Vol. 118, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 13, 1984 Pages 1-7

GLYCOPROTEIN ASSEMBLY IN LEISHMANIA MEXICANA

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Received November 9, 1983

SUMMARY: The oligosaccharide transferred from a dolichol-P-P derivative to proteins in the assembly of N-linked glycoproteins in Leishmania mexicana appeared to be $Man_6GlcNAc_2$. It was found that this compound underwent transient glucosylation once bound to protein but that $Man_6GlcNAc_2$ was the oligosaccharide present in mature glycoproteins. No complex type saccharides were detected. The structure of the oligosaccharide appeared to be similar to that of the core of compounds transferred from dolichol-P-P derivatives in protein glycosylation in Trypanosoma cruzi or animal cells.

The pathway of protein glycosylation in mammalian, avian, insect and fungal cells involves the transfer of $Glc_3Man_9GlcNAc_2$ from a dolichol-P-P derivative to asparagine residues (1). The protein-bound oligosaccharides are then processed to form high mannose or complex type oligosaccharides. The study of the mechanism of protein glycosylation in pathogenic protozoa is of special interest as glycoproteins have been shown to be the main antigenic determinants in several parasites. We have recently described that in two trypanosomatids, Trypanosoma cruzi and Crithidia fasciculata, unglucosylated saccharides were transferred from dolichol-P-P derivatives to proteins (2-5). The compounds involved in the transfer reactions were $Man_9GlcNAc_2$ and $Man_7GlcNAc_2$ in the above mentioned protozoa, respectively. Trypanosomatids of the genus Leishmania are the causative agents of a variety of cutaneous, mucocutaneous and visceral human diseases. In this paper, we are reporting for the first time the mechanism of protein glycosylation and the structure of the protein-bound oligosaccharide occurring in a pathogenic parasite of the genus Leishmania.

ABBREVIATION: Endo-H: endo-β-N-acetylglucosaminidase H.

0006-291X/84 $1.50

MATERIALS AND METHODS

Materials.- The strain of Leishmania mexicana was kindly provided by Dr. Rodrigo Zeledon, Universidad de San Jose, Costa Rica. [U-^{14}C]glucose (284 Ci/mol) was from New England Nuclear. Jack Bean α-mannosidase was from Sigma or from Boehringer Mannheim. The enzyme obtained from the latter source was contaminated with β-mannosidase as it catalyzed the hydrolysis of Manβ(1,4)GlcNAc (5). The standards used in paper chromatography were obtained as described previously (2-6).

Methods.- Acetolysis was as described by Kocourek and Ballou (7), but the reaction was carried out for 4 h at 37°C and 5 h at 30°C in acetic acid/acetic anhydride/sulfuric acid (10:10:0.75). The solvents employed in paper chromatography were: A, 1-propanol/nitromethane/water (5:2:4); B, 1-butanol/pyridine/water (10:3:3); and C, 1-butanol/pyridine/water (4:3:4). Other methods were as described previously (2-6).

Labeling procedure.- L. mexicana promastigotes were grown at 26°C in liquid medium containing Brain Heart Infusion (Difco): 34 g/l; Tryptose (Difco): 2 g/l; Na_2HPO_4: 3 g/l; KCl: 0.4 g/l; Hemine (Sigma): 0.02 g/l and 20% Fetal Calf Serum. The parasites (2.5 g) were harvested at the late logarithmic phase, washed twice with 30 ml of ice-cold Eagle's solution containing 5 mM sodium pyruvate and no unlabeled glucose (8) and resuspended in 8 ml (total volume) of the same solution. [U-^{14}C]glucose (750 μCi) was added after 5 min at 26°C and aliquots were withdrawn after 10 min (3 ml), 20 min (2 ml), 60 min (1 ml) and 120 min (2 ml) at the same temperature. Dolichol-P-P and protein-linked oligosaccharides were isolated as described previously (2-5).

RESULTS

Dolichol-P-P-bound oligosaccharides.- Dolichol-P-P- oligosaccharides isolated from L. mexicana cells incubated for 10 and 20 min with [U-^{14}C]glucose were subjected to mild acid hydrolysis and the water-soluble substances thus produced were treated with endo-β-N-acetylglucosaminidase H (Endo H) and run on paper chromatography. As depicted in Fig. 1A,B a single peak was obtained in both samples. The saccharide, which will be referred to as compound I, migrated

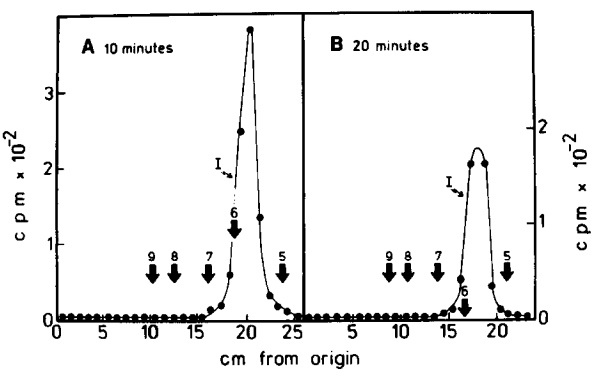

Fig. 1.- Paper chromatography of dolichol-P-P-bound oligosaccharides. Oligosaccharides isolated from dolichol-P-P derivatives obtained from cells incubated with [U-^{14}C]glucose for indicated times were run on paper chromatography with solvent A. Standards: 9, Man9GlcNAc; 8, Man8GlcNAc; 7, Man7GlcNAc; 6, average migration of two Man6GlcNAc isomers; 5, Man5GlcNAc.

slightly ahead of the average migration of two $Man_6GlcNAc$ standards. As shown in Fig. 2D, one of the $Man_6GlcNAc$ isomers used as standards migrated the same as compound I.

Protein-bound oligosaccharides.- Cells were labeled with [U-^{14}C]glucose for 10, 20, 60 and 120 min and the proteins denatured with organic solvents and treated with a protease. The digested material was then filtrated through a Biogel P-6 column and the glycopeptides treated with Endo H. The oligosaccharides thus liberated were run on paper chromatography. The main oligosaccharide obtained after 10 min of labeling migrated behind the average migration of the $Man_6GlcNAc$ standards (Fig. 2A). This substance will be referred to as compound II. A shoulder in the position of compound I could be observed in the same figure. On longer incubation periods, however, compound II gradually disappeared and compound I practically became the only oligosaccharide present in the sample withdrawn after 120 min of incubation (Fig. 2B-D). Although in the 60 and 120

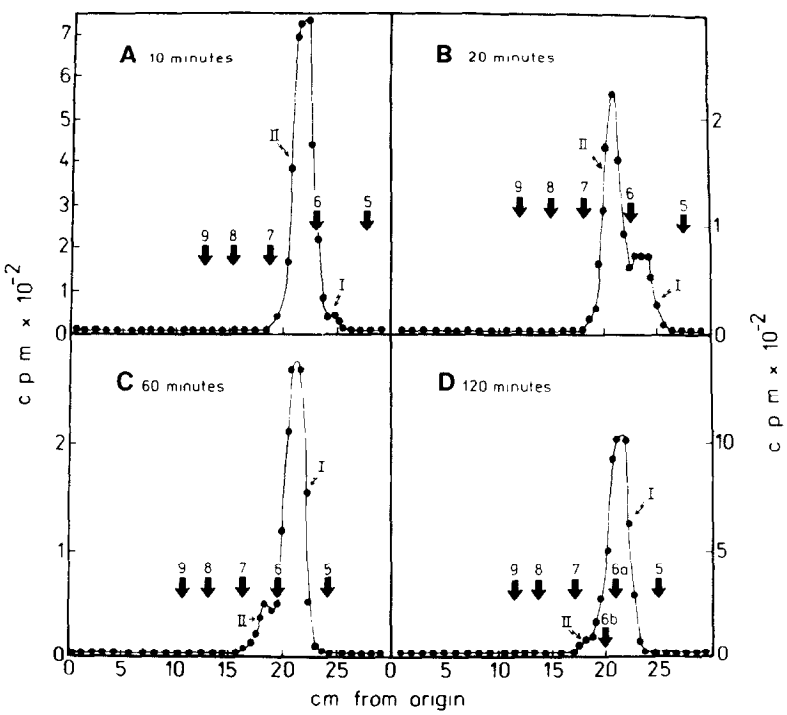

Fig. 2.- Paper chromatography of protein-linked oligosaccharides. Oligosaccharides were isolated from glycoproteins obtained from cells incubated with [U-^{14}C]glucose for indicated times and run on paper chromatography with solvent A. For standards see legend to previous figure. 6a and 6b indicate the positions where the $Man_6GlcNAc$ isomers migrated.

min samples there was labeled material that eluted from the Biogel P-6 column in the position of glycopeptides and that was resistant to Endo H, less than 3% of the label present in it migrated as mannose upon strong acid hydrolysis and paper chromatography. It was concluded, therefore, that no Endo H-resistant oligosaccharides were present in L. mexicana cells.

Monosaccharide composition.- The reader is directed to the structure depicted in Fig. 5 in order to better understand the following results. As shown in Fig. 3A, total acid hydrolysis and paper chromatography of compound I isolated from samples depicted in Fig. 1A,B revealed the exclusive presence of labeled mannose residues in the lipid-bound oligosaccharide. Apparently, the N-acetylglucosamine units were not labeled after short incubation periods. On the other hand, total acid hydrolysis and paper chromatography of protein-linked compound I isolated from the 60 min sample (Fig. 2C) revealed the presence of labeled mannose and N-acetylglucosamine units in that substance (Fig. 3C). Protein-bound compound II isolated from the 20 and 60 min samples (Fig. 2B,C) appeared to contain labeled glucose and mannose residues (Fig. 3B).

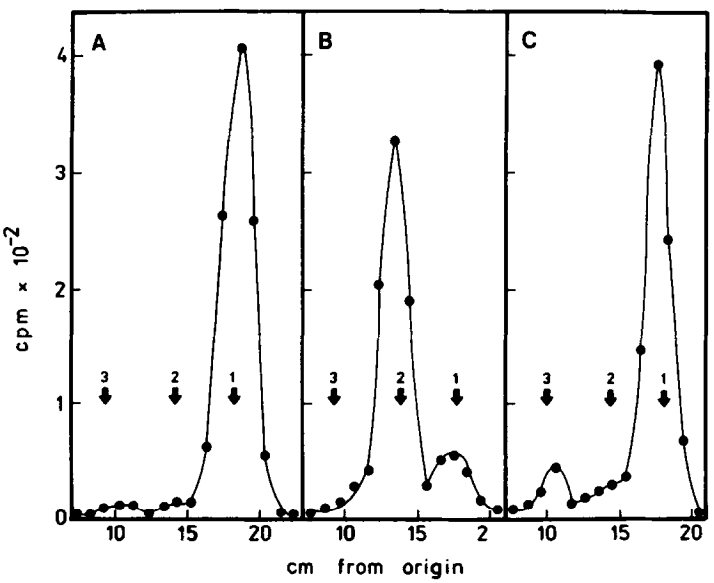

Fig. 3.- Monosaccharide composition. Oligosaccharides were subjected to strong acid hydrolysis followed by paper chromatography with solvent B. Treated compounds were A, I in Fig. 1A,B; II in Fig. 2B,C and C, I in Fig. 2C. Standards: 1, mannose; 2, glucose and 3, glucosamine.

Degradation with mannosidases.- Treatment of compound II isolated from the 10 min sample (Fig. 2A) with α - and β -mannosidases produced substances migrating as $Glc_1Man_4GlcNAc$ and $Glc_1Man_5GlcNAc$ standards as well as minute amounts of mannose (Fig. 4A). Elution and retreatment of $Glc_1Man_5GlcNAc$ with the mixture of mannosidases produced $Glc_1Man_4GlcNAc$. Treatment of compound I isolated from the 120 min sample with α - and β -mannosidases produced labeled mannose and N-acetylglucosamine in the ratio 5.9 to 1 (Fig. 4B). On the other hand, degradation of compound I with α -mannosidase produced mannose as well as $Man_1GlcNAc$, $Man_2GlcNAc$ and $Man_3GlcNAc$ (Fig. 4C). It should be noted that no free N-acetylglucosamine was produced in the absence of β -mannosidase.

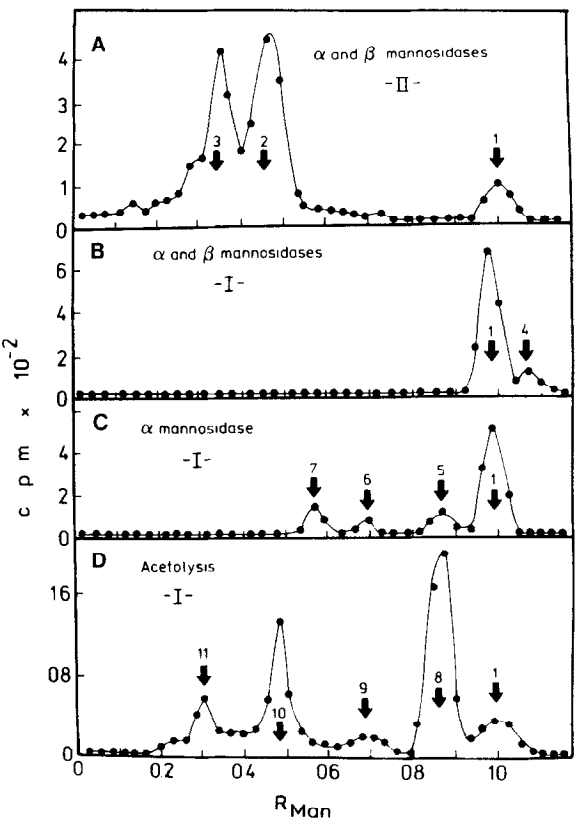

Fig. 4.- Structural analysis. Oligosaccharides were incubated with glycosidases or subjected to acetolysis as indicated in panels. The reaction products were run on paper chromatography with solvent C. Treated compounds were: A, II in Fig. 2A; B, I in Fig. 2D; C, I in Fig. 2C and D, I in Fig. 2D. Standards: 1, mannose; 2, $Glc_1Man_4GlcNAc$; 3, $Glc_1Man_5GlcNAc$; 4, N-acetylglucosamine; 5, $Man_1GlcNAc$; 6, $Man_2GlcNAc$; 7, $Man_3GlcNAc$; 8, Man_2; 9, Man_3; 10, $Man_4GlcNAc$ and 11, $Man_6GlcNAc$.

Acetolysis.- Acetolysis of compound I under extremely mild conditions rendered substances migrating as the original compound ($Man_6GlcNAc$) as well as $Man_4GlcNAc$ and mannobiose standards (Fig. 4D). This procedure breaks preferentially $\alpha(1,6)$ bonds between mannosyl residues. The substance migrating on paper chromatography as the mannobiose standard did not migrate when reduced with $NaBH_4$ and subjected to paper electrophoresis in sodium molybdate, pH 5.0, thus indicating a 1,3 bond between the mannosyl residues (5). A reduced Man $\alpha(1,2)$Man standard migrated to the anode under similar conditions.

DISCUSSION

As in other trypanosomatids, an unglucosylated oligosaccharide appeared to be transferred from a dolichol-P-P derivative to protein in L. mexicana: compound I migrated as a $Man_6GlcNAc$ standard, did not contain glucose residues, produced Man_2 and $Man_4GlcNAc$ upon acetolysis and the ratio of label in mannose to that in N-acetylglucosamine residues in saccharides isolated from cells incubated for a long period with [U-^{14}C]glucose was 5.9. It may be concluded, therefore, that the composition of compound I was $Man_6GlcNAc$. It should be noted that the compound bound either to dolichol-P-P or to protein has an additional N-acetylglucosamine residue. The protein-bound oligosaccharide was then transiently glucosylated (Fig. 5). We have previously reported the occurrence of transient glucosylation of protein-bound $Man_9GlcNAc_2$, $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ in T. cruzi and calf thyroid cells (5,9). The role of transient glucosylation is presently unknown, but our unpublished results performed with rat liver microsomes tend to suggest that this process might protect protein-linked

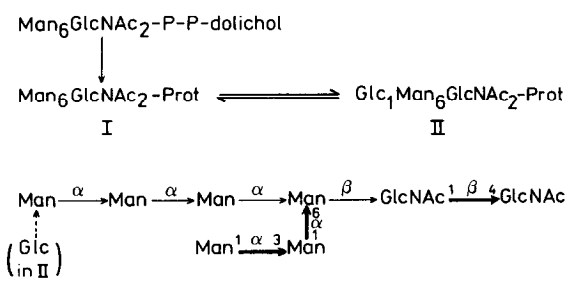

Fig. 5.- The mechanism of protein glycosylation and structure of protein-bound oligosaccharides in L. mexicana.

oligosaccharides from hydrolysis by α-mannosidases during the transit of glycoproteins through the endoplasmic reticulum. No Endo H-resistant saccharides appeared in cells incubated with [U-^{14}C]glucose for 120 min and $Man_6GlcNAc_2$ was the only protein-linked compound present in those cells. The overall mechanism of protein glycosylation in L. mexicana was, therefore, the one depicted in Fig. 5. It is different from those known to occur in other eucaryotic cells. Results presented here suggest the structure shown in the same figure for dolichol-P-P or protein-linked $Man_6GlcNAc_2$. A similar structure is present in the core of the oligosaccharides transferred in protein glycosylation in T. cruzi and animal cells (5,10). In addition, the glucose unit present in the transiently glucosylated saccharide has been found to be linked to the same mannose residue as in $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ of T. cruzi and thyroid cell glycoproteins (5,9). A recent report has shown that antigenic glycoproteins present in the outer surface of Leishmania tropica cells interacted with Concanavalin A but not with lectins recognizing N-acetylgalactosamine, fucose, external N-acetylglucosamine or galactose residues (11). These results agree with our report.

ACKNOWLEDGMENT

This investigation received support from the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

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