Glycoprotein biosynthesis in animal cells grown in suspension culture

Assembly of lipid-linked saccharides and formation of protein-bound 'high-mannose' oligosaccharides

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Glycoprotein biosynthesis was studied with mouse L-cells grown in suspension culture. Glucose-deprived cells incorporated [^3H]mannose into 'high-mannose' protein-bound oligosaccharides and a few relatively high-molecular-weight lipid-linked oligosaccharides. The latter were retained by DEAE-cellulose and turned over quite slowly during pulse-chase experiments. Increased heterogeneity in size of lipid-linked oligosaccharides developed during prolonged glucose deprivation. Sequential elongation of lipid-linked oligosaccharides was also observed, and conditions that prevented the assembly of the higher lipid-linked oligosaccharides also prevented the formation of the larger protein-bound 'high-mannose' oligosaccharides. In parallel experiments, [^3H]mannose was incorporated into a total polyribosome fraction, suggesting that mannose residues were transferred co-translationally to nascent protein. Membrane preparations from these cells catalysed the assembly from UDP-*N*-acetyl-D-[6- ^3H]glucosamine and GDP-D-[U- ^{14}C]mannose of polyisoprenyl diphosphate derivatives whose oligosaccharide moieties were heterogeneous in size. Elongation of the *N*-acetyl-D-[6- ^3H]glucosamine-initiated glycolipids with mannose residues produced several higher lipid-linked oligosaccharides similar to those seen during glucose deprivation *in vivo*. Glucosylation of these mannose-containing oligosaccharides from UDP-D-[6- ^3H]glucose was restricted to those of a relatively high molecular weight. Protein-bound saccharides formed *in vitro* were mainly smaller in size than those assembled on the lipid acceptors. These results support the involvement of lipid-linked saccharides in the synthesis of asparagine-linked glycoproteins, but show both *in vivo* and *in vitro* that protein-bound 'high-mannose' oligosaccharide formation can occur independently of higher lipid-linked oligosaccharide synthesis.

Several distinct types of oligosaccharide side chains are present on animal glycoproteins (Spiro *et al.*, 1974). The 'complex' and 'high-mannose' types of asparagine-linked oligosaccharides share a common 'core' oligosaccharide structure with a major lipid-linked oligosaccharide that, on the basis of kinetic studies, has been suggested as their precursor (Robbins *et al.*, 1977b). The assembly of such *N*-linked glycoproteins is envisaged to proceed by the 'en bloc' transfer of this common lipid-linked oligosaccharide to protein (Robbins *et al.*, 1977b; Staneloni & Leloir, 1979). The timing of this transfer has been studied with vesicular-stomatitis-virus G protein as a model, in which transfer of carbohydrate to the nascent peptide chain has been

achieved by using membrane-supplemented protein-synthesizing systems (Rothman & Lodish, 1977), and is accompanied *in vivo* by elaborate processing of the newly transferred oligosaccharide (Kornfeld *et al.*, 1978). Mature vesicular-stomatitis-virus G protein possesses mainly oligosaccharides of the 'complex' type (Reading *et al.*, 1978). Similar processing events have been found during the synthesis of mouse immunoglobulin M molecules and histocompatibility antigens, where initial glycosylation occurs while the protein is in the rough endoplasmic reticulum, whereas subsequent elaboration occurs during passage to the cell surface, probably in the Golgi complex (Tartakoff *et al.*, 1978; Dobberstein *et al.*, 1979). These events are summarized diagrammatically in Fig. 1.

Recent reports of variant lipid-linked oligosaccharides occurring in cells with abnormally glycosylated glycoproteins (Trowbridge & Hyman,

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Fig. 1. Involvement of lipid-linked saccharides in the synthesis of asparagine-linked 'complex' and 'high-mannose' side chains of glycoproteins

Lipid-linked oligosaccharide assembly, followed by co-translational oligosaccharide transfer and protein-bound oligosaccharide modification are thought to lead to the assembly of a protein-bound 'core' saccharide of structure $\text{Asn-GlcNAc}_2\text{Man}_3$, which is the precursor of asparagine-linked 'complex' chains by the further addition of *N*-acetylglucosamine, galactose and sialic acid residues (Robbins *et al.*, 1977b; Kornfeld *et al.*, 1978). Asparagine-linked 'high-mannose' chains may be formed by: (1) direct addition of completed non-glycosylated lipid-linked oligosaccharides to protein acceptors (Chen & Lennarz, 1976); (2) action of glucose-trimming enzymes on initially-transferred protein-bound 'G'-oligosaccharides (Trowbridge & Hyman, 1979; Gibson *et al.*, 1980); or (3) addition of further mannose residues to the protein-bound 'core' oligosaccharide. Abbreviations used: RER, rough endoplasmic reticulum; AcNeu, *N*-acetylneuraminate.

1979) suggest the control of the assembly of the initial lipid-linked oligosaccharide to be of some importance, since abnormalities expressed at this level remain uncensored and are reflected in abnormally glycosylated glycoproteins. The role of such lipid-linked saccharides in the synthesis of 'high-mannose'-type asparagine-linked oligosaccharides is as yet uncertain.

We have previously isolated lipid-linked oligosaccharides from rapidly dividing mouse L-cells (Bailey *et al.*, 1979) and demonstrated the biosynthesis *in vivo* of a lipid-linked oligosaccharide similar to that described by Robbins *et al.* (1977b). In the present paper, we examine the biosynthesis of this lipid-linked oligosaccharide in relationship to the assembly of protein-bound 'high-mannose' oligosaccharides both *in vivo*, with cells grown under a variety of physiological conditions, and *in vitro*, with isolated membrane preparations incubated with nucleotide sugars.

Experimental

Animal cell culture, membrane preparation and assay *in vitro*

Mouse L-cells were maintained as a continuous

suspension culture as described by Birch & Pirt (1970), at an average cell density of 5×10^5 cells/ml. To obtain glucose-depleted cells, the cells were grown in glucose-free media for 6–24 h.

A membrane fraction was obtained from the cells by the method used in previous studies (Bailey *et al.*, 1979). Cells were homogenized in 0.1 M-Tris/HCl, pH 7.5, containing 5 mM-dithioerythritol, 10 mM-MgCl₂ and 0.4 M-sucrose at 4°C with a Polytron tissue homogenizer (Brinkmann Instruments) for 20 s at half-maximum force. The homogenate was centrifuged (1000g for 10 min) and a total membrane preparation was obtained from this supernatant by centrifugation at high speed (48 000g for 60 min). The membrane preparation was resuspended in homogenization buffer at a protein concentration of approx. 10 mg/ml and incubated with radioactive substrates {GDP-D-[U-¹⁴C]mannose (Amersham; sp. radioactivity 173 Ci/mol, final incubation concentration 20 μM); UDP-*N*-acetyl-[6-³H]glucosamine (New England Nuclear; sp. radioactivity 10 Ci/mmol; final incubation concentration 3 μM); UDP-D-[6-³H]glucose (Amersham, sp. radioactivity 3.1 Ci/mmol; final incubation concentration 9 μM)} in 60 μl total volume at 25°C. The reactions were terminated by addition of chloro-

form/methanol (1:1, v/v) to bring the final concentration of chloroform/methanol/water to 1:1:0.3 (by vol.).

Characterization of lipid- and protein-bound saccharides

Chloroform/methanol/water (1:1:0.3, by vol.) extracts containing lipid-bound saccharides were fractionated into neutral, monophosphoryl and diphosphoryl lipid classes by DEAE-cellulose chromatography (modified from Lehle & Tanner, 1975). Extracts were washed three times with 0.5% (w/v) KCl, and the lower phase and interphase material were redissolved by adding a few drops of methanol and applied to a small column (10 ml; 18 mm × 20 mm) of DEAE-cellulose (Whatman DE-52) which was sequentially eluted with chloroform/methanol/water (1:1:0.3, by vol.), chloroform/methanol/5 mM-ammonium formate (1:1:0.3, by vol.) and finally chloroform/methanol/140 mM-ammonium formate (1:1:0.3, by vol.) (Bailey *et al.*, 1979). Total extracts and DEAE-cellulose-purified fractions were hydrolysed in 0.1 M-HCl at 90°C for 30 min (see Herscovics *et al.*, 1978). The water-soluble oligosaccharides were then separated by Bio-Gel-P2 (200–400 mesh) and Bio-Gel-P4 (–400 mesh; Bio-Rad Laboratories) gel-permeation chromatography in columns (120 cm × 1.2 cm) eluted with 0.1 M-Tris/HCl, pH 7.4, containing 0.2 M-NaCl and 0.05% NaN₃. Fractions (approx. 0.8 ml) were collected at a flow rate of approx. 2 ml/h. Bromocresol Purple or Phenol Red were included in each run as internal standards, eluting at fractions 107 and 109 respectively. Mannose was eluted from such a column at fraction 87, whereas the void volume occurred at fractions 30–32.

The residual material after lipid extraction contained the bulk of the cellular protein as well as other polymeric materials such as glycosaminoglycans and glycogen. Hydrazinolysis was used to release protein-bound oligosaccharides, as described by Yosizawa *et al.* (1966) and Heath & Northcote (1971). Freeze-dried samples were heated with hydrazine containing 1% (w/v) hydrazine sulphate for 10 h at 105°C. Fractionation of protein-bound saccharides was performed as described for lipid-linked oligosaccharides above.

Labelling of cells to measure mannose uptake and incorporation into polymeric products

Cells from rapidly growing glucose-supplied cultures, or from glucose-depleted cultures, were transferred to warm fresh medium at concentrations of $(2.5\text{--}3.0) \times 10^6$ cells/ml and maintained under CO₂/air (1:19). D-[1-³H]Mannose (sp. radioactivity 12.8 Ci/mmol; New England Nuclear) was added to a level of 0.4 μCi/ml and 1 ml samples were removed at suitable time intervals. The samples were centri-

fuged for 15 s in a Fisher microcentrifuge (model 59) and washed twice at room temperature with 1 ml of 0.15 M-NaCl. The cell pellet was extracted with 10% trichloroacetic acid for 30 min at 4°C to remove the acid-soluble fraction. The remaining acid-insoluble material was dissolved overnight in 0.2 M-NaOH and both fractions were counted for radioactivity in ACS scintillant (Amersham) in an Intertechnique liquid-scintillation spectrometer (model SL30).

Labelling of cells to form lipid-linked and protein-bound oligosaccharides

Cells from glucose-depleted cultures were transferred to warm fresh glucose-free medium at concentrations of approx. 5×10^6 cells/ml and maintained under CO₂/air (1:19). D-[1-³H]Mannose was added, as described above to a level of 1 μCi/ml and 10 ml samples were removed at suitable time intervals. In pulse-chase experiments, unlabelled mannose was added to a concentration of 10 mM. Cells were cooled on ice and harvested by centrifugation at 1000 g for 5 min at 4°C before the addition of chloroform/methanol/water (1:1:0.3, by vol.). In some experiments cells were washed and pre-incubated in phosphate-buffered saline (0.2 g of KCl/litre, 0.2 g of KH₂PO₄/litre, 8.0 g of NaCl/litre, 1.15 g of Na₂HPO₄/litre, adjusted to pH 7.4) and subsequently labelled with D-[1-³H]mannose also in phosphate-buffered saline.

Polyribosome isolation and analysis

Immediately after labelling with D-[1-³H]mannose, cells were rapidly cooled by the addition of frozen medium to 0°C and harvested by centrifugation at 0°C for 5 min at 1000 g. The pellet was thoroughly mixed with pre-cooled disruption buffer [0.15 M-Tris/HCl (pH 8.5)/30 mM-magnesium acetate/50 mM-KCl/0.5% (w/v) Nonidet NP40 (Bethesda Research Laboratories)/5 mM-2-mercaptoethanol/20 mM-EGTA/0.4 M-sucrose], and rapidly centrifuged (for 2 min at 20 000 g in a Sorvall RC-2B refrigerated centrifuge) to remove any insoluble material. The supernatant was layered over a heavy sucrose cushion [50 mM-Tris-HCl (pH 8.5)/20 mM-KCl/10 mM-magnesium acetate/4 mM-2-mercaptoethanol/1.5 M-sucrose] and centrifuged for 3 h at 280 000 g in a type 65 rotor (Beckman) at 4°C to pellet a total polyribosomal fraction. This pellet was resuspended in a small volume of water and a portion was analysed on a 15–40% (w/v) sucrose gradient containing 20 mM-KCl, 2 mM-magnesium acetate, 2 mM-2-mercaptoethanol and 50 mM-Tris/HCl, pH 8.5. Centrifugation was at 225 000 g for 45 min in an SW 50.1 rotor (Beckman) at 4°C. Fractions were collected from the bottom of the gradient and assayed for absorbance at 260 nm and radioactivity.

Results

Formation of mannose-labelled lipid-linked oligosaccharides and glycoproteins by whole cells

A glucose-containing medium is usually provided to obtain L-cells in an actively dividing state (Birch & Pirt, 1970). D-[1-³H]Mannose incorporation into 10% trichloroacetic acid-soluble and 10% trichloroacetic acid-insoluble fractions in the presence of such a glucose-containing medium was poor (Fig. 2a). To stimulate mannose uptake, L-cells were grown for several generations in glucose-free media and were subsequently labelled with D-[1-³H]mannose while still suspended in glucose-free medium (see the Experimental section). Substantially higher specific incorporation of D-[1-³H]mannose into both trichloroacetic acid-soluble and trichloroacetic acid-insoluble pools was observed (Fig. 2a). Mannose incorporation into intracellular trichloroacetic acid-soluble pools was approximately linear, whereas trichloroacetic acid-insoluble pools became saturated after about 30 min. This may reflect an equilibrium between secreted and intracellular mannose-containing glycoproteins; the former would be lost during the manipulations described in these experiments (see the Experimental section), resulting in a plateau-like effect.

Parallel experiments showing the time course of D-[1-³H]mannose incorporation into lipid-linked saccharide and glycoprotein products in glucose-depleted cultures are shown in Fig. 2(b). In these

experiments, unlabelled mannose (final concentration 10 mM) was added after 60 min incubation with D-[1-³H]-mannose to 'chase' label from intermediates into products. Analyses of the lipid- and protein-bound oligosaccharides formed under these conditions are shown in Figs. 3 and 4 respectively. The lipid-linked oligosaccharide profile was essentially invariant, consisting of a group of peaks of similar size, eluting from the Bio-Gel-P4 column at fractions 45–55 (Fig. 3). Turnover of these lipid-linked saccharides during the chase with unlabelled mannose (Figs. 2b and 3) was relatively slow, taking place over a period of 60 min.

The protein-bound saccharides that accumulated during incubations with D-[1-³H]mannose (Fig. 4) were considerably smaller than the lipid-linked saccharides, but corresponded closely to those formed by chick embryo cells infected with Sindbis virus (Robbins *et al.*, 1977b), which have been characterized as 'high-mannose' (S4)-type asparagine-linked oligosaccharides (Burke & Keegstra, 1979). Throughout this study hydrazinolysis has been used to liberate such protein-bound saccharides. Similar techniques have been used in studies of highly complex protein-bound saccharides with no apparent problems associated with oligosaccharide degradation (Heath & Northcote, 1971; Mizuochi *et al.*, 1978), and, indeed, offer certain advantages when compared with the limited release of oligosaccharides achieved with digestion by endoglycosaminidase H. However, de-N-acetylation of

Fig. 2. Time courses of synthesis of mannose-labelled trichloroacetic acid-soluble, trichloroacetic acid-insoluble, lipid-linked and protein-bound products formed by glucose-deprived cells

Mouse L-cells grown in the presence of a glucose-containing medium (Birch & Pirt, 1970) were incubated with D-[1-³H]mannose in normal medium with glucose added (filled symbols, Fig. 2a), whereas those grown in medium containing no glucose for several hours were incubated with D-[1-³H]mannose in glucose-free medium (open symbols, Fig. 2a). Cells were washed and extracted with 10% trichloroacetic acid to determine incorporation into trichloroacetic acid-soluble pools (■ and □) and trichloroacetic acid-insoluble fractions (▲ and △). In a parallel experiment using cells grown in the absence of glucose for 6 h (Fig. 2b) incorporation of D-[1-³H]mannose into mild-acid-labile lipid-linked (●) and hydrazinolysis-labile protein-bound (○) oligosaccharide fractions was determined. Unlabelled mannose (10 mM) was added after 60 min as a cold chase to observe lipid-linked saccharide turnover.

Fig. 3. Bio-Gel-P4 gel-filtration analysis of lipid-linked saccharides formed by glucose-depleted cells

Mild-acid hydrolysates of lipid-linked saccharide fractions from the experiment shown in Fig. 2(b) were separated by Bio-Gel-P4 (–400 mesh) permeation chromatography (for chromatography details see the Experimental section). (a), (b), (c) and (d) represent lipid-linked saccharides formed by cells during 10, 20, 30 and 60 min pulses respectively with D-[1-³H]mannose, and (e) (f) and (g) represent saccharides formed after 10, 20 and 60 min chases respectively with unlabelled 10 mM-mannose.

comparing the sizes of lipid-linked and protein-bound saccharides in such experiments. A sugar analysis of the protein-bound label showed that more than 90% remained as mannose during these experiments and a substantial proportion of the mannose residues were susceptible to digestion with jack-bean α -mannosidase (results not shown).

Similar experiments in which glucose depletion was more severe produced the results shown in Figs. 5(a) and 5(b). Under these conditions, lipid-linked saccharides of a lower molecular weight than those seen previously *in vivo* accumulated. These lipid-linked saccharides also turned over during a subsequent chase for 60 min with 10 mM-mannose (Fig. 5a). However, there was no marked change in the size of 'high-mannose' protein-bound saccharides formed under these conditions, although an increased synthesis of 'complex' protein-bound oligosaccharides was observed during the chase period (fractions 30–55, Fig. 5b).

Cells pre-incubated in phosphate-buffered saline for 10 min before the addition of D-[1-³H]mannose behaved in a similar manner to glucose-depleted cells in forming lipid-linked saccharides of a smaller size than seen in actively dividing cells (Fig. 6). Such cells incorporated D-[1-³H]mannose into lipid-linked saccharides in a sequential manner, and within 1 min several lipid-linked saccharides of low molecular weight were synthesized (Fig. 6a). By 5 min, lipid-linked saccharides that corresponded in size to those seen previously (Fig. 5a) began to accumulate. This sequential synthesis of saccharides of a progressively higher molecular weight suggests an elongation process *de novo*. Higher lipid-linked saccharides, which accumulated under normal conditions (Fig. 3), were present in decreased amounts relative to the lower intermediate-sized lipid-linked oligosaccharides, even after incubation for 20 min (Fig. 6c). All the lipid-linked saccharides that accumulated during both normal and glucose-free incubations were retained by DEAE-cellulose (results not shown).

Prolonged glucose depletion (up to 24 h) also resulted in decreased efficiency of lipid-linked oligosaccharide assembly from D-[1-³H]mannose as shown in Fig. 5(c). Only the lowest group of lipid-linked saccharides accumulated within 30 min labelling time. However, the formation of protein-bound 'high-mannose' chains proceeded quite efficiently (Fig. 5d), although the average size of such chains was distinctly smaller (eluting at fractions 60–70) than that observed normally (Fig. 5b, fractions 52–62).

Polyribosomal intermediates in glycoprotein synthesis in L-cells

In parallel experiments using D-[1-³H]mannose to label glucose-depleted cells, a total polyribosome

susceptible sugar residues within such protein-bound saccharides may produce saccharides of a lower apparent size than fully acetylated saccharides on gel filtration, and this should be borne in mind when

Fig. 4. *Bio-Gel-P4* filtration analysis of protein-bound saccharides formed by glucose-depleted cells. Protein-bound saccharides were released by hydrazinolysis of the D-[1-³H]mannose-labelled chloroform/methanol/water (10:10:3, by vol.)-insoluble residues from the experiment shown in Fig. 2(b). Repeated evaporation of the hydrazinolysate in a stream of N₂ was followed by gel filtration using Bio-Gel-P4, as described in the Experimental section. (a)–(d) represent protein-bound saccharides formed by the cells during 10, 20, 30 and 60 min pulses respectively with D-[1-³H]mannose, and (e) and (f) represent saccharides formed after 10 and 30 min chases respectively with unlabelled 10 mM-mannose.

fraction was isolated as described in the Experimental section. Sucrose-gradient analysis of the labelled polyribosomal species (Fig. 7) showed that D-[1-³H]mannose labelling was associated only with the higher polyribosomes and not with mono-ribosomes, suggesting that at least some mannose residues are transferred co-translationally to nascent proteins under these conditions, and that it is the polyribosomal species, active in protein synthesis, that act as acceptors. The size and heterogeneity of such polyribosome-bound saccharides requires further investigation.

Formation of lipid-linked and protein-bound oligosaccharides by cell-free membrane preparations

The assembly of both lipid-linked oligosaccharides and protein-bound 'high-mannose' oligosac-

charides was also investigated by using cell-free membrane preparations isolated from L-cell cultures. Such preparations, which lack the capacity for protein synthesis, should, however, retain activities that are not associated with simultaneous protein synthesis (i.e. pre- and post-translational processes).

By using similar techniques to those described previously (Bailey *et al.*, 1979) membrane preparations were tested for their ability to form polyisoprenyl diphosphate derivatives from UDP-*N*-acetyl-D-[6-³H]glucosamine (in the presence and absence of unlabelled GDP-mannose), from GDP-D-[U-¹⁴C]mannose alone or from UDP-D-[6-³H]-glucose alone. Oligosaccharides released from these DEAE-cellulose-purified lipids by mild acid hydrolysis were separated by Bio-Gel-P4 gel filtration (Fig. 8). The relative elution volumes of the major

Fig. 5. Lipid-linked and protein-bound saccharides formed by L-cells after prolonged glucose depletion

L-cells were grown for 12 h (a and b) and 24 h (c and d) in glucose-free medium, and were subsequently labelled in fresh glucose-free medium at a concentration of approx. 5×10^6 cells/ml with D-[1- 3 H]mannose (1 μ Ci/ml), for 60 min (a and b) and 30 min (c and d) in a total volume of 10 ml. For cells grown in glucose-free medium for only 12 h, and subsequently labelled with D-[1- 3 H]mannose for 60 min, parallel incubations were continued for a further 60 min in the presence of 10 mM-mannose (O, a and b). Lipid-linked oligosaccharides were released from 0.5% (w/v) KCl-washed chloroform/methanol/water (10:10:3, by vol.) extracts of these cells by mild acid hydrolysis, and protein-bound oligosaccharides were released by hydrazinolysis of the residual insoluble material (see the Experimental section). The oligosaccharides thus released were analysed by Bio-Gel-P4 gel filtration. Results for lipid-linked saccharides are shown in (a) and (c), and those for protein-bound saccharides are shown in (b) and (d). Approximate elution volumes of 'complex' and 'high-mannose' protein-bound saccharides (derived from Robbins *et al.*, 1977b) are indicated in (b).

oligosaccharides labelled from GDP-mannose and UDP-glucose are indicated above the profiles by the letters M and G respectively. To obtain an exact correlation of their elution volumes, a separate chromatographic run was performed in which 3 H-labelled glucosaccharides and 14 C-labelled mannosaccharides were mixed before chromatography (results not shown).

The higher lipid-linked oligosaccharides formed by the membranes from GDP-D-[U- 14 C]mannose alone (Fig. 8c) appear similar in size to those formed from UDP-N-acetyl-D-[6- 3 H]glucosamine in the presence of unlabelled GDP-mannose (Fig. 8d), suggesting that most, if not all, the mannose-containing oligosaccharides possess N-acetylglucosamine or chitobiose at their reducing ends. However, the glucosylated oligosaccharides are clearly higher in molecular weight than the major mannosaccharide (Fig. 8f). This is consistent with an

elongation of the mannosylated oligosaccharides by terminal glucose units, as reported by Robbins *et al.* (1977a).

Products formed during incubations of membrane preparations with UDP-N-acetyl-D-[6- 3 H]glucosamine, GDP-D-[U- 14 C]mannose and UDP-D-[6- 3 H]glucose were extracted with chloroform/methanol/water (1:1:0.3, by vol.) and the insoluble polymeric material remaining was degraded by hydrazinolysis, a procedure that breaks peptide linkages, while leaving glycosidic bonds intact (Heath & Northcote, 1971). The water-soluble oligosaccharides released by hydrazinolysis (which would include both protein-linked saccharides and true polysaccharides) were then separated by Bio-Gel-P4 gel filtration (Fig. 8). The polymeric products formed during membrane incubations with both UDP-N-acetyl-D-[6- 3 H]glucosamine and GDP-D-[U- 14 C]mannose (Figs. 10b and 10e) bear rela-

Fig. 6. Lipid-linked saccharides formed by L-cells after PBS-preincubation

L-cells grown in the presence of glucose-containing medium were harvested by centrifugation, washed once with phosphate-buffered saline, and resuspended in phosphate-buffered saline at a concentration of approx. 5×10^6 cells/ml. D-[1- 3 H]-Mannose was added at $1 \mu\text{Ci/ml}$ and cells were incubated with D-[1- 3 H]mannose for 1, 5 and 20 min. Lipid-linked oligosaccharides were prepared and separated by Bio-Gel-P4 gel filtration as described in the Experimental section. Results are

tively large amounts of low molecular weight hydrazinolysis-labile saccharides in addition to smaller amounts of 'high-mannose' oligosaccharides (eluting at fractions 52–65). Both substrates also label a variable amount of high-molecular-weight material. The latter does not correspond to the 'complex' oligosaccharide fraction seen during experiments *in vivo* (see, for example, Fig. 5b and Robbins *et al.*, 1977b), which is more heterogeneous in size, and probably represents small amounts of undegraded glycoprotein material.

In marked contrast, the protein-bound saccharides formed during membrane incubations with UDP-D-[6- 3 H]glucose (Fig. 5c) are much more heterogeneous. It is clear that glucose residues are donated to acceptors of widely differing molecular weight in the membranes, most remaining at

Fig. 7. Analysis of D-[1- 3 H]mannose-labelled polyribosome species formed by glucose-depleted L-cells

L-cells grown in the absence of glucose for 12 h were labelled with D-[1- 3 H]mannose ($1 \mu\text{Ci/ml}$) in glucose-free medium for 60 min. A total polyribosomal fraction (containing both free and membrane-bound species) was isolated as described in the Experimental section, and analysed on 15–40% (w/v) sucrose gradients. Fractions were analysed for A_{260} (■) and radioactivity (●). Fractions are numbered sequentially from the top to the bottom of the gradient.

shown for the respective time points in (a), (b) and (c). The labelled oligosaccharides were assembled in sequence in groups, indicated by (A), (B) and (C).

Fig. 8. Lipid-linked and protein-bound saccharides formed from UDP-*N*-acetyl-D-[6-³H]glucosamine, GDP-D-[U-¹⁴C]mannose and UDP-D-[6-³H]glucose by isolated L-cell membrane preparations

L-cell membrane preparations were incubated at room temperature for 60 min with UDP-*N*-acetyl-D-[6-³H]glucosamine (3 μ M; *a*, *b* and *d*), GDP-D-[U-¹⁴C]mannose (20 μ M; *c* and *e*) or UDP-D-[6-³H]glucose (9 μ M; *f* and *g*) in the presence of 0.1 M-Tris/HCl, pH 7.5, containing 5 mM-dithioerythritol, 10 mM-MgCl₂ and 0.4 M-sucrose. Unlabelled GDP-mannose (10 mM) was added to the incubation shown in (*d*) for an additional 30 min after 60 min incubation with UDP-*N*-acetyl-D-[6-³H]glucosamine. Bio-Gel-P4 gel-filtration analyses of the lipid-linked saccharides [released by mild acid hydrolysis of DEAE-cellulose purified, chloroform/methanol/water (10:10:3, by vol.)-soluble, polyisoprenyl diphosphate lipids] formed under these conditions are shown in (*a*), (*c*), (*d*) and (*f*), and protein-bound saccharides [released by hydrazinolysis of the chloroform/methanol/water (10:10:3, by vol.)-insoluble residues] are shown in (*b*), (*e*) and (*g*). The major lipid-linked saccharide formed during incubations with GDP-D-[U-¹⁴C]mannose is designated M (*c*) and the major saccharides formed from UDP-D-[6-³H]glucose are designated G₁ and G₂ (*f*). Elongation of *N*-acetylglucosamine-initiated lipid-linked saccharides in the presence of GDP-mannose produced three groups of saccharides of increasing size, shown as (A), (B) and (C) in (*d*). Chromatographic details are as described in the Experimental section.

relatively high molecular weight after hydrazinolysis, suggesting them to be polysaccharide in nature. In this context, several forms of membrane-bound glycogen synthase exist in mammalian cells (Hers, 1976) and it is thought that a glycoprotein acceptor is involved in the initial events of glycogen synthesis (Whelan, 1976). The presence of such a polysaccharide-synthesizing enzyme system in L-cell membranes may explain the abundance of the hydrazinolysis-resistant products formed after incubation of such membrane preparations with UDP-D-[6-³H]glucose.

A closer examination of the process of sequential addition of mannose units to the *N*-acetylglucosamine-initiated lipid-linked saccharides was undertaken. Membranes were incubated for 10 min with UDP-*N*-acetyl-D-[6-³H]glucosamine, followed by the addition of GDP-mannose (10 mM) to the incubations for periods ranging from 60 s to 50 min. Time courses of lipid-linked saccharide assembly and glycoprotein product formation from UDP-*N*-acetyl-D-[6-³H]glucosamine in the presence and absence of unlabelled GDP-mannose are shown in Fig. 9. The lipid-linked oligosaccharides formed by

Fig. 9. Time courses of synthesis of lipid-linked and protein-bound saccharides formed from UDP-*N*-acetyl-D-[6-³H]glucosamine by isolated L-cell membrane preparations

L-cell membrane preparations were incubated at room temperature with UDP-*N*-acetyl-D-[6-³H]glucosamine (3 μM), in the presence (●) or absence (O) of unlabelled GDP-mannose (10 mM, added at 10 min, indicated by arrows). Lipid-linked saccharides, extracted with chloroform/methanol/water (10:10:3, by vol.), were washed with 0.5% (w/v) KCl and directly hydrolysed with mild acid. Lipid-extracted residues were subjected to hydrazinolysis (see the Experimental section) and repeatedly evaporated from water to remove excess hydrazine. Portions were counted to determine total incorporation of radioactivity into lipid-linked saccharide fractions (a) and protein-bound saccharide fractions (b).

60 min with UDP-*N*-acetyl-D-[6-³H]glucosamine alone, produced a profile (Fig. 10a) in which a dominant lipid-linked oligosaccharide was formed. The elution volume of this saccharide, on both Bio-Gel-P2 (Bailey *et al.*, 1979) and Bio-Gel-P4, together with the work of others using related techniques (Lucas *et al.*, 1975; Chen *et al.*, 1975), suggest it to be chitobiose. The minor components of higher molecular weight than the dominant oligosaccharide may represent *N*-acetylglucosamine labelled components that have been elongated by trace amounts of GDP-mannose or dolichyl monophosphate mannose endogenous to the membrane preparation rather than oligosaccharides containing only *N*-acetylglucosamine.

Addition of unlabelled GDP-mannose to the UDP-*N*-acetyl-D-[6-³H]glucosamine-initiated incubations for periods as short as 60 s before terminating the reactions by the addition of chloroform/methanol resulted in the generation of a complex mixture of higher oligosaccharides at the expense of the initially-formed *N*-acetylglucosamine-containing saccharides (Fig. 10b). The elongation reaction observed was essentially complete within 5 min and no further change in the relative abundance of the major peaks was observed at later times (Figs. 10c and 10d). In all such experiments, three groups of oligosaccharides of a characteristic intermediate size (labelled A, B and C; Fig. 10c) were seen to accumulate. Their elution volumes corresponded closely to those of similar lipid-linked oligosaccharides formed during labelling experiments *in vivo* (for example, Fig. 6), suggesting that the assembly processes observed during such incubations *in vitro* reflect those that occur *in vivo*.

In experiments in which the elongation of *N*-acetylglucosamine-initiated saccharides was impaired by lowering the concentration of GDP-mannose added to the UDP-*N*-acetyl-D-[6-³H]glucosamine-initiated incubations, several new intermediate-sized oligosaccharides could be observed (bracketed as peak A; Figs. 10e and 10f). These did not accumulate under the conditions used in previous experiments in which GDP-mannose concentrations were not limiting.

Discussion

The present study of glycoprotein biosynthesis in mouse L-cells has investigated the assembly of lipid-linked oligosaccharides containing mannose, *N*-acetylglucosamine and glucose both *in vivo*, using intact cells labelled under different physiological conditions, and *in vitro*, using isolated membrane preparations supplied with nucleotide sugars. In intact cells supplied with normal concentrations of glucose, the formation of protein-bound 'high-mannose' oligosaccharides from exogenous D-[1-³H]man-

elongation of the *N*-acetylglucosamine-initiated species were analysed by Bio-Gel-P4 gel filtration. Control incubations in which no GDP-mannose was added, but in which incubation was continued for

Fig. 10. Bio-Gel-P4 gel filtration analysis of lipid-linked saccharides formed from UDP-N-acetyl-D-[6-³H]glucosamine by isolated L-cell membrane preparations

Mild-acid hydrolysates of lipid-linked saccharide fractions from the experiment shown in Fig. 9 were separated by Bio-Gel-P4 gel filtration (see the Experimental section for chromatographic details). (a) Lipid-linked saccharides formed during control incubations with UDP-N-acetyl-D-[6-³H]glucosamine alone for 60 min. (b) Lipid-linked saccharides formed during incubations with UDP-N-acetyl-D-[6-³H]glucosamine for 10 min, followed by addition of unlabelled GDP-mannose (10 mM) for a further 60 s. (c) as (b), except that incubation was for 5 min with GDP-mannose. (d) As for (b), except that incubation was for 20 min with GDP-mannose. Lipid-linked saccharides formed in parallel experiments in which incubation with UDP-N-acetyl-D-[6-³H]glucosamine alone for 10 min was followed by incubation with 1 μ M and 10 μ M unlabelled GDP-mannose for a further 60 min are shown in (e) and (f) respectively. (A), (B) and (C) above the profiles designate the groups of oligosaccharides characteristically produced during such elongation reactions (see also Fig. 8d).

nose is accompanied by the synthesis of high-molecular-weight lipid-linked 'G'-oligosaccharides, analogous to those previously reported by other workers (Fig. 3; Robins *et al.*, 1977b). However, under conditions of glucose deprivation, the synthesis of such high-molecular-weight lipid-linked oligosaccharides is inhibited, and those of a lower size begin to accumulate (Fig. 5a). When glucose deprivation is severe, only the lowest lipid-linked saccharides are synthesized from D-[1-³H]mannose during a 30 min labelling period (Fig. 5c). These intermediate-sized lipid-linked oligosaccharides may represent precursors of the normal G-oligosaccharide of such cells. The fact that they are biosynthetic intermediates is suggested by their turnover in the presence of unlabelled 10 mM-mannose (Fig. 5a).

Studies *in vitro* with N-acetylglucosamine-initiated lipid-linked oligosaccharides have demonstrated that their elongation with further mannose residues is dependent on the concentration of GDP-mannose present during such incubations (Fig.

10). The size of such intermediates assembled in the presence of 10 mM-GDP-mannose (Fig. 10d) is quite close to that of those synthesized from UDP-glucose *in vitro* (Fig. 8f) and D-[1-³H]mannose *in vivo* (Fig. 3). Earlier observations of distinct differences in size between lipid-linked oligosaccharides assembled from GDP-D-[U-¹⁴C]mannose *in vitro* and those assembled from D-[1-³H]mannose *in vivo* might be explained by the presence in the incubations of GDP-mannose at concentrations that were inadequate for the complete elongation of mannose-containing chains, as well as an absence of terminal glucosylation events (discussed by Speake & White, 1978). Recent findings with rat enterocytes (Herscovics & Bugge, 1980) suggest that such differences may also arise as a consequence of varying activities of GDP-mannose-degrading enzymes, which may decrease GDP-mannose concentrations during incubations *in vitro*, resulting in incomplete synthesis of the highest lipid-linked saccharides. These results suggest that the ability of cells to assemble such lipid intermediates is critically dependent on the intra-

cellular concentration of GDP-mannose. Lowered concentrations of GDP-mannose will result in the assembly of smaller lipid-linked oligosaccharides.

Animal cells grown in suspension culture initially synthesize mainly 'high-mannose'-type protein-bound oligosaccharides when supplied with D-[1-³H]mannose (Robbins *et al.*, 1977b). Earlier results using cells infected with wild-type vesicular-stomatitis virus (Kornfeld *et al.*, 1978) as well as more recent results using temperature-sensitive vesicular-stomatitis-virus mutants blocked in intracellular transport (Zilberstein *et al.*, 1980) suggest that such oligosaccharides represent the first of a series of protein-bound intermediates. Processing of initial high-molecular-weight oligosaccharides, donated co-translationally from lipid carriers to nascent protein chains while the latter are being transferred across the membrane of the rough endoplasmic reticulum, results in the formation of a protein-bound 'core' oligosaccharide, which may subsequently be elaborated to form 'complex'-type side chains (see Fig. 1 and Staneloni & Leloir, 1979). A central feature of this hypothesis is that the complex sequence of mannosyl transfer reactions involved in the assembly of both protein-bound 'high-mannose' side chains and the trimmed 'core' region of 'complex' side chains takes place while these saccharides are lipid-bound, emphasizing the importance of the correct assembly of such lipid intermediates. The synthesis of such protein-bound 'high-mannose' saccharide chains concomitant with vectorial segregation of the nascent proteins within the endoplasmic reticulum may be one of several processes that control the subsequent post-translational modification, intracellular transport and enzymic activation of such segregated proteins (Gibson *et al.*, 1980; Hasilik, 1980).

Trowbridge & Hyman (1979) have observed an abnormal lipid-linked oligosaccharide involved in the biosynthesis of glycoproteins in class E Thy-1⁻ mutant mouse lymphoma cells. Characterization of this intermediate (Chapman *et al.*, 1979) has shown that it corresponds to a biosynthetic precursor of the normal lipid-linked G-oligosaccharide of Chinese hamster ovary cells and has the structure Man₅GlcNAc₂, whereas characterization of the processing intermediates shows that the initial size of the protein-bound 'high-mannose' chains is decreased in these mutants compared with those of normal cell lines (Kornfeld *et al.*, 1978). Our results may be similarly interpreted: the assembly of smaller lipid-linked saccharides during glucose deprivation is accompanied by the appearance of less elaborate protein bound-saccharides (Figs. 5c and 5d). There have been many reports of assembly and transfer of small lipid-linked saccharides *in vitro*, for example the transfer of β -mannosyl-chitobiose from dolichyl diphosphate *N*-acetylglucosaminyl-*N*-acetylglucos-

aminylmannose to endogenous membrane-protein acceptors (Chen & Lennarz, 1976). The apparently wide specificity *in vitro* of the glycosyltransferase responsible for the transfer of saccharides from lipid intermediates to protein acceptors may thus reflect a physiologically relevant process.

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