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## Preliminary communication

# Presence of *O*-glycosylated glycoproteins in the *Plasmodium falciparum* parasite*

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Malaria is a widespread disease caused by the protozoan parasite Plasmodium. About one third of the world's population lives in malaria endemic areas. Of the four species that infect man, *Plasmodium falciparum* is the most virulent, causing considerable morbidity and high mortality, particularly among children[1]. The asexual erythrocytic stage is responsible for the disease and associated pathology. Protein and glycoprotein antigens of asexual erythrocytic development forms have been indentified[1,2]. The presence of carbohydrates on the surface of *P. falciparum* cells, particularly merozoites, has been ascertained by *in vitro* incorporation of radiolabeled carbohydrates[3,4], enzymic release of radiolabeled sugars[5], lectin binding[6], and inhibition studies[7]. Because of failure to produce a plasmodial antigen-based malarial vaccine by recombinant DNA technology[8], growing interest is being focused on the carbohydrates of integral antigens[5]. The glycoproteins of the parasite have not been structurally investigated yet, and their composition and nature still remain unclear.

In the present study, *P. falciparum* strain M25/Zaire was cultured asynchronously in human $A^+$ erythrocytes in RPMI medium containing human serum from $A^+$ donors. The cells were labeled with D-[6-$^3$H]- or D-[1-$^{14}$C]-glucosamine, D-[6-$^3$H]galactose, and D-[2,6-$^3$H]mannose, separately as well as in a mixture. Labeled parasites were harvested after 6- and 16-h incubations at 37° in a candle jar[9]. Infected-erythrocyte-containing parasites were enriched on 60% Percoll. This cell fraction was lysed in 20 vols. of 2% NP-40 in TNE (50mM Tris·HCl, 100mM NaCl, and 5 mM EDTA) containing protease inhibitors [leupeptin, chymostatin, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone (TPCK), and α-toluenesulfonyl fluoride (PMSF)]. The lysate was centrifuged at 100 000*g* and the supernatant was

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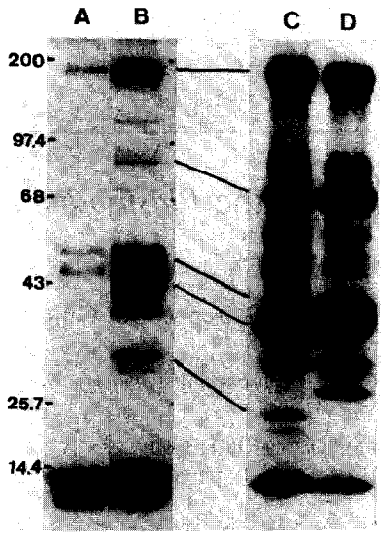


Fig. 1. Biosynthetic labeling of *P. falciparum* glycoproteins and immunogenicity in human hosts. Autoradiographic analysis of D-[$^3$H]mannose (A) and D-[$^3$H]glucosamine-labeled glycoproteins (B, C, and D). A and B: total labeled glycoproteins; C and D: immunoprecepitates with human immune sera. Lanes A and B are 10% poly(acrylamide) gels, and lanes C and D are 6–20% poly(acrylamide)-gradient gels. Mobilities of bands in lanes C and D have been aligned to those in lanes A and B on the basis of mobilities of standard molecular-weight markers shown on the left of the figure.

examined by sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis (SDS-PAGE) and by immunoprecipitation. The labeled components in SDS-PAGE (Fig. 1) showed the presence of glycosylated proteins of molecular sizes between 195 and 10 kDa, labeled either with D-[$^3$H]mannose (lane A) or D-[$^3$H]glucosamine (lane B). The major labeled species were also immunoprecipitated by two different immune human sera from adults living in malaria-endemic areas of West Africa (Fig. 1, lanes C and D), indicating that these species are immunogenic in humans and represent antigenic targets for the human immune response against *P. falciparum*. Nonimmune human sera do not recognize any of these glycoproteins (not shown).

Because of low uptake of sugars into *P. falciparum*-glycosylated proteins and the small amounts of purified infected cells available after labelling, surface glycoproteins were isolated by use of TPCK-trypsin[10] (20 $\mu$g/mL, shaken for 15 min at 0°). Several glycopeptides were separated by gel chromatography on Bio-Gel P-30 and Bio-Gel P-60. Attempts to purify these glycopeptides were limited owing to the very minute quantity of isolated material. Chemical and enzyme studies were, therefore, performed on the mixture of glycopeptides obtained by TPCK-trypsin treatment, followed by chromatography on Bio-Gel P-30. These glycopeptides were treated with NaOH–$NaBH_4$, and then processed as described earlier[11]. After acid hydrolysis in 2M HCl for 16 h at 98°, and separation by p.c. [solvents: (*a*) 5:5:1:1 ethyl acetate–pyridine–acetic acid–water; (*b*) 4:1:5, upper layer butanol–ethanol–water], radiolabeled components having the mobility of 2-acetamido-2-deoxyglucitol, galactose, and a small amount of 2-acetamido-2-deoxygalactitol were observed.

In a separate experiment, glycopeptides were incubated in $NaOH–NaBH_4–NaB^3H_4$. The reaction product was processed as described above, and the acid hydrolyzate in two-dimensional p.c., solvents (*a*) and (*b*), showed the presence of a labeled component that migrated with alanine. This suggested that derived alanine had arisen from serine, linked to a hexosamine, during reductive β-elimination.

Percoll-purified, labeled, and parasitized cells ($10^9$ cells/mL) were resuspended in phosphate-buffered saline solution (PBS) containing protease inhibitors (leupeptin, chymostatin, TLCK, TPCK, PMSF, and phenanthroline), and disrupted by sonication four times at 0° for 30 s with 30-s intervals betweeen each sonication cycle. After centrifugation at 20 000*g* for 30 min, the supernatant was removed, and the pellet washed twice with PBS containing protease inhibitors and centrifuged as described above. The supernatants were mixed and centrifuged at 100 000*g* to remove cellular debris, and then extensively dialyzed to remove exchangeable radioactivity and lyophilized. The residue was dissolved in 10mM phosphate buffer, pH 6.4, and treated with O-Glycanase (2.4 mU; EC 3.2.1.97; Genzyme, Boston, MA) for 20 h at 37°. The enzyme-treated product was extensively dialyzed at 4° and freeze-dried. The residue was dissolved in water and de-ionized by chromatography on AG 50 ($H^+$) and AG 1 ($AcO^-$) ion-exchange resins. The column washings (water) were combined and freeze-dried. The residual material showed in p.c., in solvent (*a*), the presence of radiolabeled components that migrated with standard *N*-acetylglucosamine and *N*-acetylgalactosamine. The dialyzate contained over 60% of the labeled material, whereas, in the control-treated sample, the dialyzable radioactively-labeled material did not exceed 10%. These results suggested that the O-Glycanase cleaved *O*-glycosyl-linked sugars from the protein core. The O-Glycanase is known to cleave the *O*-(2-acetamido-2-deoxy-D-galactopyranosyl)-(1→3)-L-serine or -L-threonine linkage[12] and its activity towards the *O*-(2-acetamido-2-deoxy-D-glucopyranosyl) linkage has not yet been explored.

Labeled glycopeptides were incubated with N-Glycanase (0.25 U; EC 3.5.1.52; Genzyme, Boston, MA) in 0.2M sodium phosphate buffer, pH 8.5, at 37° for 16 h. The enzyme-treated material was chromatographed on a column of Bio-Gel P-30, calibrated with labeled glycopeptides. The N-Glycanase-treated glycopeptides were recovered unchanged, with only a slight decrease in c.p.m., suggesting the resistance of these glycopeptides to N-Glycanase and the lack of asparagine-linked oligosaccharides in the malarial glycoproteins. A similar observation, *i.e.*, absence of asparagine-linked oligosaccharides, has been reported earlier[6], though the studies were performed under different conditions.

The carbohydrate composition of the malarial glycopeptides, as determined by β-elimination–reduction, and O-Glycanase and N-Glycanase treatments, suggested the presence of an *O*-glycosyl-type linkage in the *P. falciparum* asexual-form glycoproteins. The labeled glycoproteins with *O*-glycosyl linkage are the biosynthetic products of the parasite and not of erythrocytes, since the latter do not incorporate radiolabeled compounds[4] in culture.

To our knowledge, this is the first report of the existence of *O*-glycosylated glycoproteins in the malaria parasite. The structure and function of plasmodial gly-

coproteins are not known, and their contribution to the immunogenicity and antigenicity of plasmodial antigens is unclear, although their significance towards the development of a malaria vaccine has been emphasized[13].

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