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Characterization of GDPMannose: Man_2 $GlcNAc_2$-PP-Dolichol Mannosyltransferase from Bovine Mammary Gland

15

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Abstract

Glycosylation represents the most common conjugation of both membrane-bound and secreted proteins of animal cells. Among the different types of glycosylation, the N-linked attachment of sugars to the polypeptide backbone is by far the most abundant modification. In higher eukaryotes the N–glycosylation is the major post translational process, initiated by the synthesis of a large lipid oligosaccharide, Glc_3–Man_9–$GlcNAc_2$–PP–Dolichol. This represents the immediate precursor of carbohydrate unit involved in the biosynthesis of glycoproteins. Glycosyltransferase are the enzymes involved in synthesis of above moiety. Glycosyltransferases are species– specific as well as tissue–specific. The glycosyltransferases involved in the initial processes are therefore of great interest In the present study we have characterized the above mentioned enzymes in mammary glands which is developmentally regulated. The acceptor substrate specificity using polyprenols of various carbon chain length was done ,the properties and kinetic parameters of the enzyme catalyzed reaction were studied and,metal ion requirement was also observed .

Keywords: *Glycosyaltion, glycosyltransferase, mammary glands,*

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Introdution

Glycosylation represents the most common conjugation of both membrane-bound and secreted proteins of animal cells. Among the different types of glycosylation, the N-linked attachment of sugars to the polypeptide backbone is by far the most abundant modification. The biosynthesis of the precursor carbohydrate unit of these proteins is initiated by a stepwise assembly of Glc3Man9GlcNAc2P-P-Dol in the dolichol cycle, its transfer en bloc to the nascent polypeptide in the rough endoplasmic reticulum (RER), followed by excision of the glucosyl residues by processing-specific enzymes, glucosidase I and II, also resident in the endoplasmic reticulum. Additional posttranslational modifications of the carbohydrate moiety in the RER, Golgi, and trans-Golgi network, differ for individual glycoproteins for the completion of final products as high mannose, complex or hybrid glycoproteins en route to their final destinations in the secretory pathway (Sharma *et.al.* 1974).

In all normal eukaryotic cells, the N-glycosylation of proteins is initiated by the *en bloc* transfer of the core oligosaccharide, $Glc_3Man_9GlcNAc_2$ to the asparagine residue of Asn-X-Ser/Thr sequences of the nascent polypeptide chains from the lipid-linked oligosaccharide, $Glc_3Man_9GlcNAc_2$-PP-dolichol (Kornfeld*et.al.*1985). The precursor lipid-linked oligosaccharide is assembled in the endoplasmic reticulum, by the sequential addition of monosaccharides to the lipid carrier dolichyl phosphate catalyzed by various glycosyltransferases (Struck *et.al.1980*). It is now well established that the first seven sugar residues (two N - acetylglucosaminyl and five mannosyl) are derived directly from the corresponding sugar nucleotide (UDP-GlcNAc and GDP Mannose), while the next four mannosyl (Man_{5-9}) and three terminal glucosyl residues come from dolichylphosphomannose (Dol-P-Man) and dolichylphosphoglucose (Dol-P-Glc), respectively (Sharma *et.al.* 1981,1991). Although the complete sequence of reactions and structures of all the lipid-linked oligosaccharide intermediates of N-glycosylation pathway, popularly known as "dolichol cycle" have been worked out, (Kornfeld 1980). The regulation and the enzymology of this pathway still remain as the unsolved challenging problem.

Materials And Methods

Pig (swine) mammary gland was obtained from the local slaughter house. All other chemicals were obtained from reliable chemical sources and were of best grade available.

All operations were performed at 0-4°C. The swine mammary gland tissue (500g) was used to prepare the crude microsomal pellet as described

(Prakash 1984) and the enzyme was partially purified (Sharma, 2014).

Preparation of Lipid-Linked Oligosaccharide Acceptor Substrate (Man_2-$GlcNAc_2$-PP-Dol)

Crude microsomes, prepared from swine mammary gland, were used for the preparation of acceptor lipid-saccharides essentially as described by Rosner *et. al.* (1982) with slight modification. 120 tubes each containing 350ìl of microsome suspension (20mg/ml), 25ìM UDP-GlcNAc, 10mM $MgCl_2$, 0.14M sucrose, 17mM NaCl, 1mM EDTA, 1mM DTT and 50mM Tris-HCl, pH 7.2, in a final volume of 700ìl were incubated for 12 min at 37°C . After addition of 10 ìl of 350 ìM of GDP-Mannose, the incubation was continued for another 10min. Parallel incubations containing 5ìM of UDP- with all other components in the reaction mixture were carried out in order to monitor the reaction and also to prepare the lipid linked oligosaccharide were use to monitor the column fractions during the purification of lipid saccharides. After incubation, total GlcNAc-containing lipids in each tube were extracted with 20ml $CHCl_3$ - CH_3OH (3:2) for 10 min and centrifuged. The pellet was again extracted with 10 ml of the same solvent and combined with the first extract. The combined extract $CHCl_3$-CH_3OH was phase partitioned by the addition of 6 ml of 4mM $MgCl_2$, followed by vigorous vortexting and centrifugation .The lower ($CHCl_3$) layer which contained a mixture of the smaller lipid-linked oligosaccharides (Man_{0-5}-$GlcNAc_2$-PP-Dolichol) was collected and washed three times with theoretical upper phase, CH_3OH-H_2O-$CHCl_3$ (48:47:3 by vol.) as described previously (Sharma*et.al.*, 1990). The washed lower phase ,which contained largely smaller lipid-linked oligosaccharide intermediates (smaller than Man_5-$GlcNAc_2$-PP-Dolichol) was evaporated to dryness by rotary evaporator at 37°C and redissolved in a minimum volume of $CHCl_3$-CH_3OH (2:1,by vol.) and purified by repeated chromatography (two times) on DEAE-cellulose (acetate form) columns (1.5 x 30cm) in $CHCl_3$-CH_3OH-H_2O (10:10:3, by vol.) and the charged lipids were eluted with gradients of ammonium formate (0-100mM). A small amount of purified GlcNAc-containing lipids was added to the lipid sample loaded on the column to help locate and identify the lipid-linked oligosaccharide fractions. The fractions containing acceptor lipid were pooled, adjusted to $CHCl_3$-CH_3OH-H_2O (10:10:10, by vol.) by addition of an appropriate volume of H_2O. After thorough mixing the phases were separated by centrifugation. The lower layer was washed three times as before and then the washed $CHCl_3$ layer was dried on a rotary evaporator, redissolved in small volume of $CHCl_3$-CH_3OH-H_2O

(10:10:3) and rechromatographed on DEAE cellulose column (acetate form) as above except that the aliquots from each fraction were assayed for their ability to serve as acceptors of mannose from GDP-mannose into the lipid-oligosaccharide by the NP-40 solubilized enzyme preparation from swine mammary gland.

Fractions that stimulated the mannose incorporation into lipid-linked oligosaccharides were pooled, partitioned into chloroform to remove salt. The chloroform layer containing acceptor lipid oligosaccharide substrates was dried by rotary evaporator, redissolved in CM (2:1) and used as lipid- linked oligosaccharide acceptor substrates for the mannosyltransferases that catalyze the transfer of mannose to the acceptor lipid from GDP-mannose.

Assay of Mannosyltranferase III

The activity of mannosy ltransferase was measured by determining the amount of mannose transferred to the lipid-linked oligosaccharide, accepter substrate, from GDP- mannose, essentially as described previously (Mudgapalli *et.al.* 1994) with slight modification. The incubation mixture contained the following component in a final volume of 70ìl; 2.8ìM of lipid linked oligosaccharide dried under N_2 in the reaction tube, 0.1% NP-40, 50mM sodium phosphate buffer, pH 6.0, 12mM $MgCl_2$, 5mM DTT, 2.5mM AMP, 4.5ìM GDP-mannose, 1 mM EDTA and various amounts of enzyme at various stages of purification. After 20 min incubation at 37°C, the reaction was terminated by the addition of 2 ml of chloroform-methanol (3:2,v/v), vortexed vigorously and centrifuged to remove denatured proteins and other insoluble materials. To the clear supernatant 0.4ml of 4mM $MgCl_2$ were added, thoroughly mixed by vigorous vortexting, and the phases were separated by centrifugation. The upper phase (aqueous methanol) was removed and discarded. The lower ($CHCl_3$) layer which contained the lipid-linked oligosaccharides was washed with Folch method as described (Sharma *et.al.* 1982). An aliquot of the washed chloroform layer was transferred to screw capped glass tubes, dried under stream of N_2 gas and subjected to mild acid hydrolysis as described. The mannose-containing oligosaccharide released from the lipid-oligosaccharide was estimated by DNS method, using mannose as standard.

Protein was determined by the method of Bradford, (1976). SDS-gel electrophoresis was performed under reduced condition in 10% acrylamide gels as described (Laemmli 1970).

Results

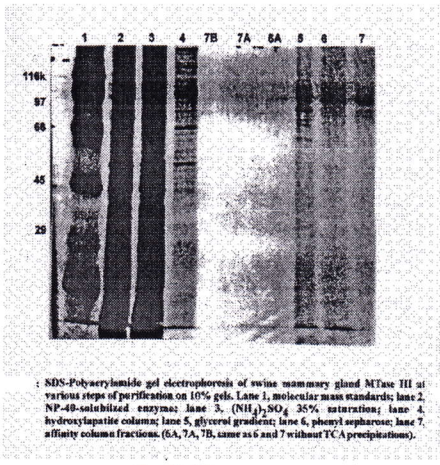
Product analysis

In order to obtain a sufficient amount of product for identification, standard

incubation mixtures were scaled up at least 10 times, and the lipid-linked oligosaccharides were isolated as described above. The oligosaccharides were released from the lipid by mild acid hydrolysis in 40% n-propanol containing 0.01N HCl at 95°C for 30 min as previously described (Forsee 1979). The released oligosaccharides were concentrated to dryness several times under a stream of nitrogen gas to remove HCl and were neutralized with ammonia. The oligosaccharides were then identified by descending paper chromatography in ethyl acetate/pyridine/aceticacid/water, 5:5:1:3 (by vol.) using oligosaccharides standards for size determination.

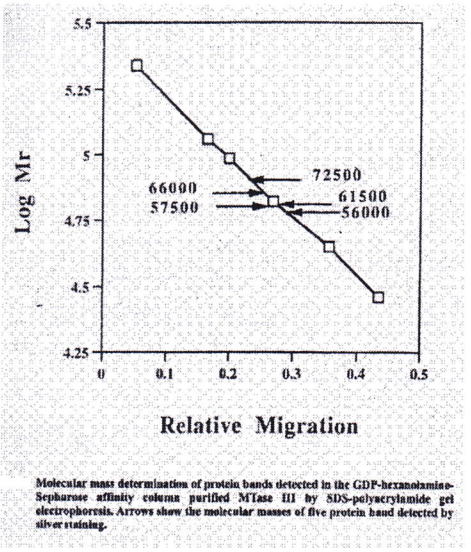
Molecular Mass and Homogeneity of MTase III:

Fig shows SDS-PAGE separation of MTase III at various steps of the purification scheme. In the most purified enzyme preparation from the affinity column five protein bands were detected (fig 1A)with silver staining corresponding to apparent molecular mass of 72, 66, 61.6, 57.5, and 56 kDa (fig .1 B). It should be noted that of these five bands the 72 kDa protein was the most prominent, followed by 56kDa band and surprisingly the enzyme preparation from the phenyl–Sepharose column also gave the same protein band, suggesting that these protein bands may represent the subunits of the same enzyme.



: SDS-Polyacrylamide gel electrophoresis of swine mammary gland MTase III at various steps of purification on 10% gels. Lane 1, molecular mass standards; lane 2, NP-40-solubilized enzyme; lane 3, $(NH_4)_2SO_4$ 35% saturation; lane 4, hydroxylapatite column; lane 5, glycerol gradient; lane 6, phenyl sepharose; lane 7, affinity column fractions. (6A, 7A, 7B, same as 6 and 7 without TCA precipitations).

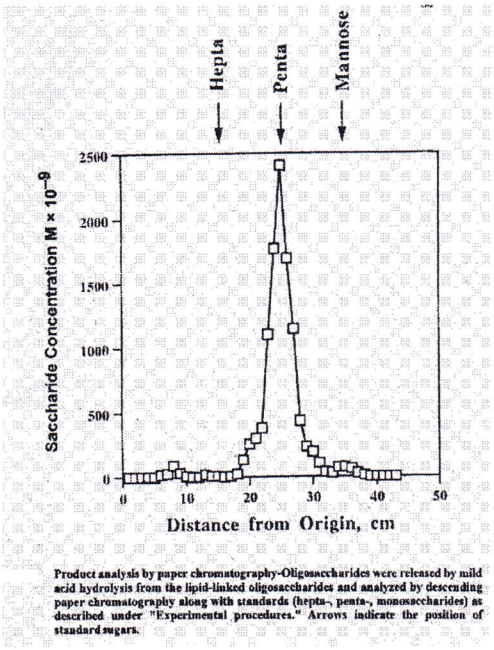
fig(1A)



Molecular mass determination of protein bands detected in the GDP-hexanolamino-Sepharose affinity column purified MTase III by SDS-polyacrylamide gel electrophoresis. Arrows show the molecular masses of five protein band detected by silver staining.

(fig1B)

Figure 2 shows the identification of the oligosaccharide released by mild acid hydrolysis from the lipid- linked oligosaccharide formed by the hydroxylapatite –purified enzyme by paper chromatography as described (Shailubhai *et.al.*,1988). It can be seen that only one major peak corresponded to pentasaccharide, $Man_3GlcNAc_2$ standard.

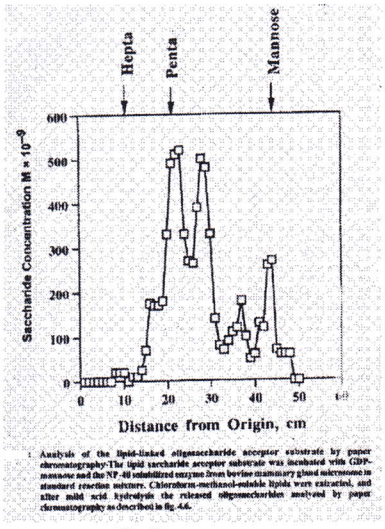


Product analysis by paper chromatography-Oligosaccharides were released by mild acid hydrolysis from the lipid-linked oligosaccharides and analyzed by descending paper chromatography along with standards (hepta-, penta-, monosaccharides) as described under "Experimental procedures." Arrows indicate the position of standard sugars.

(fig2)

Identification of the Oligosaccharide Components of the Lipid-Linked Oligosaccharide Acceptor Substrate

In order to identify the oligosaccharide components, the mixed lipid-linked oligosaccharide acceptor substrate was incubated with GDP-mannose and the detergent solubilized enzyme preparation from swine mammary gland microsomes, using standard assay reaction mixture as described (Kaushal *et.al.* 1986) The $CHCl_3$-CH_3OH souble lipid-linked oligosaccharide thus obtained was subjected to mild acid hydrolysis to release the oligosaccharide components from the glycolipid, which were then identified by paper chromatography. The results are shown in Figure 3 . As expected the major oligosaccharides obtained were tetra-and pentasaccharides with small amount of hexa-and heptasaccharides. These results indicated that the original lipid-linked oligosaccharide acceptor substrate mainly contained Man-$GlcNAc_2$PP-Dolichol and Man_2-$lcNAc_2$-PP-Dolichol with only small amounts of lipid-linked penta- and probably hexasaccharides present.



Analysis of the lipid-linked oligosaccharide acceptor substrate by paper chromatography-The lipid saccharide acceptor substrate was incubated with GDP-mannose and the NP-40 solubilized enzyme from bovine mammary gland microsomes in standard reaction mixture. Chloroform-methanol-soluble lipids were extracted, and after mild acid hydrolysis the released oligosaccharides analyzed by paper chromatography as described in fig. 4A.

(fig3)

Donor substrate specificity

The swine mammary gland MTase III is also highly specific for GDP-Manose and showed no activity towards UDP-glucose, UDP-GlcNAc or UDP-galactose

MTase III activity as a function of pH

The MTase III from swine mammary gland has a sharp pH optimum of 6.5, which is on the acidic side of the scale.

Discussion

The enzyme has been extensively purified to near homogeneity is supported by the fact that on SDS-PAGE under fully reducing conditions five closely migrating protein bands corresponding to molecular masses from 52 to 72 KDa were located on the gel with silver staining (Fig1). Furthermore, this pattern was consistently found in enzyme preparations from both phenyl-Sepharose and GDP-hexanolamine-Sepharose columns. Whether these five protein brands are subunits of MTase III is not clear at the moment. However, sedimentation of the enzyme activity to 50/30 percent glycerol gradient during centrifugation indicates that MTase III is a high molecular protein and may contain subunits. In fact after the phenyl-Sepharose step (Sharma 2014) the protein had to be precipitated with 10% TCA in order to see the bands on gel by silver staining.

. SDS-PAGE gave five closely migrating protein bands corresponding to apparent molecular masses of approximately 72.4, 66.0, 61.6, 57.5 and 56 kDa, suggesting the possibility of multisubunit structure of the enzyme. MTaseIII shows specificity towards GDP-Mannose and has pH optimum of 6.5. The purified enzyme is extremely labile, losing almost total activity within 24h when stored either at 0°C or at -70°C.

The experiments were designed to follow the approach for identifying additional glycosyltransferase so that studies can be undertaken to delineate the regulation of protein and glycosylation during the hormonally modulated growth and differentiation of mammary gland. This tissue synthesizes and secretes massive amount of some of well characterized proteins some of which are asparagine-linked glycopoteins. As a tissue that is intensely modulated by a variety of hormones during its ontogeny and possess enormous secretary capacity, the mammary gland offers unlimited potential as a bioreactor for the synthesis and secretion of biomedically significant glycoproteins.

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