Formation of Lipid Oligosaccharides in a Tunicamycin Resistant Mutant of Chinese Hamster Ovary Cells

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The synthesis of lipid oligosaccharide intermediates of a tunicamycin resistant mutant of Chinese hamster ovary cells was investigated both *in vivo* and *in vitro*. It was shown that tunicamycin resistant mutant cells had a significantly lower rate of synthesis of lipid oligosaccharide *in vivo* and their pool of lipid oligosaccharide was smaller.

The formation of lipid oligosaccharides *in vitro* using microsomes, however, did not show any defect in the enzymes which were involved in this reaction. The rate of transfer of [^3H]glucose from UDP-[^3H]glucose into oligosaccharide of lipid intermediates was the same in wild type and mutant cells while that into glycoproteins was higher in mutant cells than in wild type cells.

All high-mannose and complex-asparagine-linked oligosaccharides have in common a core structure of pentasaccharides.^{2,3)} Recent studies have shown that the core structure is synthesized as a lipid-linked precursor, a dolichol-P-P-GlcNAc₂Man₉Glc₃, and this intermediate is transferred *en bloc* to asparagine residues of nascent polypeptides.^{4,18)} The chemical structure of the lipid oligosaccharides has been conserved from yeast to mammalian cells.^{5~7)} However, little is known about the enzymes involved in the synthesis of lipid oligosaccharides and the regulation of their synthesis in intact cells except the microsomal localization of the biosynthetic enzymes.^{8~12)}

Tunicamycin resistant (TM^R) cells, obtained in this laboratory¹⁶⁾ offer a feasible genetic system to examine the regulatory mechanism of glycoprotein biosynthesis. Tunicamycin is an antibiotic which specifically inhibits the first step in the synthesis of lipid oligosaccharides by the following reaction:

TM^R cells were assumed to have some defects or modification in the biosynthesis and/or regulation of lipid oligosaccharides. As reported previously, TM^R cells incorporate less radioactive glucosamine into glycoproteins.^{1,16)} In this paper, we show the decreased rate of synthesis of lipid oligosaccharides at the cellular level. However, the decreased rate was not due to the defect in the enzymes which were involved in the synthesis of lipid oligosaccharide.

MATERIALS AND METHODS

Cells. The wild type CHO cells were obtained from Dr. L. Siminovitch, the Hospital for Sick Children, Toronto, Canada and are auxotrophic for glycine, adenosine, and thymidine and are designated Gat-CHO (B-2). The wild type and mutant cells were routinely maintained as monolayer cultures in α -MEM medium supplemented with 10% fetal calf serum and antibiotics (streptomycin and kanamycin), with passages every three days. The tunica-

Abbreviations: TM, tunicamycin; CHO, Chinese hamster ovary; TM^R, tunicamycin resistant; Dol-P, dolichol phosphate; GlcNAc, N-acetylglucosamine; Man, mannose; Glc, glucose; UDP, uridine diphosphate; GDP, guanosine diphosphate.

mycin resistant cell line used in this study was clone TM^R 205.

Radioactive labeling of cells. Wild type and TM^R cells were grown to a density of $1 \sim 2 \times 10^6$ cells/plate (60 mm diameter) and they were exposed to either to 0.1 mCi/ml of [³H]glucosamine or to 0.05 mCi/ml of [³H]mannose in a medium containing 1 mM glucose. At the times indicated for each experiment, the radioactive medium was removed. Cells were then scraped and extracted with chloroform-methanol-4 mM MgCl₂ (3:2:1 by volume) immediately.

Preparation of microsomes. Wild type and mutant cells were grown in spinner culture flasks in a water bath at 37°C. The concentration of cells was $3 \sim 5 \times 10^5$ cells/ml. Approximately 2×10^8 cells were used in this study for each microsomal preparation.

Cells were harvested by centrifugation at $1000 \times g$ for 10 min. The cell pellet was washed by resuspending in 20 mM Tris-HCl, 0.15 M NaCl, pH 7.4 and centrifuged at $1000 \times g$ for 10 min at room temperature. The cell pellet was resuspended in a small volume of Tris-buffered saline ($2 \sim 4 \times 10^7$ cells/ml) by gentle pipetting and added dropwise to 9 volumes of 20 mM Tris-HCl, pH 7.4 at 4°C and left to swell for 20 min at 4°C. The cells were broken by 20~25 manual strokes by a tight fitting Dounce homogenizer and 0.1 volume of 30 mM MgCl₂ and 0.1 M NaCl was added. Unbroken cells and nuclei were removed by centrifugation at $1000 \times g$ for 5 min. The resulting supernatant was collected and centrifuged for 1 hr at 4°C at $100,000 \times g$. The membranous pellet was resuspended in Tris-buffered saline and the protein content was measured. Finally, the suspension of microsomes was adjusted to a concentration of 10 mg protein/ml and used as an enzyme solution.

Measurement of enzyme activity.

Preincubation: To a sample of 0.03 ml of the membrane suspension, GDP-mannose, MgCl₂, and MnCl₂ were added in 20 mM Tris-HCl, pH 7.4, and 0.05 M NaCl to a final concentration of 0.72 μM, 0.46 mM, and 0.45 mM, respectively, in a final volume of 0.035 ml. This mixture was then incubated at 37°C for 20 min.

Assay: The mixture was then diluted with assay components to a final volume of 0.08 ml and the incubation was continued for various times at 37°C. The final concentrations in the assay mixture were 0.4 mM MgCl₂, 0.4 mM MnCl₂, 20 mM Tris-HCl (pH 7.4), 0.15 M NaCl, and various nucleotide sugars, 75 μM UDP-[³H]*N*-acetylglucosamine for the assay of *N*-acetylglucosaminyl transferases, 75 μM UDP-*N*-acetylglucosamine and 2.0 μM GDP-[¹⁴C] mannose for the assay of mannosyltransferases, and 75 μM UDP-*N*-acetylglucosamine, 2.0 μM GDP-mannose, and 1.8 μM UDP-[³H]glucose for the assay of glucosyltransferases. The mixture contained 0.14 mM dolichol phosphate when mentioned. The reaction was

stopped by the addition of 20 volumes of chloroform-methanol (2:1, v/v).

Extraction of lipid oligosaccharides. Lipids were extracted by the method of Hubbard and Robbins.¹⁷⁾ The materials which were extracted with chloroform-methanol-4 mM MgCl₂ (3:2:1 by volume) and chloroform-methanol-water (10:10:3 by volume) were combined.

Mild acid hydrolysis. The lipid oligosaccharide fractions were pooled and transferred to a screw-cap tube. The solvent was evaporated at 30~40°C under a stream of nitrogen. The residue was resuspended in 1.2 ml of 0.01 N HCl in 50% propanol and the tube was capped. After 20 min at 100°C, the tube was cooled and lipid-free oligosaccharides were obtained after partition.

Gel filtration. Bio-Gel P-4 column (200~400 mesh, 1×120 cm, Bio Rad) was used to resolve the oligosaccharides. The elution buffer was 0.1 M Tris-HCl (pH 8.0) containing 0.5 mM sodium azide or 0.1 M ammonium bicarbonate (pH 8.0). The oligosaccharides migrated in the same position in both solvents. Samples were put on the column in a volume of 200 μl and 0.45 ml fractions were collected. Markers for exclusion (500 μg of bovine serum albumin) and inclusion (1000 cpm of [³H]mannose) volumes were added in each run. The column was calibrated with oligosaccharide markers kindly provided by Dr. C. Hubbard of M.I.T., Cambridge, Mass., U.S.A.

Chemicals. 1,6-[³H]Glucosamine-HCl (6.6 Ci/mmol), D-2-[³H]mannose (16 Ci/mmol), UDP-D-[6-³H]glucose (8.6 Ci/mmol), UDP-[U-¹⁴C]glucosamine (60.8 mCi/mmol) were purchased from Amersham. UDP-D-[6-³H(N)]*N*-acetyl-D-glucosamine (24 Ci/mmol), GDP-[U-¹⁴C]mannose (269 mCi/mmol), and UDP-[U-¹⁴C]*N*-acetyl-D-glucosamine (306 mCi/mmol) were purchased from New England Nuclear. Dolichol phosphate (Grade 3), *N,N*-diacetylchitobiose, UDP-*N*-acetylglucosamine, and GDP-mannose were purchased from Sigma Chemicals Co.

RESULTS

Incorporation of [³H]mannose and [³H]glucosamine into lipid oligosaccharides and glycoproteins

To compare the formation of lipid oligosaccharides in TM^R cells with wild type cells, both were labeled with [³H]mannose for 30 min. As was shown in Table I, TM^R cells incorporated less radioactivity into lipid oligosaccharide than wild type, while incorporation of [³H]mannose into glycoproteins in TM^R cells

TABLE I. DISTRIBUTION OF RADIOACTIVITY IN CELLS LABELED WITH $[^3\text{H}]\text{MANNOSE}$ AND $[^3\text{H}]\text{GLUCOSAMINE}$

$[^3\text{H}]$	Cells	Lipid	
		Oligosaccharide	Glycoproteins
Mannose	Wild type	20.8	2.32
	TM ^R cells	2.75	2.02
Glucosamine	Wild type	1.32	5.19
	TM ^R cells	0.21	1.27

Unit: $\times 10^{-1}$ cpm. Values were the average of six culture.

The extraction procedure is described in MATERIALS AND METHODS.

FIG. 1 (a) AND (b). Bio-Gel P-4 Chromatogram of Lipid Oligosaccharides Isolated from Wild Type (a) and TM^R205 (b) Labeled with $[^3\text{H}]\text{mannose}$ for 30 min.

Lipid oligosaccharides were extracted, hydrolyzed, and chromatographed as described in MATERIALS AND METHODS. The markers from the left are 1) $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$, 2) $\text{Man}_5\text{GlcNAc}_2$.

was 80~90% of the wild type level. Since the total uptake of $[^3\text{H}]\text{mannose}$ into TM^R cells was similar to that into wild type cells¹⁾ and the incorporation of radioactivity into the lipid oligosaccharide fraction reached a plateau within 10 min in both types of cells, the data were assumed to represent the decreased amount of lipid oligosaccharide in TM^R cells. On the other hand, when the cells were labeled with $[^3\text{H}]\text{glucosamine}$, TM^R cells incorporated less radioactivity not only into lipid oligosaccharide but also into the glycoprotein fraction (Table I).

Analysis of sugar moieties of lipid oligosaccharides on Bio-Gel P-4 column

Lipid oligosaccharides extracted from

$[^3\text{H}]\text{mannose}$ labeled cells were hydrolyzed with mild acid and the released oligosaccharide moieties were fractionated on a Bio-Gel P-4 column. The chromatographic profile of wild type cells is shown in Fig. 1a. Most of the radioactivity was found in a peak migrating in the position of the standard marker $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ with a K_d of 0.280 (K_d value = $(V_e - V_o)/(V_i - V_o)$, V_e ; elution volume, V_o ; void volume, V_i ; internal volume). A minor peak with a K_d of 0.449 was also detected. By its K_d value, this compound is $\text{Man}_5\text{GlcNAc}_2$.

The chromatographic pattern of the sample obtained from the mutant is shown in Fig. 1b. The peaks are $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ (K_d ; 0.280), $\text{Man}_8\text{GlcNAc}_2$ (K_d ; 0.368), and $\text{Man}_5\text{GlcNAc}_2$ (K_d ; 0.449). These oligosaccharides were further characterized by endo- β -N-acetylglucosaminidase H and α -mannosidase treatment, indicating the structure of lipid oligosaccharides of TM^R cells is identical to that of wild type cells (data not shown).

Since GlcNAc -lipid and $(\text{GlcNAc})_2$ -lipid could not be detected as lipid oligosaccharide intermediates in $[^3\text{H}]\text{mannose}$ labeled cells, both types of cells were labeled with $[^3\text{H}]\text{glucosamine}$ and the sugar moieties of lipid intermediates were analyzed. The pool size of GlcNAc -lipid, $(\text{GlcNAc})_2$ -lipid and the other intermediates shorter than $(\text{Man})_5(\text{GlcNAc})_2$ -lipid seemed to be so small that most of the counts was incorporated rapidly into the longer lipid oligosaccharides

FIG. 2. (a). Bio-Gel P-4 Chromatogram of lipid Oligosaccharides from Wild Type and TM^R Cells Labeled with $[^3H]$ Glucosamine for 3 min.

Extracted oligosaccharides were hydrolyzed and chromatographed on Bio-Gel P-4 (minus 400 mesh). The markers from the left are $Glc_3Man_9GlcNAc_2$, $Man_5GlcNAc_2$ and Man. Wild type (—●—), TM^R205 (—○—).

in both types of cells. Most of the counts was recovered in $(Man)_5(GlcNAc)_2$ -lipid within 3 min both in wild type and TM^R cells (Fig. 2a). In wild type cells, a minor peak in the position of $Glc_3Man_9GlcNAc_2$ was also detected. In the mutant cells the peak of this compound was scarcely detectable. There was no great difference in the amount of other shorter intermediates.

Pulse chase experiment on the formation of lipid oligosaccharides

To measure the turnover of the lipid oligosaccharides, a pulse chase experiment was done. Both types of cells were labeled for 3 min with $[^3H]$ glucosamine and cells were washed and reincubated with MEM medium supplemented with 10% fetal calf serum. Cells were harvested several times and lipid oligosaccharides which were extractable with chloroform-methanol-water (10:10:3 by volume) were prepared. As shown in Fig. 2b, incorporation of radioactivity into lipid oligosaccharides proceeded for 10 min in wild type cells, while in TM^R cells, the formation reached a plateau after 3 min.

The results of the pulse chase experiment demonstrate that in mutant cells the pool size of lipid oligosaccharide intermediates was smaller and the formation of $(Glc)_3(Man)_9$ -

FIG. 2 (b). Pulse Chase Experiment on the Formation of $[^3H]$ Glucosamine Labeled Lipid Oligosaccharides.

After both types of cells were labeled with $[^3H]$ glucosamine for 3 min, they were washed and reincubated with the medium without $[^3H]$ glucosamine for 0, 3, 10 and 30 min. Wild type (—●—), TM^R205 (—○—).

$(GlcNAc)_2$ -lipid was reduced dramatically compared to wild type cells.

Formation of glucosamine containing lipid oligosaccharides in vitro

One possible explanation for the significant reduction of lipid oligosaccharide synthesis in TM^R cells would be some defect in the enzymes involved in the synthesis. It has been reported that the microsomal fraction contains the enzyme activity for lipid oligosaccharide synthesis and glycosylation of proteins. Since tunicamycin inhibits the first step of the synthesis of lipid oligosaccharides, this reaction was compared using the microsomal fractions from both types of cells.

The profile of sugar moieties of lipid oligosaccharide obtained in 2 min of incubation with the microsomal fraction, UDP-*N*-acetylglucosamine and dolichol phosphate is shown in Fig. 3. Both types of cells showed similar profiles. Typical Michaelis-Menten kinetics with V_{max} values of 9.2 and 8.2×10^2 cpm/mg protein/10 min for wild type and mutant cells, respectively, was obtained. From their Lineweaver-Burk plots, the K_m values of $33.3 \mu M$ for wild type and $31.3 \mu M$ for mutant cells were calculated (data not shown). The

FIG. 3. Bio-Gel P-2 Chromatogram of Glucosamine Containing Lipid Oligosaccharide Synthesized *in vitro*.

Microsomal fractions from wild type (—○—) and TMR²⁰⁵ (—●—) cells were incubated with UDP-[³H]*N*-acetylglucosamine for 5 min in the presence of dolichol phosphate. Lipid oligosaccharides were extracted, hydrolyzed, and chromatographed. Markers from the left are *V*₀ (bovine serum albumin), *V*_c (*N,N*-diacetylchitobiose) and *V*_n (*N*-acetylglucosamine).

data implied that transferases with the same properties were working in both types of membranes.

To follow the conversion of GlcNAc-lipid and (GlcNAc)₂-lipid into lipid oligosaccharide a pulse chase experiment was done. The microsomal fraction was incubated with UDP-[³H]-acetylglucosamine for 5 min, and then a large excess of UDP-*N*-acetylglucosamine and GDP-mannose was added to the mixture. In a 5-min pulse, two peaks corresponding to *N*-acetylglucosamine and *N,N*-diacetylchitobiose were observed in both types of cells. After a 10-min chase, these precursors were further converted into oligosaccharide, Man_{1~7}GlcNAc₂ (data not shown). The membranes of mutant cells synthesized these compounds at a similar rate to the membranes of wild type cells. The result indicates that no significant difference in the process of conversion of GlcNAc-lipid and (GlcNAc)₂-lipid into oligosaccharides up to (Man)_{6~7}(GlcNAc)₂-lipid exists between membranes of wild type and mutant cells. Formation of mannose-containing oligosaccharide *in vitro* was also compared between wild type and the mutant cells. The microsomal fraction was incubated with UDP-*N*-acetyl-

glucosamine and GDP-mannose as was described in MATERIALS AND METHODS. No great difference was observed in the amount of the incorporation into lipid oligosaccharide and the profile of gel filtration. In addition, the rate of incorporation of mannose into protein by membranes was the same for both types (data not shown).

Formation of glucose-containing lipid oligosaccharides

The incorporation of [³H]glucose from UDP-[³H]glucose into the lipid fraction, lipid oligosaccharide, and protein was examined. The result is shown in Fig. 4. Incorporation of [³H]glucose into lipid and lipid oligosaccharides was the same in both types of membranes. In contrast, an increased rate of glycosylation of proteins by membranes of the mutant cells was observed. The oligosaccharide released from lipid oligosaccharide by mild acid hydrolysis was Glc₃Man₉GlcNAc₂. This implies that the final product in formation of lipid oligosaccharides was produced in the reaction and the transfer of oligosaccharide into protein proceeded.

DISCUSSION

It was found that TMR^R cells had a significantly decreased pool of lipid oligosaccharide. Although some defect in the synthesis of lipid oligosaccharide or their precursors affects the pool size of lipid oligosaccharide in the mutant, they can still provide sufficient sugar moieties for glycoprotein synthesis. In TMR^R cells the rate of glycosylation seems not to be controlled by the size of the lipid oligosaccharide pool but is dependent on some other factors, probably on the rate of synthesis of acceptor proteins themselves. The observed increase in the rate of transfer of oligosaccharide into acceptor polypeptide (Fig. 4) may be the secondary effect of that.

We did not find out why lipid oligosaccharide synthesis is decreased in the mutant cells since there was no reduction in the enzymatic activity involved in the synthesis of lipid

FIG. 4. Kinetics of Incorporation of $[^3\text{H}]$ Glucose from UDP- $[^3\text{H}]$ Glucose into Various Fractions.

The incubation was done as described in MATERIALS AND METHODS. The reaction mixture contained dolichol phosphate. Wild type (—○—) and TM^{R} cells (—▲—).

oligosaccharide. In addition, the first reaction which is a target of tunicamycin remained sensitive to tunicamycin.²¹⁾ Since a preliminary experiment suggested that the rate of synthesis of UDP-*N*-acetylglucosamine was the same in the mutant cells as in wild type cells (unpublished), we considered that the lesion of $\text{TM } 205^{\text{R}}$ is in a step after the synthesis of UDP-*N*-acetylglucosamine.

The localization and translocation of lipid oligosaccharide in the endoplasmic reticulum have been studied.^{19,20)} Hanover and Lennarz showed that $(\text{GlcNAc})_2$ -lipid is in the luminal phase of the endoplasmic reticulum¹⁹⁾ and they suggested the existence of pool of UDP-*N*-acetylglucosamine or its precursor in the luminal phase of endoplasmic reticulum.²¹⁾ If the same mechanism is operating in CHO cells, one possibility is that TM^{R} cells has the lesion in this system, for example, in the transport of UDP-*N*-acetylglucosamine across the endoplasmic reticulum. Such a lesion may cause the reduction in lipid oligosaccharide without any change in the enzymes involved in their synthesis and may explain the resistance of the mutant to tunicamycin because it is an ana-

logue of UDP-*N*-acetylglucosamine and may be translocated using the same transport system.

As is shown in Table I, the incorporation of $[^3\text{H}]$ glucosamine into glycoproteins in TM^{R} cells was approximately one fourth of wild type incorporation. The result is different from the data on $[^3\text{H}]$ mannose in which the incorporation of the radioactivity into glycoproteins in TM^{R} cells was 80~90% of wild type level. The discrepancy must be due to the difference of the pathways through which both compounds are incorporated into glycoproteins. $[^3\text{H}]$ Mannose is incorporated into glycoproteins *via* a lipid oligosaccharide intermediate. $[^3\text{H}]$ Glucosamine is incorporated into glycoproteins not only from lipid oligosaccharide but also directly from UDP-*N*-acetylglucosamine by terminal transferases. It is possible that the observed decrease in $[^3\text{H}]$ glucosamine in corporation into glycoproteins is the decrease in the terminal transfer of $[^3\text{H}]$ glucosamine.

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