Effects of UDP-glucose Addition on the Synthesis of Mannosyl Lipid-linked Oligosaccharides by Cell-free Fibroblast Preparations*

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(Received for publication, September 7, 1976)

The pattern of mannosyl lipid-linked oligosaccharides synthesized by cell-free enzyme preparations from cultured fibroblasts is altered substantially when 0.2 μM UDP-glucose is added to the incubation medium. Inclusion of UDP-glucose results in the appearance of a new labeled oligosaccharide, which is 1 or 2 glucose units larger than the lipid-linked oligosaccharide synthesized in the presence of only GDP-mannose (2 μM) and UDP-N-acetylgalactosamine (20 μM). Label from UDP-[3H]glucose is incorporated into the same larger oligosaccharide size class. The results can be explained most easily by assuming that the new mannosyl lipid-linked oligosaccharide contains 1 or 2 glucose residues in addition to 5 to 6 mannose residues. The results are compatible with the recent finding by Spiro et al. (Spiro, M. J., Spiro, R. G., and Bhoitroo, V. D. (1976) J. Biol. Chem. 251, 6400-6408; Spiro, R. G., and Spiro, M. J., Spiro, R. G., and Bhoitroo, V. D. (1976) J. Biol. Chem. 251, 6420-6425) that naturally occurring mannosyl lipid-linked oligosaccharides contain glucose.

In addition to being incorporated into lipid oligosaccharides, glucose residues are also incorporated into endogenous glycoproteins. Incorporation of glucose into glycoproteins that give rise to pronease glycopeptides of the typical asparagine-linked size classes is almost completely dependent on the presence of GDP-mannose.

There is a generally held opinion that the complex glycoproteins of serum and plasma membrane lack glucose (see, for example Ref. 1). In his classical review on nucleotide sugars and transglycosylation reactions, Ginsburg (2) makes the point that "a teleological argument can be made for the absence of n-glucose in that it is the only sugar found free in most tissue fluids. The efficiency of recognition surfaces' composed of n-glucosyl residues would be impaired by free n-glucose much as antigen-antibody interactions are inhibited by haptenes. Evolutionary selection against this impairment, however slight, might thus be expected to eliminate n-glucose as a component of cell surfaces."

Whatever the merits of this teleological argument and of published data concerning the presence or absence of glucose in cell surface glycoproteins there are a number of facts that at least indirectly implicate n-glucose in the synthesis of cell surface macromolecules. These include numerous demonstrations that cell-free systems will catalyze the incorporation of glucose from UDP-glucose into lipid-linked structures with properties similar to those of the mannosyl lipid components which now appear to be intermediates in glycoprotein synthesis. Dolichol monophosphate-glucose has been synthesized with preparations from rat liver, hen oviduct, Tetrahymena pyriformis, and cotton fibers, as well as other sources (3). It has also been shown that lipid-linked glucosyl oligosaccharides can be synthesized by many of the same preparations (for recent reviews see Ref. 3). In general, these glucose-containing lipid-linked oligosaccharides have not been characterized in detail.

Among characterized glycoproteins only collagen and related structural proteins have been clearly shown to contain glucose. These proteins have hydroxylsine residues which carry O-α-D-glucosylgalactose disaccharides (4-6). Although unrelated to the glycoprotein question it should be pointed out that most complex glycophospholipids contain a single glucosyl moiety attached directly to sphingosine.

A major new contribution to this area of research has been the discovery by Spiro et al. (4-8) of lipid-linked oligosaccharides containing both glucose and mannose. These materials have been isolated from thyroid, oviduct, kidney, thymus, liver, and pancreas. All appear to contain glucosamine and mannose, with small amounts of glucose. The glucose residue(s) has been demonstrated to be an integral part of the oligosaccharide structure. In the present paper we show that extremely small amounts of UDP-glucose can modify the nature of lipid-linked oligosaccharides synthesized from GDP-mannose in a cell-free system, and that glucose is probably incorporated into these same structures.

MATERIALS AND METHODS

Cell Culture and Membrane Preparation

NIL-8 cells were grown to confluency in glass roller bottles (Bellco Glass, Inc.) at 37° in Dulbecco's modified Eagle's medium (Grand Island Biological Company) with 5% fetal calf serum (Flow Labora-
Cells from one roller bottle (5 x 10^10 cells) were transferred to five bottles during passage and cells were routinely harvested 4 days after transfer.

Cells were scraped from the roller bottles in phosphate-buffered saline (1.4 x 10^{-1} M NaCl, 7 x 10^{-3} M KCl, 1 x 10^{-3} M potassium phosphate) with calcium (1 x 10^{-7} M) and magnesium (1 x 10^{-6} M), pH 7.4. They were washed once in 2.5 x 10^{-3} M sucrose, 5 x 10^{-3} M Tris/HC1, 2 x 10^{-4} M MgCl2, pH 7.4, and resuspended in this buffer for nitrogen cavitation (9). The cell lysate was centrifuged at 1000 x g for 7 min. The pellet was discarded and the supernatant fluid was 

The original pellet was dried thoroughly under a stream of nitrogen. The dried material is referred to as the particulate enzyme preparation. All the experiments were done using ethyl acetate/pyridine/water (8/2/1, by volume, 36 h) as the solvent system. Internal sugar standards (Sigma Chemical Co.) and 2 x 10^{-6} M GDP-[-^14C]mannose (8 x 10^6 cpm, 150 mCi/mmol, New England Nuclear). Some incubation mixtures also contained 2 x 10^{-6} M UDP-[H]glucose (2.7 x 10^6 cpm, 4.86 Ci/mmol, New England Nuclear). Approximately 200 μg of the membrane preparation was added to initiate the reaction. The assay was begun in a shaking water bath set at 37°C. The reaction was terminated by adding 2 ml of a chloroform/methanol mixture (2/1, by volume) to the incubation mixture. After at least 15 min at room temperature, the extracted mixture was separated into supernatant and pellet by centrifugation at 700 x g for 5 min.

The pellet was re-extracted with 2 ml of the chloroform/methanol (2/1) mixture and the resultant supernatant was combined with the first extract. The 4 ml of chloroform/methanol 2/1 extract were partitioned into two phases by the addition of 1 ml of 0.9% NaCl; the solutions were mixed (30 s), chilled for 10 min, and centrifuged (700 x g) for 5 min at room temperature. The upper phase was discarded; the lower phase, mostly chloroform, was again partitioned as described after the addition of 1.5 ml of 0.9% saline/methanol (2/1, by volume).

The resultant lower phase (Fraction II) was washed free of non-lipid material in chloroform/methanol (2/1) mixture and the resultant supernatant was combined with the first extract. Mild Acid Hydrolysis -The procedure used to prepare samples for gel filtration chromatography was as follows: the sample was dissolved in 0.2 ml of 0.5 N HCl in tetrahydrofuran (1/4, by volume) and was heated at 56°C for 5 h. After cooling the sample, 0.2 ml of 0.1 N NaOH in 0.02 N sodium phosphate was added. The solution was then evaporated to dryness under reduced pressure. The oligosaccharide was dissolved in water and applied to the column.

**RESULTS**

Most of the experiments reported in this paper were carried out with particulate enzyme preparations from NIL-8 fibroblasts. The particles were incubated with 2 μM GDP-[14C]mannose and 0.2 μM UDP-[H]glucose. All incubations also contained unlabeled UDP-N-acetylglucosamine (30 μM). The effect of varying nucleotide sugar concentrations is discussed below. The fractions isolated following the incubation were as follows: Fraction I: material soluble in chloroform/methanol (2/1). This fraction contains dolichol monophosphate-phate-glycose derivatives and other components; Fraction II: material soluble in chloroform/methanol/water (1/1/0.3). The principal labeled materials in this fraction are lipid-linked oligosaccharides, presumptive intermediates in glycoprotein synthesis; Fraction III: insoluble material. The bulk of the labeled material in this fraction is converted to compounds with the properties of glycopeptides by pronase digestion.

The monosaccharides released from Fractions II and III by strong acid hydrolysis were shown by paper chromatography to be exclusively [14C]mannose and [3H]glucose. Paper chromatography was carried out in a system which clearly separates galactose, glucose, and mannose. Labeled galactose was not found.

**Incorporation Kinetics**

Fig. 1 presents the kinetics of incorporation of label into Fractions I, II, and III when the two nucleotide sugars were incubated separately or together. In all of these experiments the initial concentrations of GDP-mannose and UDP-glucose were 2 and 0.2 μM, respectively. All incubations contained 20 μM unlabeled UDP-N-acetylglucosamine. As can be seen, the

1 S. S. Krag, and P. W. Robbins (1977) J. Biol. Chem., in press.
UDP-glucose and Mannosyl Lipid Synthesis

Fig. 1. Rates of incorporation of GDP-[14C]mannose (GDPM-[14C]) and UDP-[3H]glucose (UDPG-[3H]) into Fractions I, II, and III. A, B, and C represent chloroform/methanol (2:1)-soluble, chloroform/methanol/water (1:1:0.3)-soluble, and residual nonextractible materials, respectively.

Small amount of UDP-glucose (2 x 10^{-6} M) does not have a significant effect on the rate or extent of [14C]mannose incorporation into the chloroform/methanol, chloroform/methanol/water, or insoluble residue fractions. GDP-mannose (2 x 10^{-6} M), on the other hand strongly inhibits the incorporation of UDP-glucose into the lipid fraction, and stimulates its incorporation into the oligosaccharide-lipid and residue fractions.

Since in every case the UDP-glucose incorporation was maximal at 5 to 10 min, the 10-min time point was chosen for the isolation and characterization studies described below. The relatively short duration of UDP-glucose incorporation may reflect complete utilization of the small amount of added substrate or other limiting factors. Higher concentrations of UDP-glucose were not used since, in general, they led to heterogeneity in the number and size of the oligosaccharide products (see "Discussion"). These points are currently being investigated.

Fraction I: Chloroform/Methanol (2:1) - When the chloroform/methanol (2:1) extract from doubly labeled particles is subjected to chromatography on DEAE-cellulose, several components are displayed. A component which elutes with chloroform/methanol alone contains only glucose label and could be glycosphingolipid or a glycosyl glyceride but has not been characterized. The components eluting at the beginning of the ammonium acetate gradient are in the position of dolichol monophosphate-glucose (mannose and glucose). A third pair of peaks eluting at higher ammonium acetate concentrations, could be oligosaccharide lipid derivatives. All of these materials are now being studied in detail and will be the subject of a future publication.

Fraction II: Chloroform/Methanol/Water (1:1:0.3) - When subjected to DEAE-cellulose chromatography, the material in this fraction behaves in the manner expected of oligosaccharide pyrophosphate lipid derivatives, as described by previous workers (4-6). Essentially all of the radioactivity is retained by the column and is eluted at approximately 50 mM ammonium acetate (Fig. 2).

Mild acid hydrolysis converts this material to water-soluble oligosaccharides which have been fractionated by chromatography on Bio-Gel P-6. A typical set of profiles for singly and doubly labeled materials prepared from samples incubated for 10 min is shown in Fig. 3. The striking result is the alteration in the mannose oligosaccharide profile produced by UDP-glucose addition. Although the total yield of [14C]mannose oligosaccharide material is the same in 3A and 3B (cf. also Fig. 1), approximately half of the mannose label appears in a

Fig. 2. DEAE-column chromatogram of doubly labeled material extracted into chloroform/methanol/water (1:1:0.3). A 0 to 100 mM ammonium acetate gradient was applied after eluting the column with chloroform/methanol/water (1:1:0.3). Fractions (9 ml) were collected and transferred to scintillation vials, dried, and counted in 1 ml of 1% sodium dodecyl sulfate, 8 ml of scintillation fluid.

larger oligosaccharide when 0.2 μM UDP-glucose is added to the incubation mixture. Radioactive glucose from UDP-glucose is incorporated into this same larger oligosaccharide. Some 3H is incorporated into the larger oligosaccharide area in the absence of GDP-mannose but this incorporation is stimulated 2- to 3-fold by GDP-mannose addition.

FIG. 3 (left). Bio-Gel P-6 chromatogram of oligosaccharides released from the chloroform/methanol/water (1/1/0.3) fraction by mild acid hydrolysis. Acid hydrolysis and Bio-Gel P-6 chromatography were carried out as described in the text. GDP-l[4C]mannose; UDPG-3H, UDP-[3H]glucose.

FIG. 4 (right). Bio-Gel P-6 chromatogram of doubly labeled oligosaccharides prepared by 30-min enzymatic incubation. The incubation, extraction, and mild acid hydrolysis were carried out as described in Fig. 3. GDPM-14C, GDP-1[4C]mannose; UDPG-3H, UDP-[3H]glucose.

FIG. 5. High voltage paper electrophoresis of doubly labeled oligosaccharide phosphate. Doubly labeled lipid oligosaccharide was hydrolyzed in a sealed tube in 1 × NaOH at 100° for 1 h. The sample was cooled, neutralized, and a portion treated with Escherichia coli alkaline phosphatase. The treated (not shown) and untreated (shown) portions were streaked separately on Whatman No. 3MM paper and subjected to high voltage paper electrophoresis in pyridinium acetate. The sample was started at 0 and the anode is to the right. The electrophoreograms were cut into 2-mm strips, eluted into 1 ml of water, and counted. The anionic region of electrophoreogram shown above contained materials completely sensitive to alkaline phosphatase. Non-phosphatase-sensitive materials, presumably alkaline degradation products, occurred in the cationic and in more anionic areas.

FIG. 6. Bio-Gel P-6 chromatogram of glycopeptides derived by pronase treatment of Fraction III. The nonextractable residue fractions prepared from 10-min incubations were treated with pronase by a modification of the procedure described by Sefton and Keegstra (13). The residue fractions were suspended in 250 μl of 1 × 10^-3 M Tris/HCl pH 8, 1 × 10^-2 M CaCl₂. A 1% solution of predigested (13) pronase in the same medium (Calbiochem, A grade) was added as follows: 100 μl at zero time, 100 μl after 24 h, and 50 μl after 48 h. The digestion was carried out at 50-60°, and the preparations were protected from bacterial contamination by periodic addition of a drop of tolune. After 60 to 72 h the samples were concentrated to 200 to 300 μl under a stream of nitrogen and applied directly to a Bio-Gel P-6 column. The vertical arrows indicate the positions of the exclusion volumes and Sindbis virus glycopeptides S-2, S-3, and S-4. The carbohydrate portions of S-4 and S-3 have molecular weights of 1600 and 1800, respectively. S-2 and S-1 are similar to S-3 except that they contain, respectively, 1 and 2 residues of sialic acid. The inclusion volume occurs at Fractions 68 to 70. The amount of material in this fraction varied from experiment to experiment. GDPM-14C, GDP-1[4C]mannose; UDPG-3H, UDP-[3H]glucose.
Other experiments have shown that UDP-N-acetylglucosamine stimulates glucose incorporation into the lipid oligosaccharide fraction only in the presence of GDP-mannose (data not shown). The Bio-Gel P-6 columns used for these experiments have been calibrated with glycopeptides of known molecular weight. Calculation based on these calibrations shows that the larger oligosaccharide contains 1 or 2 more glucose units than the smaller component.

The P-6 pattern of oligosaccharides derived from Fraction II changes with incubation time. Fig. 3 shows a typical 10-min profile. A typical 30-min profile for doubly labeled material is shown in Fig. 4. As may be seen by comparing Figs. 3 and 4, the major change produced by extended incubation is an increase in the size of the lower molecular weight oligosaccharide peak. In addition there may be a broadening of both the 3H and 14C peaks which could reflect an increase in the complexity of the oligosaccharide mixture.

In order to resolve the oligosaccharide mixture further, high voltage paper electrophoresis separations were carried out on the oligosaccharide phosphate esters derived by alkaline cleavage of the lipid oligosaccharides. A typical electrophoresis pattern for doubly labeled material is shown in Fig. 5. A major component containing both glucose and mannose is evident in addition to the usual 14C-mannosyl oligosaccharide phosphate. In addition, there is a suggestion of component(s) containing intermediate ratios of glucose and mannose (Fractions 40 to 45). The resolution of these components was seen more clearly in other electrophoreograms (data not shown). All of these materials were converted to neutral species by hydrolysis of the oligosaccharide phosphate esters derived by alkaline phosphatase and following phosphatase treatment the free oligosaccharides gave single sharp peaks in the appropriate position on P-6 columns. Component A corresponds to the larger oligosaccharide and Component B to the smaller oligosaccharide (see Fig. 3). These electrophoretic separation studies are continuing.

Fraction III: Nonextractable Residue - Strong acid hydrolysis of the residue fraction releases 3H-glucose and 14C-mannose. No detectable label is present in galactose or other monosaccharides. Label in the residue fraction is largely solubilized by pronase digestion. Typical Bio-Gel P-6 profiles of pronase digests of singly and doubly labeled materials are shown in Fig. 6. The position of the exclusion volume (V_e) is marked as well as the calibrated positions of Sindbis virus glycopeptides S-2, S-3, and S-4 (13). As is true for the lipid-linked oligosaccharides, the presence of UDP-glucose in incubation mixtures alters the pattern of mannose-labeled glycopeptides. There is stimulation of mannose incorporation into the oligosaccharides in the S-2 to S-4 size region. There is significant incorporation of glucose into glycopeptides of the same size class only in the presence of GDP-mannose.

In addition to the demonstration of incorporation into Fraction III, we have shown that glucose can be incorporated into the well characterized glycopeptides of Sindbis virus. These studies were carried out with membrane fractions of infected chick embryo fibroblasts. The data are presented in Table I. Both direct and indirect Sindbis specific antibody precipitates were isolated. As may be seen, the ratio of glucose to mannose label is the same in Fraction III and the specific precipitates. Sodium dodecyl sulfate-gel electrophoresis patterns of antibody precipitates prepared from these labeled materials show a single radioactive component with a mobility similar to that of the viral glycoproteins.

**DISCUSSION**

The experiments described strongly support and extend the observations of Spiro et al. (4–8) concerning the incorporation of glucose in naturally occurring (in vivo labeled) lipid-linked mannose oligosaccharides. Although the materials synthesized in our *in vitro* system are probably smaller by several glucose units than the oligosaccharides isolated by Spiro et al., this result may be explained by differences between the *in vitro* and *in vivo* synthesis systems, or may be a reflection of tissue specificity. We have not, as yet, examined the oligosaccharide lipids made by fibroblasts in vivo. We should emphasize that we have not yet proven, in our system, that mannose and glucose are present in the same molecule although the evidence points strongly in this direction. The relevant experiments are being carried out now and will be the subject of a separate publication.

Calculations based on glycopeptide calibrations of the P-6 columns indicate that the presumptive mannosylglucosyl oligosaccharide is 1 or 2 glucose units larger than the mannosyl oligosaccharide. Such calculations are uncertain, however, since P-6 elution position depends on both molecular size and shape. Calculations of sugar ratios based on the specific activities of the nucleotide sugar precursors are even more precarious because of the possible presence of unlabeled nucleotide sugars in the enzyme preparation and the presence of some glucose label in the larger oligosaccharide peak area when

**Table I**

|                          | Nonextractible residue Fraction III | Direct antibody precipitate | Indirect antibody precipitate |
|--------------------------|------------------------------------|-----------------------------|-------------------------------|
|                          | 14C  | 3H  | 14C  | 3H  | 14C  | 3H  |
| Uninfected GDP-[14C]Man  | 33,000 | <50 | 1400 |
| Uninfected GDP-[14C]Man + UDP-[3H]Glc | 50,700 60,300 | <50 | <50 | 2400 6000 |
| Infected GDP-[14C]Man  | 39,600 | 1550 (4%) | 10,600 (27%) |
| Infected GDP-[14C]Man + UDP-[3H]Glc | 61,200 54,400 | 2730 (4.5%) 2170 (4%) | 15,400 (25%) 14,000 (26%) |
incubation is carried out in the absence of GDP-mannose (see Fig. 3). However, such specific activity calculations give values which are compatible with the presence of 1 or 2 glucose residues for each 4 to 8 mannose residues. The higher \(^4\)H than \(^3\)C counts per min in the larger oligosaccharide simply reflects the higher specific activity of the UDP-glucose (1.7 × 10^7 cpm/\(\mu\)mol UDP-\[^{14}\)C]mannose). The striking feature of the UDP-glucose effect on mannose oligosaccharide synthesis is that it occurs in the range of 10^-7 M UDP-glucose. By contrast, the \(K_m\) for UDP-glucose in glycogen synthesis is on the order of 10^-2 M (14). 3 to 4 orders of magnitude higher. The UDP-glucose effect is manifest in the presence of a 10-fold higher concentration of GDP-mannose and 100-fold higher concentration of UDP-\(^N\)acetylglucosamine. As the level of UDP-glucose is increased beyond 1 × 10^-4 M, the pattern of glucose containing products becomes more complex but little, if any, increase is observed in the ratio of large/small mannose containing oligosaccharides. Whether the UDP-glucose effect would be altered by changing the ratio of large/small mannose containing oligosaccharides.

A major unanswered question concerns the role of the mannose-glucose oligosaccharides in glycoprotein synthesis. One finding that may be significant is that although UDP-glucose does not stimulate quantitatively the incorporation of mannose into the lipid-linked oligosaccharide fraction, it does, in fact, appear to stimulate to some extent the incorporation of mannose into glycoprotein material that gives rise to glycopeptides of the typical asparagine-linked size classes after pronase digestion (seeFig. 6).

A final point that may bear on possible functions of the mannose-glucose lipid-linked intermediates is that our in vitro system incorporates mannose and glucose (in the same ratio as that found in crude "residue") into well characterized proteins, namely the Sindbis virus glycoproteins (see Table I). Since these glycoproteins each contain an A type and B type glycopeptide of known structure, it seems possible that the products synthesized in vitro are precursors of at least one type of well known glycopeptide. It is of interest to note that Sefton and Keegstra (13) found that PE2, the cellular precursor of viral protein E2, contained glycopeptides that differed significantly in size from those of the mature viral glycoprotein. Furthermore, it is likely that some carbohydrate is lost during the process of maturation of PE2 into the viral protein (15).

If it is true that glycosylation plays a role in glycoprotein synthesis, this fact might provide an explanation for the puzzling inhibitory effects of glucosamine and 2-deoxyglucose on glycoprotein synthesis in vitro. Recent publications from the Scholtissek group (16, 17) suggest that 2-deoxyglucose may interfere with mannose metabolism, but interference with glucose pathways is clearly also possible. In the case of the glucosamine block, it is known that the principle metabolic effect is a sequestering of the cellular uridine pool as UDP-N-acetylglucosamine (18). The lack of availability of UTP would clearly interfere with UDP-glucose synthesis, but would not, presumably, block GDP-mannose metabolism. Glucosamine-blocked cells should, in fact, have ample UDP-N-acetylglucosamine and GDP-mannose available for glycoprotein synthesis.

We are currently undertaking experiments to investigate this problem.

Taken together the results and systems discussed above suggest some obvious directions for future research. Those that we are currently emphasizing are:

1. Structural studies on the glucose-mannose-containing lipid linked oligosaccharides and glycopeptides.
2. Studies on the transfer of isolated lipid-linked oligosaccharides to endogenous and exogenous protein acceptors.
3. A search for "maturation" reactions which alter the carbohydrate structure of glycoproteins formed in vitro. For these studies we plan to use the Sindbis virus system since in this system only two glycoprotein species are synthesized in vivo, and considerable information already exists concerning the structure of both the cell-bound and mature forms of the viral proteins.

Acknowledgment—We would like to thank Ms. Dyann Wirth for the Sindbis antibody preparation.
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P W Robbins, S S Krag and T Liu

J. Biol. Chem. 1977, 252:1780-1785.

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