490 nm.

Affinity analysis of mAb V binding to glycosylated and deglycosylated Pfs48F1 variants

The K_d of mAb V binding to glycosylated and deglycosylated (*in vitro* and *in vivo*) variants of Pfs48F1 were assessed using the KinExA 3200 instrument (Sapidyne, Boise, ID; Blake *et al.*, 1999) by determining the amount of free antibody remaining in the solution after equilibration with the respective Pfs48F1 binding partner was reached. mAb V concentrations were held constant while the selected glycosylated or deglycosylated Pfs48F1 protein was serially diluted. mAb V was mixed with glycosylated Pfs48F1 at 107 nM, 69 nM and 22 nM; with *in vitro* deglycosylated Pfs48F1 at 42.4 nM, 16.2 nM and 4 nM; and with *in vivo* deglycosylated Pfs48F1 at 42.4 nM, 10.6 nM and 4 nM. Running buffer was 1x PBS, pH 7.5 with 0.02% sodium azide as a preservative. The samples and the secondary antibody were diluted using running buffer augmented with 1 mg/mL bovine serum albumin (BSA). The secondary antibody was Dylight 649-conjugated goat anti-rat (Jackson ImmunoResearch, West Grove, PA) used at 0.5 µg/mL. PNGase-treated Pfs48F1 was used as the coating reagent for the flow cell bead pack (PMMA beads; Sapidyne) in all experiments. The flow rate for all samples and for the labelling antibody was 0.25 mL/min. Sample volume ranged from 0.35 to 3.0 mL.

Titration data resulting from three different antibody concentrations equilibrated with each Pfs48F1 were fit to a global 1 : 1 binding model included in the KinExA software (version 3.1.2; Sapidyne, Boise, ID). Data generated for mAb V and Pfs48F1 were fit to the standard 1 : 1 binding model where K_d , active binding site concentration (ABC), and signal maximums and minimums were determined. In this case, the concentration of Pfs48F1 (glycosylated) was determined by densitometry vs. BSA using Coomassie-stained SDS–PAGE. Data generated for mAb V with either deglycosylated version of Pfs48F1 were fit to the same 1 : 1 binding model, but using an unknown antigen concentration model (Xie *et al.*, 2005). In this model, the K_d

and signal parameters were determined from the fit as well as the LCM. The LCM relates an unknown concentration of the ligand to the determined ABC (from the experiment with mAb V and Pfs48F1). This allows for directly comparing binding data when the concentration of antigen is less well characterized or unknown. In this case, the concentration of *in vivo* deglycosylated Pfs48F1 was estimated using Western blotting, so the binding analysis was standardized to the concentration of glycosylated Pfs48F1.

Qualitative analysis of mAb III inhibition by glycosylated and deglycosylated Pfs48F1 variants

Qualitative analysis of mAb III inhibition by the Pfs48F1 variants (glycosylated and PNGase F-deglycosylated *in vitro* and *in vivo*) was performed using the KinExA 3200 instrument. Solutions containing mAb III at 140 nM binding site and 70 nM nominal protein concentrations and one of the Pfs48 variants (glycosylated or PNGase F-deglycosylated) at a final concentration of 250 nM were prepared and incubated, and the amount of free mAb III in each reaction mixture was determined using the KinExA instrument. The initial concentration of each Pfs48F1 variant in the reaction mixture was based on the LCM value determined in the mAb V experiments. The signal generated by mAb III in the solution without Pfs48F1 was considered as 100%.

Acknowledgements

The authors are grateful to Dr. Stephen J. Streatfield for useful discussion and Dr. Natasha Kushnir for editorial assistance. This study was supported by Fraunhofer USA Center for Molecular Biotechnology. The authors have no conflict of interest to declare.

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