The Primary Glycosylation Defect in Class E Thy-1-negative Mutant Mouse Lymphoma Cells Is an Inability to Synthesize Dolichol-P- Mannose*

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Thy-1" mutant mouse lymphoma cells of the class E complementation group are unable to synthesize the normal GlcManGlcNAc2 lipid-linked oligosaccharide, but instead accumulate a smaller lipid-linked species with the structure Man1 → 2Man1 → 2Man1 → 3(Man1 → 6)Manf1 → 4GlcNAcβ1 → 4GlcNAc (Trowbridge, I., and Hyman, R. (1979) Cell 17, 503-508 and Chapman, A., Trowbridge, I., Hyman, R., and Kornfeld, S. (1979) Cell 17, 509-515). The present study demonstrates that the primary defect in the Thy-1" cells is an inability to synthesize dolichol-P-mannose. Thus, intact Thy-1" cells incubated with [3H]mannose failed to incorporate any detectable radioactivity into dolichol-P-mannose and crude membrane preparations of Thy-1" cells were unable to transfer mannose from GDP-[3H]mannose to dolichol-P. However, these membrane preparations did incorporate [3H]mannose into lipid-linked oligosaccharides up to the size of Man6GlcNAc2, suggesting that these species are formed from mannosyl donors other than dolichol-P-mannose. When class E Thy-1" membrane preparations were incubated with exogenous dolichol-P-[3H]mannose, lipid-linked oligosaccharides ranging in size from Man6GlcNAc2 to Man9GlcNAc2 were formed. This result demonstrates that the mutant cells have the α1,3-mannosyltransferase necessary for the conversion of the Man4GlcNAc2 species to Man9GlcNAc2, but lack the appropriate mannosyl donor (dolichol-P-mannose) necessary for the formation of the larger lipid-linked oligosaccharides. We conclude that at least two mannosyl donors are involved in the synthesis of lipid-linked oligosaccharides. GDP-mannose is the probable donor for the formation of the Man9GlcNAc2 species, while dolichol-P-mannose donates the 6th mannosyl residue and probably mannosyl residues 7 through 9.

The biosynthesis of asparagine-linked oligosaccharides is initiated by the "en bloc" transfer of a GlcManGlcNAc2 species from dolichol pyrophosphate to the nascent polypeptide. Following transfer, the oligosaccharide is processed to form either high mannose or complex type oligosaccharides (1-3). The assembly of the lipid-linked GlcManGlcNAc2 species occurs by the highly ordered addition of the mannosyl residues (4, 5). In this pathway, the Man4GlcNAc2 intermediate is converted to Man9GlcNAc2 by the action of a α1,3-mannosyltransferase as shown in Scheme 1.

![Scheme 1](image)

Recent studies have demonstrated that Thy-1" mutant mouse lymphoma cells of the class E complementation group have a block in the synthesis of the high molecular weight lipid-linked oligosaccharide precursor and accumulate the Man9GlcNAc2 species shown in Scheme 1 (6, 7). One possible explanation for this block is that the mutant cells lack the α1,3-mannosyltransferase necessary for the formation of the Man9GlcNAc2 species. Alternatively, the α1,3-mannosyltransferase could be normal, but the appropriate mannosyl donor necessary for the formation of the Man9GlcNAc2 species might be missing.

In this study, we demonstrate that the Thy-1" E mutant cells have the α1,3-mannosyltransferase but are unable to synthesize dolichol-P-mannose. When exogenous dolichol-P-mannose is added to in vitro assays, the defect in the cells is corrected. The implications of these findings for the synthesis of lipid-linked oligosaccharides are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—GDP-[3H]mannose (10 Ci/mmol), GDP-[6-3H]mannose (0.16 Ci/mmol), dolichol-P-[3H]mannose (0.9 Ci/mmol), and n-[2-3H]mannose (14 Ci/mmol) were from New England Nuclear Corp. Dolichol-P was from Sigma Chemical Co., St. Louis. The 3a70 scintillation fluid was purchased from Research Products International Corp., Elk Grove Village, Ill. Siilica Gel G (250 μm thick) plates were from Analtech, Inc. Whatman No. 1 chromatography paper and DEAE-cellulose (DE52) were from Whatman. The DEAE-cellulose was converted to the acetate form as described by Rouser et al. (8) and stored dry. All other chemicals were of reagent grade and were obtained from commercial sources.

**Cell Lines and Culture Conditions**—The wild type mouse lymphoma cell line BW5147.3 (Thy-1") and the class E mutant lines, BW5147.3 (Thy-1e) 0.10 and (Thy-1e) 0.23, were kindly provided by Drs. Ian Trowbridge and Robert Hyman of the Salk Institute. The cells were grown in suspension in plastic roller bottles (Corning in α-modified Eagle's medium (Flow Laboratories) supplemented with 10% fetal calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin (GIBCO).

**Oligosaccharide Standards**—The [3H]mannose-labeled
Defective Dolichol-P-Mannose Synthesis

ManGlcNAC2 to GlcManGlcNAC2 were isolated from the lipid-linked oligosaccharide fraction of Chinese hamster ovary cells as previously described (5). The ManGlcNAC2 species was isolated in a similar fashion from a mouse MOPC 315 variant kindly provided by Dr. Scott Hickman of this institution. Man1→2Manα1→2Man1→2Manβ1→4GlcNacβ1→GlcNac, Man1→2Manα1→3Man, and Man1→2Man were isolated from an acetolysate of ManGlcNAC2.

Preparation of Crude Membranes—Cells (2 to 5 x 10^9) in exponential growth (less than 1 to 2 x 10^10 cells/ml) were sedimented at room temperature by centrifugation and washed once with chilled Dulbecco's phosphate-buffered saline (9). The cell pellet was resuspended in 1 ml of ice-cold 0.02 M Tris-HCl, pH 7.5, containing 0.15 M NaCl, 1 mM MnCl2, and 1 mM MgCl2 (Buffer A) and subjected to sonication with the microprobe of a Biosonix II sonicator (Brownell Scientific Co.) using two 15-s pulses at a probe intensity of 10. The resulting cell lysate was sedimented at 130,000 x g for 60 min. The membrane pellet was resuspended in 0.5 to 1.0 ml of Buffer A containing either 0.2% or 0.5% Triton X-100 and homogenized with a Teflon pestle Potter-Elvehjem tissue homogenizer. An aliquot of the membrane suspension was assayed for protein by the method of Lowry et al. (10).

Formation of Lipid-linked Oligosaccharides by Crude Membranes—Reaction mixtures containing 0.5 mg of crude membrane protein in 45 μl of Buffer A with 0.5% Triton X-100 were incubated at 30°C for 3 min prior to the addition of the labeled mannosyl donor to ensure temperature equilibrium. The reactions were then initiated by the addition of a 5-μl aliquot of either Dolichol-P[^3H]Man (50,000 cpm) or GDP[^3H]Man (10,000 cpm). The reaction mixtures were then incubated at 30°C for the indicated times and the reactions were stopped by the addition of 2 ml of chloroform/methanol (2:1). The reaction mixtures were extracted three times each with chloroform/methanol (2:1), water, and chloroform/methanol/water (10:10:3) as described by Sfetoor (11). The combined chloroform/methanol/water (10:10:3) extracts were passed over a column of Sephadex G-25 suspended in chloroform/methanol/water (10:10:3) in order to remove trace amounts of GDP[^3H]Man and[^3H]Man which are extracted into the chloroform/methanol/water (12). Aliquots of the samples were transferred to scintillation vials, dried under a stream of N2, and assayed for radioactivity. Acetolysis of the oligosaccharides was performed as previously described (16).

α-Mannosidase Digestion— α-Mannosidase was prepared from jack bean meal by the method of Li and Li (17). The oligosaccharide samples were incubated with 1 unit of α-mannosidase in 0.05 ml of sodium citrate, pH 4.5, for 16 h at 37°C under a nitrogen atmosphere. The free mannose was separated from residual oligosaccharide by descending paper chromatography.

RESULTS

Transfer of Mannose from GDP-Mannose and Dolichol-P-Mannose to Lipid-linked Oligosaccharides—The accumulation of the lipid-linked ManGlcNAC2 species in the class E Thy-1- mutant cells suggested that there might be a defect in the α,1,3-mannosyltransferase which is responsible for the formation of the ManGlcNAC2 species. To test this possibility, we assayed for lipid-linked oligosaccharide biosynthesis using crude microsomal membranes of parent and mutant cells as a source of enzyme and endogenous lipid-linked oligosaccharide and either GDP[^3H]mannose or dolichol-P[^3H]mannose as the mannosyl donor. Membrane preparations from both cell lines were able to incorporate[^3H]mannose into lipid-linked oligosaccharides soluble in chloroform/methanol/water (10:10:3) using either GDP[^3H]mannose or dolichol-P[^3H]mannose as donors (data not shown). The reactions were linear for 5 to 10 min.

We next examined the size of the lipid-linked oligosaccharides formed in these in vitro experiments. The lipid-linked oligosaccharides were subjected to mild acid hydrolysis to release the oligosaccharide moieties which were then separated by descending paper chromatography. The spectrum of the oligosaccharides formed from GDP[^3H]mannose by parent and mutant extracts is shown in Fig. 1, A and B. The parent extract formed oligosaccharides ranging in size from ManGlcNAC2 to ManGlcNAC6. In contrast, the largest oligosaccharide formed by the mutant extract migrated in the position of ManGlcNAC5, which is the size of the lipid-linked oligosaccharide that accumulates in the intact cells. The pattern of oligosaccharides formed from dolichol-P-mannose is shown in Fig. 2, A and B. With this mannosyl donor, oligosaccharides ranging in size from ManGlcNAC2 to ManGlcNAC6 are formed with both cell extracts. These results indicate that the mutant cells have the α,1,3-mannosyltransferase necessary for the formation of the ManGlcNAC2 species, but this activity is only expressed when the mannosyl donor is dolichol-P-mannose.

Further Characterization of the Lipid-linked Oligosaccharides Formed from Dolichol-P-Mannose—When the ManGlcNAC2 to ManGlcNAC6 species formed from dolichol-P[^3H]mannose by both cell extracts were subjected to α-mannosidase digestion, all of the radioactive material was released as free mannose, demonstrating that all of the mannose residues transferred from the dolichol-P-mannose were linked α. The ManGlcNAC2 species formed by the mutant cell extract was next subjected to acetolysis, a procedure which preferentially cleaves mannose residues linked 1,6. The major radioactive

chemical methods—Mild acid hydrolysis of the lipid-linked saccharide samples was performed in 1 ml of 0.1 N HCl at 50°C for 60 min (2). The hydrolyzed material was desalted by passage over a column (0.5 x 5 cm) of Amberlite MB-3 and then analyzed by descending paper chromatography. Saponification was performed essentially as described by Admao et al. (18). Samples were mixed vigorously and the upper and lower phases of the resulting portion were transferred to scintillation vials, dried under a stream of N2, and assayed for radioactivity. Acetolysis of the oligosaccharides was performed as previously described (16).

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Defective Dolichol-P-Mannose Synthesis

4443

FIG. 1. Paper chromatograms of the oligosaccharides formed from GDP-[3H]mannose by membrane preparations of parent and class E Thy-1- cells. Reaction mixtures containing 130,000 x g particulate enzyme and 2 x 10^5 cpm of GDP-[3H]mannose were incubated for 20 min at 30°C and extracted as described under "Experimental Procedures." The chloroform/methanol/water (10:10:3) extracts were subjected to mild acid hydrolysis and the released oligosaccharides were separated by descending paper chromatography in ethyl acetate/pyridine/acetic acid/water (5:5:1:3) for 4 days. A. [3H]mannose-labeled oligosaccharides formed by parent Thy-1- membranes; B. [3H]mannose-labeled oligosaccharides formed by mutant Thy-1- membranes. The arrows indicate the position of the standards: M5, Man3GlcNAc2; M6, Man2GlcNAc2; M7, ManGlcNAc2; M8, ManGlcNAc2; M9, Man4GlcNAc2; M10, ManGlcNAc2.

FIG. 2. Paper chromatograms of the oligosaccharides formed from dolichol-P-[3H]mannose by membrane preparations of parent and class E Thy-1- cells. Reaction mixtures containing 200 µl containing 130,000 x g particulate enzyme, 5 x 10^5 cpm of dolichol-P-[3H]mannose, and 0.5% Triton X-100 were incubated for 20 min at 30°C and extracted as described under "Experimental Procedures." The chloroform/methanol/water (10:10:3) extracts were subjected to mild acid hydrolysis and the released oligosaccharides were separated by descending paper chromatography in ethyl acetate/pyridine/acetic acid/water (5:5:1:3). A. [3H]mannose-labeled oligosaccharides formed by parent Thy-1- membranes; B. [3H]mannose-labeled oligosaccharides formed by mutant Thy-1- membranes. The migration of the standard compounds is indicated by the arrows. The standards are the same as those described in the legend to Fig. 1.

FIG. 3. Paper chromatogram of the [3H]mannose-labeled acetylase fragments of the Man4GlcNAc2 species formed in vitro by class E Thy-1- membranes from dolichol-P-[3H]mannose. The chromatogram was developed in ethyl acetate/pyridine/acetic acid/water (5:5:1:3) for 19 h. The arrows indicate the position of the standards: M1, GlcNAc2; M2, Man1+2Man1+2Man; M3, Man1+2Man; M4, Man1+2Man; M5, Man1+2Man; M6, Man1+2Man; M7, Man1+2Man; M8, Man1+2Man; M9, Man1+2Man; M10, Man1+2Man; M11, Man1+2Man; M12, Man1+2Man; M13, Man4GlcNAc2. The inset shows the structure of the normal Man4GlcNAc2 species. The dashed lines indicate the expected cleavages during acetylation. The mannose residues indicated as "Man" would be expected to be labeled if the Man4GlcNAc2 species was formed by the transfer of [3H]mannose from dolichol-P-[3H]mannose to endogenous lipid-linked Man4GlcNAc2 acceptor.
Defective Dolichol-P-Mannose Synthesis

FIG. 4. DEAE-cellulose chromatography of chloroform/methanol (2:1)-extractable lipids formed by parent Thy-1+ and class E Thy-1- cells when incubated with 2-[3H]mannose. Cells in 15 ml of complete media were incubated with 1 mCi of 2-[3H]mannose for 30 min and extracted with chloroform/methanol (2:1) as described under "Experimental Procedures." The lipid extracts were loaded into columns of DEAE-cellulose equilibrated with chloroform/methanol (2:1). The columns were washed with this solvent until no more radioactivity was eluted and then the columns were sequentially eluted with chloroform/methanol/water (10:10:3), chloroform/methanol/0.01 M ammonium formate (1:10:3), and chloroform/methanol/0.1 M ammonium formate (10:10:3) as indicated by the arrows. Fractions of 1.0 ml were collected and aliquots tested for radioactivity. The peaks of radioactivity were pooled as indicated by the brackets. The large arrow (1) indicates where standard dolichol-P-mannose elutes. A, parent Thy-1+ cell extracts; B, class E Thy-1- cell extracts.

In Vitro Synthesis of Dolichol-P-Mannose from GDP-[14C]mannose—We next tested membrane preparations from parent and mutant cells for their ability to synthesize dolichol-P-mannose from GDP-mannose. As shown in Fig. 5, membranes from parent cells incorporated [14C]mannose from GDP-[14C]mannose into chloroform/methanol (2:1)-extractable lipids and this incorporation was greatly stimulated by the addition of exogenous dolichol-P. In contrast, membranes from class E Thy-1- cells incorporated very little [14C]mannose into chloroform/methanol-extractable material even in the presence of dolichol-P. The effect of dolichol-P concentration on this reaction is shown in Fig. 6. Parent membranes were optimally stimulated at a dolichol-P concentration of 100 μg/ml, whereas membranes from the mutant cells were not stimulated at any dolichol-P concentration. These experiments show that the defect in the mutant line is not an inability to synthesize dolichol-P, but rather an inability to transfer mannose from GDP-mannose to dolichol-P.

The chloroform/methanol-extractable material synthesized by parent membranes in the presence of dolichol-P was shown to be predominantly dolichol-P-mannose by the following criteria. The labeled material bound to DEAE-cellulose (acetate form) and was eluted with chloroform/methanol/0.01 M ammonium formate. This material was resistant to mild base treatment, susceptible to mild acid hydrolysis, and co-chromatographed with authentic dolichol-P-mannose on thin layer chromatography using chloroform/methanol/water (65:35:6) as solvent. When the small amount of radiolabeled material present in the chloroform/methanol (2:1) extracts of mutant cell membrane incubations was analyzed by DEAE-cellulose chromatography, none of the label eluted with chloroform/methanol/0.01 M ammonium formate, demonstrating that even the low level of [14C]mannose incorporation which occurred in these reactions was not reflecting dolichol-P-mannose synthesis. Rather, the majority of the labeled material eluted from the column with chloroform/methanol/0.1 M ammonium formate, which is where Man1-GlcNAc2-P-P-dolichol compounds should elute.

One possible reason for the inability of the mutant cells to
Defective Dolichol-P-Mannose Synthesis

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<th>Enzyme source</th>
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<tr>
<td>Thy-1</td>
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sythesize dolichol-P-mannose is that the cells contain an inhibitor of GDP-mannose: dolichol-P-mannosyltransferase. In order to test this possibility, the mixing experiment shown in Table I was performed. When 1:1 mixtures of parent and mutant membranes were assayed for dolichol-P-mannose synthesis, the amount of dolichol-P-mannose formed was significantly greater than the amount formed by the parent membranes alone. While the mechanism of this stimulation is not known, clearly there is no detectable inhibitor of dolichol-P-mannose synthetase in the class E Thy-1 membrane preparations.

**Discussion**

These experiments demonstrate that class E Thy-1 negative mouse lymphoma cells are unable to synthesize dolichol-P-mannose. This finding has several interesting implications for the biosynthesis of lipid-linked oligosaccharides. First of all, since the mutant cells are able to synthesize lipid-linked oligosaccharides up to Man,GlcnGlcNAc2 in size, the Man to Man-Man species must be formed from some mannolysyldonor other than dolichol-P-mannose. Since GDP-mannose can be used to form the Man,GlcnGlcNAc2 species in vitro, this may be the mannolysyldonor for the formation of the Man, GlcNAc to GlcNAc residue 6 and probably for mannose residues 7 through 9.

This proposal agrees well with studies by Chambers et al. (18), Forsey et al. (19), and Kang et al. (20) in which the in vitro synthesis of lipid-linked oligosaccharides from GDP-[H]mannose or dolichol-P-[H]mannose was studied using pig aorta or rabbit liver microsomal membranes as a source of enzyme. These studies showed that a series of lipid-linked oligosaccharides, ranging in size from 5 to 15 glucose units, could be formed from either GDP-mannose or dolichol-P-mannose. If, however, the microsomal membranes were incubated with GDP-mannose in the presence of EDTA or the drug amphotericin, each of which prevent the formation of dolichol-P-mannose from GDP-mannose, then the largest lipid-linked oligosaccharide formed was a heptasaccharide. This suggested that GDP-mannose could serve as a donor for lipid-linked oligosaccharides up to the size of a heptasaccharide while some, or all, of the larger species were formed from dolichol-P-mannose. Prior to these studies, it had been established that GDP-mannose, but not dolichol-P-mannose, could serve as mannolysyldonor for the synthesis of dolichol-P-N-acetylgalactosaminitol mannose (21, 22).

The lipid-linked Man,GlcnGlcNAc2 species which is synthesized by the class E Thy-1 cells can be converted to GlcMan,GlcnGlcNAc2 and then transferred to protein (6, 7). Following transfer, this species can be processed to form typical complex type oligosaccharides (23). Clearly, the cells are viable and capable of synthesizing both complex and high mannose (albeit truncated) type oligosaccharides even though they lack the capacity to synthesize dolichol-P-mannose and the usual lipid-linked GlcMan,GlcnGlcNAc2 species. However, the glycosylation with the GlcMan,GlcnGlcNAc2 species rather than the GlcMan,GlcnGlcNAc2 species may be detrimental to the functioning of some glycoproteins. Trowbridge et al. (24) have shown that the Thy-1 surface antigen present in the parent cell line is not detectable on the surface of the class E Thy-1 cells. These workers found that a M, = 25,000 glycoprotein (termed T-25) which carried the Thy-1 antigenic determinant is synthesized and glycosylated in the class E cells, but is rapidly degraded within the cell and, therefore, does not reach the cell surface. One possible explanation for this phenomenon is that when the T-25 glycoprotein is glycosylated with the less bulky GlcMan,GlcnGlcNAc2 species, it undergoes a conformational change which makes it susceptible to intracellular proteases.

While the purpose of having two mannosyl donors for the synthesis of lipid-linked oligosaccharides is unknown, it is interesting to speculate that the two donors might have some regulatory function in this pathway. Since normal cells, as well as class E Thy-1 cells, are capable of synthesizing the lipid-linked GlcMan,GlcnGlcNAc2 species (5), it may be that under certain conditions it is advantageous to switch from the synthesis of GlcMan,GlcnGlcNAc2 to GlcMan,GlcnGlcNAc2. That this in fact occurs is supported by our recent observations that Chinese hamster ovary cells synthesize more lipid-linked GlcMan,GlcnGlcNAc2 and Man,GlcnGlcNAc2 and less GlcMan,GlcnGlcNAc2 when incubated in glucose-deficient medium. In addition, the GlcMan,GlcnGlcNAc2 species synthesized under these conditions is transferred to protein.

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REFERENCES


Defective Dolichol-P-Mannose Synthesis