# Glycobiology of MSP-1 and MSP-2: Potential Malaria Vaccine Candidate Glycoproteins

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**Summary**: Metabolic labelling of *Plasmodium falciparum* parasites with [$^3$H]GlcN, [$^3$H]Man, [$^3$H]Gal and [$^3$H]ethanolamine, and subsequent purification by SDS-PAGE of the labelled material provided effective labelling of the MSP-1 195 kDa, and MSP-2, 42-53 kDa, glycoproteins. Reductive β-elimination of the MSP-2 released from the gel consisted of glycopeptides containing labelled sugars. Processing of the eliminated components and identificatin of the sugar residues demonstrated the presence of *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol amongst other labelled sugars. Reductive β-elimination with soduim hydroxide-sodium borotritideborohydride showed the presence of glucosaminitol and alanine in the hydrolysis products. The MSP-2 was retained on solid phase wheat-germ agglutinin and was released from the lectin by treatment with GlcNAc. Upon treatment with *O*-glycanase the MSP-2 glycoprotein released labelled amino sugar, and derived oligosaccharides on treatment with exoglycosidases released labelled components corresponding to the metabolically incorporated sugars. Labelled Gal was incorporated into the MSP-2 glycoprotin using [$^3$H]UDP-Gal and galactosyltransferase. The galacosylated glycoprotein released labelled Gal upon treatment with β-galactosidase. The results of the present study suggest that the carbohydrate chains of the MSP-2 glycoprotein are attached to the protein backbone via GlcNAc- and GalNAc-serine/threonine in *O*-glycosyl linkage and the glycoprotein has terminal GlcNAc and Gal residues. The carbohydrate moieties of MSP-2, glycoprotein consist mainly of short chains linked to the protein core. Mannosamine inhibits biosynthesis as well as parasitemia of *P.facliparum*.

## Introduction

Malaria is a major parasitic disease of tropical and subtropical countries, and occurs in a large part of the population each year with a considerable toll of debilitating morbidity and mortality [1]. Various approaches to prevent and contain the disease are continuously made. Developments to eradicate, prevent and control the disease by vector regulation and antimalarial drugs, though of consequences, are however, restricted due to resistance of *Anopheles* mosquito to the insecticides and of the parasite to the parasitic drugs. In the recent years, an extensive interest has evolved to develop malarial vaccines to control the disease. Four species of malaria infect human, of which two, *Plasmodium falciparum* and *Plasmodium vivax* are widespread, and *Plasmodium falciparum* is the pathogen causing the most serious of the human malaria. *Plasmodium ovale* and *Plasmodium malariae* are the less common species. For each specie, there are numerous stages in the life cycle, each morphologically and antigenically discrete. Considering the stage specificities and diversity of the antigenic molecules in addition to modifications within each stage and species that would be essential candidates for vaccines, strategies to explore three major type of various stage specific, have developed. 1: Vaccine against sporozoites, i.e. the life cycle stage transmitted by the mosquito to the vertebrate host. 2: Vaccine against asexually multiplying and blood stages of parasite. These stages are solely responsible for the severity of the disease. Vaccines against these stages are, therefore, expected to prevent the disease or to provide considerable relief. 3: Vaccines against sexual forms are expected to provide useful mechanisms for controlling the spread of the disease within the population.

Malaria is currently controlled by vector control, development of chemotherapy and the formulation of vaccines. The search of malaria vaccines dates back to the 1940s but the major break through in this field have not occurred until recently. The mechanisms of protective immune responses and cloning of plasmodium genes coding for stage-specific antigens have accelerated the pace of malaria vaccine development. The chimeric vaccine Spf66 has only limited protection of minor consequences [2].

The possibility of an efficient blood stage based vaccine [3] has been clearly demonstrated. A

high degree of protection which was stage and species-specific, was induced in monkeys by immunization with enriched *P.falciparum* merozoite preparations of Freund's adjuvant [4]. Monkeys could also be protected by vaccination with highly purified *P.falciparums* Schizonts in a synthetic adjuvant. In the case of blood stage vaccines, like sporozoite vaccines, an attenuated or killed parasite vaccine is ridden with more difficulties that render it impractical at the present time. Hence the need to obtain a detailed picture of the immune mechanisms of protection operating in clinical immunity and in vaccinated non-human primates and to identify potential parasite target antigens.

A large number of blood stage antigens, in contrast to sporozoite antigens, have been characterized at the molecular level due to the efforts of many research groups during the past decade. Recently, the results of several vaccination trials using recombinant or synthetic blood stage products have become available [1,5]. As detailed below these are for the most part disappointing. However, valuable information regarding the safety and immunogenicity of a number of candidate vaccines has been obtained.

Vaccines currently tested in high endemic areas produce insufficient protection. The recombinant DNA technology used to produce vaccines against malaria have been ineffective [1,6] perhaps because of lack of native sugar moiety. Recently, extensive interest has been developed to investigate the role of sugar residues [4] present in the glycoprotein antigens that are synthesized by *P.falciparum* and are present on the merozoite surface and in the GPI anchor. The carbohydrate chains intact protein help to maintain the antigenicity of the glycoproteins.

The sugar residues in the human malarial parasite proteins has been shown by the metabolic labelling [7,8], lectin binding [9] and enzymatic digestion studies [10, 11]. The parasite proteins contain α-linked galactose residues which could influence the binding of the MSP-1 to its antibody. The amino acid sequences of the parasite proteins have *N*- or *O*-glycosylation sites of the glycoproteins [9].

Because of the strong immunological [9] specificity of the sugar incorporated molecules and nature of the incorporated carbohydrates, investigation of the role of carbohydrates in malaria is of interest. The absence of *N*-glycosylation has been shown using a specific cleavage enzyme, *N*-glycanase and by non-transfer *in vitro* of several dolichol-polyphosphate sugars and oligosaccharides [11, 12]. More recently, in contrast to earlier findings [11, 12], it has been demonstrated [13] that the malarial parasite asexual erythrocytic stage-dependent protein contain, as previously shown, *O*-glycosidically linked as well as *N*-glycosidically linked sugars. Also, it has been suggested that the antibiotic tunicamycin inhibit schizont formation due to the blockage of *N*-glycosylation.

The merozoite surface proteins, MSP-1 and MSP-2,(195 kDa & 43-58 kDa) are of particular interest for immunization because of their known immunogenic properties [14], their location at the surface of parasite and involvement in binding of the merozoite to the host erythocyte [14,15]. Glycosylation of the merozoite on the surface protein and in the GPI anchor is a post translational modification that regulate intermolecular and intercellular interactions and more importantly provide with adaptive functions in cellular metabolism [16].

Controversy as to the type of sugar linkage and location on the protein exists. It is, however, confirmed by several reports that O-glycanase and N-glycanase residue sugar moieties exist in the protein relevant to stage specificities of the asexual blood cycle. This protein modification does provide an exceptional advantage to the parasite against human immune responses [13,17,18]. In this study we have shown that O-linked carbohydrate are the synthesized glycoproteins of the *P.falciparum*. The carbohydrate chains are attached to the protein core via GlcNAc and GalNAc-Serine/threonine in *O*-glycosyl mode and the glycoproteins has terminal GlcNAc and Gal residues. The carbohydrates moieties of the MSP-1, MSP-2,glycoproteins consist mainly of short chains sugar residues.

**Results and Discussion**

The parasite cells were metabolically labelled in asynchronous mode to obtain an overall profile of

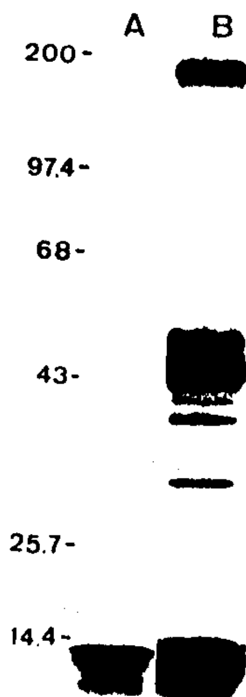



Fig. 1:

sugars incorporation of different stages. There was diversity of sugar incorporation in the different proteins (Fig.1) Ethaolamine was incorporated into the protein with selectivity. Similar observation of variable incorporation of different sugars have been reported [17,18]. The variation in the amount of sugars incorporation in different glycoproteins and the number of proteins to which sugars were added may have arisen because of the asynchronous mode of parasite culture used in this study. This mode of culture inevitably provide a broad range of labelled glycoprotein synthesized in different stages of development of the asexual blood cycle of the parasite. The metabolic labelling of *P.falciparum* cells using GlcN resulted in the incorporation of GlcNAc and GalNAc. It appears that the labelled glycoproteins were extracted from the cell parasitic glycoproteins in a stage dependent manner. The labelled glycoprotein were extracted from the cell lysates in non-ionic detergents and examined by SDS-PAGE under nonreducing condition followed by authoradiography. Fig.1 shows that several glycoproteins were labelled with GlcN (lane B:195, 100, 85, 42-53, 39, 30, and 16kDa) and Man (lane A: 195, 45, 53, and 16kDa). Few proteins were labelled with Gal and with ethanolamine (data not shown.)

The incorporation of GlcN was much more intense compared to that of Man, Gal and ethanolamine; Man incorporation was abundant relative to Gal (Fig.1). Mannose labelling was much more intense in the lower molecular size glycoproteins suggesting more mannose incorporation in MSP-$1_{19}$, *i.e.* fragment that remains anchored to the membrane during processing of MSP-1.

Carbohydrate analysis of glycoprotein showed the presence of GlcN, Man and a minute quantity of a component with the mobility of GalN. Ethanolamine was incorporated into the 195 kDa proteins. The malarial proteins were incubated with wheat-germ agglutinin -agarose beads. The MSP-1, MSP-2 glycoproteins were retained on the wheat-germ agglutinin column, and about 80% of the radioactivity in the glycoprotein was eluted with GlcNAc. Treatment of the residual material with α-galactosidase and then with α-mannosidase removed labelled components that comigrated in pc with Gal and Man. A separate treatment of the glycoprotein with α-galactosidase and subsequently with α-mannosidase and β-*N*-acetylglucosaminidase released components that comigrated in pc with Gal, Man, and GlcNAc. The endo-*O*-glycanase treatment of the glycoprotein released 40% radioactivity and labelled material that migrated in pc with GalNAc in addition to slow moving components (Fig.2).

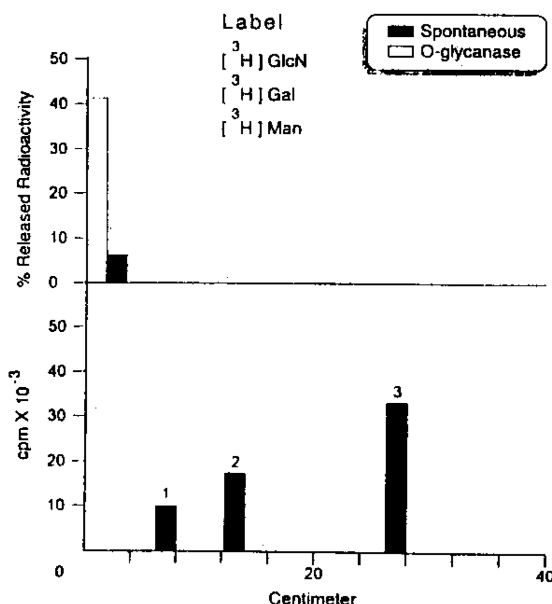



Fig. 2:

In order to identify the sugar residues and amino acids involved in the glycan-peptide linkage, the gel strips were treated with alkaline borohydride, eluted on Bio-Gel P-4 column, and the radioactivity was obtained in glycoproteins, and six fractions containing radioactivity were obtained from Bio-Gel P-4 column (Fig.3, Table-1).

Table-I: Carbohydrate composition of the MSP-2 glycoprotein and its derived products after chemical treatments

| Sugars | Glycoprotein Fractions | | | | | | |
|---|---|---|---|---|---|---|---|
| | Gly | a | b | c | d | e | f |
| N-acetylglucosamine | + | + | + | + | + | - | + |
| N-acetylgalactosamine | trace | - | - | - | - | - | - |
| N-acetylglucosaminitol | - | - | - | + | + | + | - |
| N-acetylgalatosaminitol | - | - | - | - | - | - | + |
| Mannose | + | trace | trace | - | - | - | - |
| Galactose | + | + | - | + | - | - | + |
| Arabinose* | + | + | + | + | - | - | - |
| Lyxose* | + | - | - | - | - | - | - |

Gly: acid hydrolysis products of the MSP-2 glycoprotein.

A to f: fractions 1to6 of the β-eliminated,alkaline borohydride treatment, Products of the MSP-2 glycoprotein obtained from Bio-Gel P-4 column

*sugars obtained after ninhydrin treatment of amino sugars

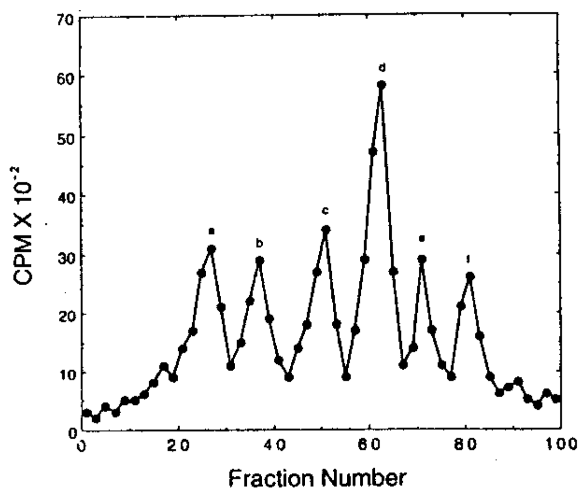



Fig. 3:

Among the fractions, fraction a showed the presence of GlcN,and a minor amount of Man and traces of Gal (Table 1). Treatment with β-N-hexosaminidase released GlcNAc. Galactose was released from this fraction upon treatment with α-galactosidase (Table II). GlcN was also present in the fraction b with traces of Man and no sugar alcohol was there (table 1). On treatment of fraction b with β-N-hexosaminidase sugar GlcNAc was released.

Sequential treatment with α-galactosidase and β-N-acetylglucosaminidase released components with mobility of Gal and GlcNAc in pc (Table II). On acid hydrolysis fraction c showed glucosaminitol, GlcN and traces of Gal, another component unidentified with Rg 1.3 was also observed. This fraction on treatment with α-galactosidase released a small quantity of a labelled material with the mobility of Gal. Minor amount of GlcNAc was released on treatment of this fraction with β-*N*-acetylglucosaminidase (Table II).The fraction d in pc showed the presence of a major component *N*-acetyl-glucosaminitol and minor components. On acid treatment presence of glucosaminitol, a minor component of GlcN and a faster moving component could be shown (Table I). Treatment of this fraction with β-N-glycanase released a component with the mobility of GlcNAc (Table II). The presence of *N*-acetyl-glucosaminitol was suggested in fraction e. Fraction f also showed the presence of *N*-acetyl-glucosaminitol and small amount of Gal. Hexosamines present in different fractions were treated with ninhydrin and corresponding pentoses were analysed in pc (Table 1). These results suggest the presence of *O*-and *N*-linked oligosaccharides, as there are glycoproteins containing glucosamine and mannose that are resistant to β-elimination.

Table II: Sugars released by glycosidases from the MSP-2 glyco-protein (Gly) and its β-eliminated products, fractions a to f, obtained from the Bio-Gel P-4 column.

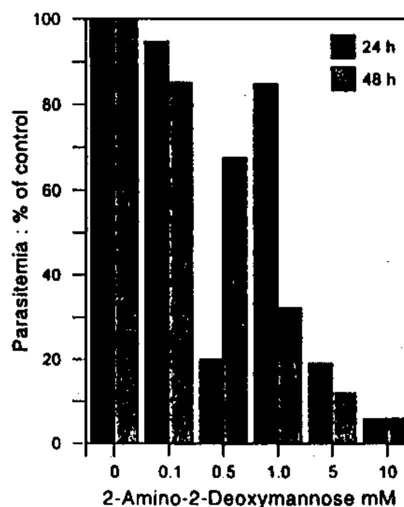
| Glycoprotein Fraction | Sugars | | | |
|---|---|---|---|---|
| | Gal | Man | GlcNAc | GalNac |
| Gly[1] | + | | + | |
| Gly[2] | + | trace | | |
| Gly[3] | + | trace | + | |
| Gly[4] | | | | + |
| A5 | + | | | |
| A[6] | | | + | |
| B[5] | + | | | |
| B[6] | | | + | |
| C[5] | + | | | |
| C[6] | | | + | |
| D[6] | | | + | |
| E | | | | |
| F | F | | | |

1. Sequentially β-galactosidase-and β-N-acetylglucosaminidase-treated.
2 Sequentially α-galactosidase-and α-mannosidase-treated after treatment as in 1.
3. Sequentially α-galactosidase-α-mannosidase and β-N-acetyl-glucosaminidase-treated.
4. O-Endoglycanase-treated
5. α-Galactosidase-treated.
6. β-N-Acetylgulcosaminidase-treated.

The exogalactosylation of the MSP-1 with [3H]-UDP-galactose and galactosyl transferase was efficient as shown by tritium incorporation. A substantial increase in tritum label, and treatment with β-galactosidase resulted in a significant loss of label, and showed the mobility of Gal. The substantial release of labelled Gal residues by β-galactosidase demonstrated the highly selective nature of galactosyl transferase reactivity and the presence of terminal GlcNAc residues.

Galactose transfer to the glycoprotein was further confirmed by deglycosylation of the glycoprotein with alkaline borohydrate. An oligosaccharide with high tritium content was obtained which on acid treatment showed the presence of labelled components with the mobility of Gal and *N*-acetylglucosaminitol. These results suggest the presence of terminal GlcNAc residues. In addition, the absence of *N*-glycosidic linkages in erythrocytic malarial glycoproteins had been reported [19] and recently confirmed [20,11,21]. Carbohydrates components of MSP-1 contribute to immunogenecity of this protein and warrant by the vaccine potential of this protein. Moreover, it was recently shown that GlcNAc residues, *O*-linked to serine and presented by major histocompatibility class I molecules, were important for glycopeptide recognition by cytolytic T lymphocytes [22]. The existence of the GlcNAc-hydroxyamino acid linkage has been reported in lymphocyte cell surface proteins [23], nuclear membrane proteins [24] and proteins from the human liver fluke, *Schistosoma mansoni* [25]. In recent studies, we investigated the effect of mannosamine on parasite survival and glycosylation by metabolic labelling using [$^3$H]-glucosamine. Mannosamine significantly reduced parasite survival and biosynthesis of glycosylated proteins (Fig. 4). Inhibition of parasite growth was closely related to the incorporation of radioactive glucosamine [26].

The sequential enzymic digestion suggested the presence of oligosaccharide chains in addition to single sugar residues on the protein core. It also revealed the presence of terminal GlcNac and Gal residues. Wheat-germ agglutinin studies suggest the presence of terminal GlcNAc, and observation similar to that reported for the erythrocytic stages of malarial glycoprotein [27]. The presence of terminal GlcNAc in animal cell glycoproteins has been




The effect of various concentrations of mannosamine on the survival of *Plasmodium falciparum* in culture. Bars represent the mean values indicated for the three experiments.

Fig. 4:

described [25]. The sugar residues in *P.falciparum* glycoproteins are present on the peptide backbone as well as in the anchor region. The data of this study suggest that the MSP-1 glycoprotein incorporated GlcNAc more abundantly than Man or Gal: GlcNAc and GalNAc were linked to serine or threonine of the protein core via an *O*-glycosyl linkage; GlcNAc was present as a terminal sugar, α-linked Gal was present as a terminal residue in the glycoprotein; Man residues were not present in β-eliminated oligosaccharides or in alditol-containing products; *N*-acetylglucosaminitol was the main sugar component amongst the β-eliminated sugar chains; and the glycoprotein contained ethanolamine.

Our observation of *O*-glycosylation in MSP-1 [28,18] and sensitivity of labelled parasite lysate glycoproteins to *O*-glycanase [17] was confirmed by other investigators [11,13]. The function of glycoproteins containing O-linked GlcNAc moieties is not yet known. These molecules have structural and functional roles [24]. It has now been shown that *O*-GlcNAc is a regulatory modification [29-31] providing means for immune evasion [22]. In *P-falciparum* the existence of such glycoproteins has recently been recognized [28,30] and preponderance of *O*-glycosyl linkage between sugar moiety and

protein core demonstrated [17,18,11]. The function of stage specific modification of malarial glycoproteins with *O*-glycosyl and *N*-glycosyl linkages has not been investigated, although it is known that these are antigenic and can bind to erythrocytes, in particular to glycophorins A and B [15,32]. It is very possible that these glycoproteins may act in appropriate biological environment as cell adhesion proteins for sialic acid containing oligosaccharides or sialyloligosaccharide-peptide complex that exist on erythrocytes.

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