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Reduced Utilization of $Man_5GlcNAc_2$-P-P-Lipid in a Lec9 Mutant of Chinese Hamster Ovary Cells: Analysis of the Steps in Oligosaccharide-Lipid Assembly

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Abstract Recently we reported that CHB11-1-3, a Chinese hamster ovary cell mutant defective in glycosylation of asparagine-linked proteins, is defective in the synthesis of dolichol [Quellhorst et al., 343:19–26, 1997: Arch Biochem Biophys]. CHB11-1-3 was found to be in the Lec9 complementation group, which synthesizes polyprenol rather than dolichol. In this paper, levels of various polyprenyl derivatives in CHB11-1-3 are compared to levels of the corresponding dolichyl derivatives in parental cells. CHB11-1-3 was found to maintain near normal levels of $Man_5GlcNAc_2$-P-P-polyprenol and mannosylphosphorylpolyprenol, despite reduced rates of synthesis, by utilizing those intermediates at a reduced rate. The $Man_5GlcNAc_2$ oligosaccharide attached to prenol in CHB11-1-3 cells and to dolichol in parental cells is the same structure, as determined by acetolysis. $Man_5GlcNAc_2$-P-P-polyprenol and $Man_5GlcNAc_5$-P-P-dolichol both appeared to be translocated efficiently in an in vitro reaction. Glycosylation of G protein was compared in vesicular stomatitus virus (VSV)–infected parent and mutant; although a portion of G protein was normally glycosylated in CHB11-1-3 cells, a large portion of G was underglycosylated, resulting in the addition of either one or no oligosaccharide to G. Addition of a single oligosaccharide occurred randomly rather than preferentially at one of the two sites. J. Cell. Biochem. 67:201–215, 1997.

Key words: dolichol; polyprenol; translocation; glycosylation sites; eukaryotic cells

N-linked glycosylation of proteins in eukaryotic cells involves a lipid carrier, dolichyl phosphate, which is synthesized from mevalonate in a series of biosynthetic steps, many in common with sterol biosynthesis [Kaiden and Krag, 1991]. An oligosaccharide of 14 sugars, $Glc_3Man_9GlcNAc_2$, is assembled on dolichyl phosphate in stepwise fashion utilizing membrane-associated enzymes, sugar nucleotides, and monoglycosylated forms of dolichyl phosphate [Cummings, 1992]. Oligosaccharide assembly is thought to involve translocation of some of the glycosylated dolichyl intermediates from the cytoplasmic to the lumenal face of the endoplasmic membrane prior to completion and transfer of the oligosaccharide to nascent protein [Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1990]. Once attached to protein, the oligosaccharide is modified to its final structure by a number of processing enzymes located in the endoplasmic reticulum and Golgi [Kornfeld and Kornfeld, 1985].

One of the final steps in the synthesis of dolichyl phosphate is reduction of polyprenyl pyrophosphate, the end product of *cis*-prenyl transferase activity, or of polyprenol itself [Sagami et al., 1993] to form functional prenol with the α-isoprenyl unit saturated. The importance of this step is evidenced by the glycosylation defect(s) of Lec9 mutants, which are deficient in polyprenol reductase activity [Stoll et al., 1988; Rosenwald and Krag, 1990].

In a recent paper, we showed that CHB11-1-3 is a member of the Lec9 complementation group

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[Quellhorst et al., 1997]. This Chinese hamster ovary (CHO) cell mutant was isolated by screening for cells with lowered amounts of intracellular lysosomal enzyme activity [Hall et al., 1986]. Lysosomal enzymes have high mannose oligosaccharides attached to their asparagine residues [Krag and Robbins, 1982] and thus are affected only by alterations early in the glycosylation pathway (e.g., in the synthesis of dolichyl phosphate or in the assembly of the oligosaccharide on dolichyl phosphate). Although incorporation of mannose into mannosylphosphoryllipid and $Man_5GlcNAc_2$-P-P-lipid appeared comparable in parental and mutant cells, incorporation into $Glc_3Man_9GlcNAc_2$-P-P-lipid was significantly reduced in CHB11-1-3 [Hall et al., 1986]. In this paper, we examine the seeming disparity between synthesis of $Glc_3Man_9GlcNAc_2$-P-P-lipid and its precursors in this mutant. In addition, the effect of decreased $Glc_3Man_9GlcNAc_2$-P-P-lipid on the glycosylation of a single protein, G protein of vesicular stomatitis virus (VSV)–infected cells, is examined.

METHODS

Cells and Culture Conditions

CHB11-1-3 (isolated as described [Hall et al., 1986] and parental CHO (WTB) [Krag, 1979] were grown attached in either α-modified minimal essential medium with 10% fetal bovine serum or Eagles #2 medium supplemented with 5% fetal bovine serum in 5% CO_2 at 34°C. Cells were usually plated 1 day before virus infection experiments in 6 cm dishes and fed 1 h prior to infection. For all other experiments, cells were usually plated 2 days prior to use. In culture, mutant cells were never allowed to exceed 4×10^6 cells per 10 cm dish, due to diminished phenotypic expression of the mutation at higher cell densities.

Labeling, Extraction, and Separation of Lipids Labeled With Mevalonate

CHO cells were plated at a density of 0.5×10^6 cells per 10 cm dish in 10 ml of α-modified minimal essential medium containing 10% fetal bovine serum. The following day, the medium was replaced with 10 ml of medium containing 20 μCi/ml [5-^{3}H]mevalonolactone (NEN, Boston, MA) (27.8 Ci/mmol; diluted to 0.3 mM final concentration) and 10 μg/ml mevinolin [Rosenwald et al., 1990]. Cells were grown in the labeling medium for 72 h at 34°C. Lipids were extracted as described previously [Rosenwald et al., 1990], and [^{14}C]dolichol and [^{14}C]dolichyl phosphate were added as internal standards. The different prenol forms were separated by thin layer chromatography as described [Kaiden and Krag, in preparation].

Labeling, Extraction of Lipids Labeled With Mannose, and Separation of the Saccharides Derived From Those Lipids

Cells grown on 6 cm dishes were incubated in either 1 ml of α-modified minimal essential medium containing 10% dialyzed fetal bovine serum or Eagles #2 medium supplemented with 5% dialyzed fetal bovine serum, 0.1 mM glucose, 5 mM pyruvate, and 200 μCi/ml [2-^{3}H]mannose (10–20 Ci/mmol) for various periods of time. Labeling was terminated by washing the cells with cold phosphate-buffered saline, and the saccharide lipids were extracted using mixtures of chloroform, methanol, and water with different ratios [Krag and Robbins, 1977]. Mild acid hydrolysis of the material soluble in chloroform:methanol 2:1 and chloroform:methanol: water 10:10:3 was performed in tetrahydrofuran:0.5 N HCl, 4:1 at 50°C for 2 h. Samples were neutralized by the addition of an equal volume of 0.1 N NaOH containing 0.02 M sodium phosphate and were dried under reduced pressure. Internal standards were added to the dried sample, and the mixture was applied to either a Bio-Gel P6 or P4 column (200–400 mesh; 1×115 cm, (Bio-RAD, Hercules, CA)) and eluted in 0.1 M Tris-HCl/0.1% sodium azide, pH 8.0. One milliliter fractions were collected and the radioactivity determined by liquid scintillation counting using Liquiscint or Hydrofluor (National Diagnostics, Manville, NJ). Internal standards consisting of dextran (40,000 daltons), mannose, and oligomers of GlcNAc were prepared and detected as described [Krag, 1979].

Acetolysis Analysis of Labeled Saccharides

$Man_5GlcNAc_2$ saccharide purified by gel filtration chromatography on a Bio-Gel P6 column was desalted using a Sephadex G-15 column (Pharmacia Biotech, Piscataway, N.J.). The oligosaccharide was then subjected to acetolysis as described [Guthrie and McCarthy, 1967]. Briefly, the oligosaccharide was acetylated with acetic anhydride in pyridine, hydrolyzed with glacial acetic acid and sulfuric acid, and then deacetylated with sodium methoxide. The products were separated on a Bio-Gel P6 column as described above.

Membrane Preparations

Postnuclear supernates from cells grown in 15 cm plates (eight plates of parental cells and 21 plates of CHB11-1-3 cells) were prepared as previously described [Rome et al., 1979] except that cells were harvested by scraping. Lysosomes and mitochondria were pelleted by a 10 min centrifugation at 6,300*g*. The supernate was spun at 175,000*g* for 65 min. Pelleted membranes were rinsed gently with cold 10 mM Tris/Cl, pH 7.4, 150 mM NaCl, and 5 μM leupeptin, and the pellets were combined in a total of about 600 μl of the same buffer. Protein was determined by the method of Lowry [Lowry et al., 1951]. Total membrane protein yield was about 4–6 mg. The integrity of membranes prepared by this technique was checked by isolating membranes from VSV-infected cells and analyzing the susceptibility of G protein to proteolysis with and without detergent.

In Vitro Translocation

To generate a maximum amount of $Man_5GlcNAc_2$-P-P-lipid, we incubated membranes in 20 mM Tris/Cl, pH 7.4, containing 150 mM NaCl, 0.46 mM $MgCl_2$, 0.46 mM $MnCl_2$, and 0.08 mM UDP-GlcNAc at 5 mg protein/2.5 ml (preparations varied from 4.8–6.0 mg) for 30 min at 37°C. To this mixture, 25 μCi [^{3}H]GDP-mannose (previously dried under N_2 and reconstituted in about 100 μl of the incubation mixture) and unlabeled GDP-mannose were added to a final concentration of 1 μM, and the UDP-GlcNAc concentration was adjusted to a final concentration of 2 μM; to prevent elongation of translocated $Man_5GlcNAc_2$-P-P-lipid, we added EDTA to a final concentration of 2 mM. The incubation of the membranes was continued for 30 min at 37°C. The total reaction mixture was layered onto a single two-step sucrose gradient (bottom (4.0–4.6 ml), 2.4 M sucrose; top (4.4 ml), 0.25 M sucrose) with 2 mM EDTA and spun in an SW40Ti at 200,000*g* for 70 min. Membranes were collected from the interface, and the volume was measured.

One-fifth of the volume in which the membranes were recovered was used in each incubation which consisted of 20 mM MES(2-[N-Morpholino] ethane sulfonic acid), pH 6.0, 10 mM $MgCl_2$, and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) with or without 20 units of α-mannosidase and with or without 1% Triton X-100 in a total volume of 800 μl. A sucrose concentration of 0.25 M was maintained by addition of supplemental sucrose. The mixtures were incubated at 30°C for 30 min. For these experiments, α-mannosidase was purified after dialysis against phosphate buffer, pH 6.0, with 5 μM $ZnCl_2$ by affinity chromatography using D-mannose immobilized on agarose beads (Pierce Chemical Co., Rockford, IL). Samples were subjected to mild acid hydrolysis by addition of 4 ml 0.5 N HCl/tetrahydrofuran (1:4) and incubation at 50°C for 2 h. Debris was sedimented by centrifugation at 700*g* (RT6000; Sorvall, Wilmington, DE) for 5 min. Supernates were removed and neutralized with 4.8 ml 0.1 N NaOH in 0.02 N Na_2HPO_4 and then frozen. Samples were evaporated to dryness under N_2. For mini-Folch extractions, 4 ml chloroform/methanol 2:1 was added to each sample, samples were vortexed, 0.8 ml water was added, and samples were vortexed 1 min and placed on ice for 10 min. Samples were spun at 700*g* for 5 min. The upper layer was removed and dried down under N_2. Dextrans, mannose, and $GlcNAc_{1–6}$ standards were added to the dried samples, which were then applied to a 1.0 × 100 cm Bio-Gel P4 column. Elution was with 0.1 M Tris/Cl, pH 8.0, containing 0.1% NaN_3. Standards were detected as described previously [Krag, 1979], and radioactivity was detected by liquid scintillation counting.

Our initial approach locating $Man_5GlcNAc_2$-P-P-lipid using α-mannosidase was thwarted because the enzyme remained active in chloroform:methanol (2:1), the method first used to terminate the reaction. The reactions were terminated by adding tetrahydrofuran/water 4:1 and heating the mixture to 50°C for 2 h. Use of acid hydrolysis made it impossible to determine the amount of mannose released by α-mannosidase because of the high background radioactivity resulting from the conversion of residual, labeled GDP-mannose to mannose by acid hydroysis.

Incubation of Virally Infected Cells With Radiolabeled Precursors

Cells were infected with 10 pfu/cell of vesicular stomatitis virus (VSV), (Indiana serotype) for 4.5 h and then rinsed 3× with the appropriate deficient medium lacking glucose for the sugar-labeling experiments or lacking the specific amino acid for the amino acid–labeling experiments. When sugar-labeling was done, cells were returned to the incubator during the last rinse for 15 min before addition of label. Infected cells were then labeled as follows: 1 mCi/ml [2-^{3}H]mannose in the presence of 0.1 mM mannose and 0.1 mM glucose for 2 h; 50

TABLE I. Steady-State Levels of the Various Prenol Lipids in Parental and CHB11-1-3 Cells*

Prenol Form	dpm/10^6 cells	% of total
Wild type		
Neutral	205 ± 37	10
OSL	1,336 ± 300	64
Dol-P	206 ± 25	10
Man-P-Dol	118 ± 29	6
Glc-P-Dol	209 ± 94	10
Total	2,074	
CHB11-1-3		
Neutral	995 ± 72	39
OSL	682 ± 94	27
Pol-P	281 ± 74	11
Man-P-Pol	199 ± 20	8
Glc-P-Pol	395 ± 227	15
Total	2,552	

*Parental and CHB11-1-3 cells were separately incubated with tritiated mevalonate for 72 h as described in Methods. Lipids were extracted, saponified, and separated into prenol forms as described in Methods. Incorporation into each fraction was corrected for recovery as determined by adding [^{14}C]dolichol and [^{14}C]dolichyl phosphate. The data represent duplicate determinations from two experiments.

μCi/ml [^{3}H]galactose or [^{3}H]glucosamine with 0.1 mM glucose for 1 h; 20 μC/ml [^{3}H]fucose with 0.1 mM glucose for 1 h; 20 μCi/ml [^{35}S]methionine with 0.4 μg/ml methionine for various times as indicated in the figure legends; [^{35}S]cysteine, 1.1 μCi/ml, for 2 h; or [^{3}H]phenylalanine, 1.1 μCi/ml, for 2 h. All incubations with labeled precursors were done in the appropriate deficient versions (see "Cells and Culture Conditions") of α-modified minimal essential medium or Eagles #2 medium, each supplemented with dialyzed fetal bovine serum. When virions were to be collected, cells were rinsed with complete growth medium (with a full complement of glucose, methionine, cysteine, and phenylalanine) and then incubated in complete medium without radiolabel for 3 h.

Treatment of Viral Proteins With Endo-β-N-Acetylglucosaminidase H

Cell-associated viral proteins in 0.02 M Tris-Cl containing 1% SDS were precipitiated with trichloroacetic acid (TCA) and subjected to reductive alkylation [Zilberstein et al., 1980] before subsequent treatment with and without endo-β-N-acetylglucosaminidase H [Hall et al., 1986].

Treatment of Virally Infected Cells With Tunicamycin

Two hours after infection with VSV, tunicamycin was added at a concentration of 2 μg/ml/dish, and treatment was continued for 4 h. A similar amount of medium was added to the control plates. Following incubation of the cells with labeling medium, the plates were rinsed in cold phosphate-buffered saline and solubilized in Tris/glycerol/SDS [Hall et al., 1986].

SDS-PAGE

In most experiments, samples were separated on 8–12% exponential gradient gel containing urea as described [Schleif and Wensink, 1981]. In the experiment presented in Figure 5A, samples were separated on a 7–11% exponential gradient gel without urea. [^{14}C]methylated protein standards (Amersham, Arlington Heights, IL) contained a mixture of phosphorylase b (97.4 kd), bovine serum albumin (BSA) (69 kd), ovalbumin (46 kd), carbonic anhydrase (30 kd), and lysozyme (14 kd). Lysozyme routinely ran at the dye front.

Purification of G Protein Forms From Virions of Parental and CHB11-1-3 Cells

Floating cells were removed from the medium of virally infected cells (previously radiolabeled and chased as above) by centrifugation at 450*g* at room temperature. Clarified medium was centrifuged at 45,000 rpm (140,000*g*) for 1.5 h in a Ti75 rotor at 4°C, and pelleted virions were suspended by brief sonication in 0.05 M sodium acetate, pH 5.5, containing 1 mM $CaCl_2$, 1 mg/ml BSA, and 0.02% NaN_3. After suspension of the virion pellet, 2 mM PMSF, 10 μM Pepstatin A, and 0.2 IU/ml *V. cholera* neuraminidase were added, and the mixture was incubated for 2 h at 37°C. The virions were again pelleted as above and then solubilized in 10 mM Tris-Cl, pH 7.4, containing 1 mM EDTA and 1% NP40. After incubation for 30 min at 37°C,

Fig. 1. Pulse-chase labeling of parental and CHB11-1-3 cells. Parental (**A**) and CHB11-1-3 (**B**) cells were either incubated with [2-^{3}H]mannose for 5 min (closed symbols) or incubated with labeled mannose for 5 min and then with unlabeled medium for 3.5 min (open symbols). Reactions were terminated by addition of methanol, and the saccharide-lipids were extracted. After release from the lipid by mild acid hydrolysis, saccharides were separated by gel permeation chromatography. Internal standards: D, dextran; M, mannose; N_{1-6}, oligomers of N-acetylglucosamine.

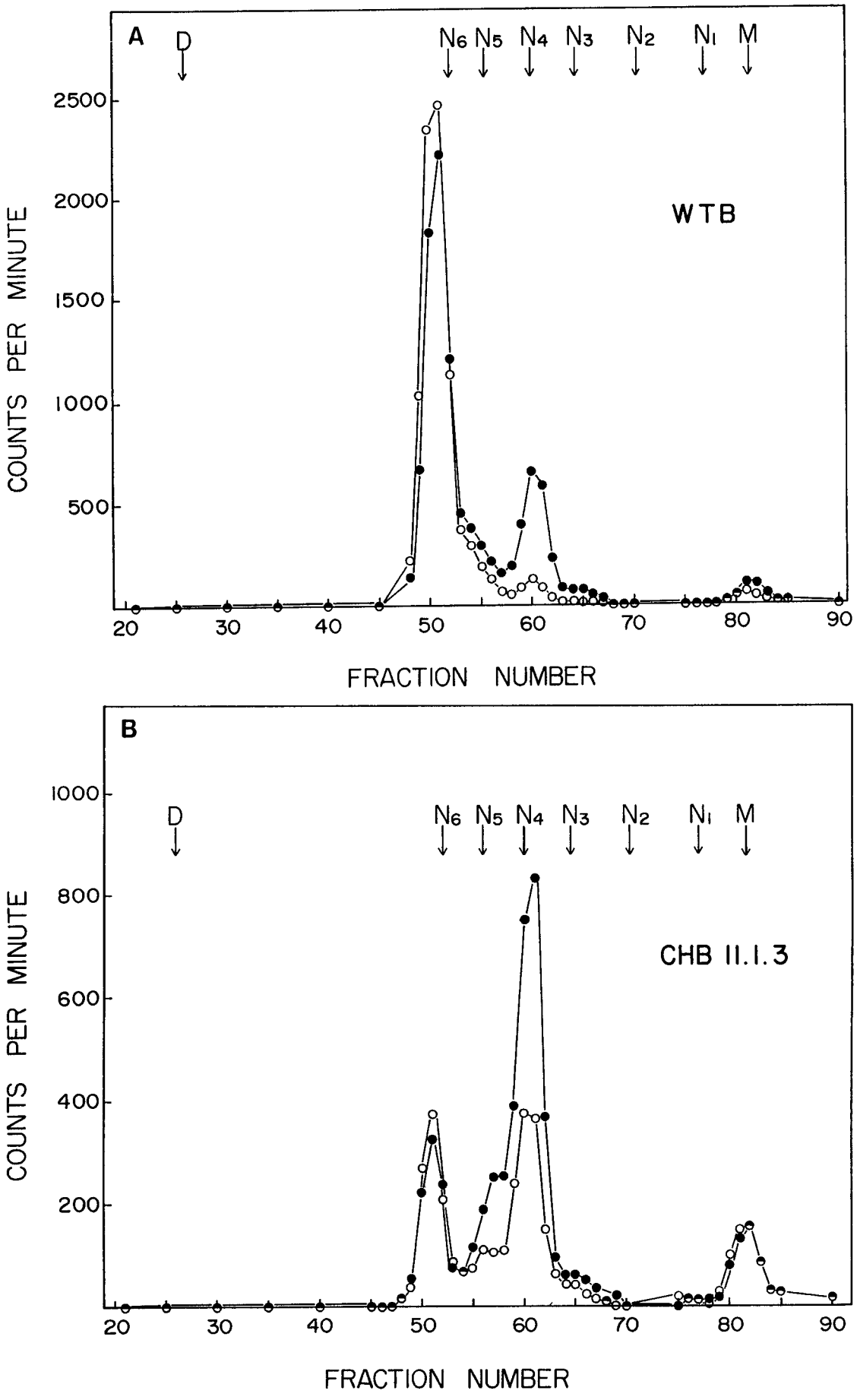

A
D
N6 N5 N4 N3 N2 N1 M
WTB
COUNTS PER MINUTE
2500
2000
1500
1000
500
20 30 40 50 60 70 80 90
FRACTION NUMBER
B
D
N6 N5 N4 N3 N2 N1 M
CHB 11.1.3
COUNTS PER MINUTE
1000
800
600
400
200
20 30 40 50 60 70 80 90
FRACTION NUMBER

Figure 1.

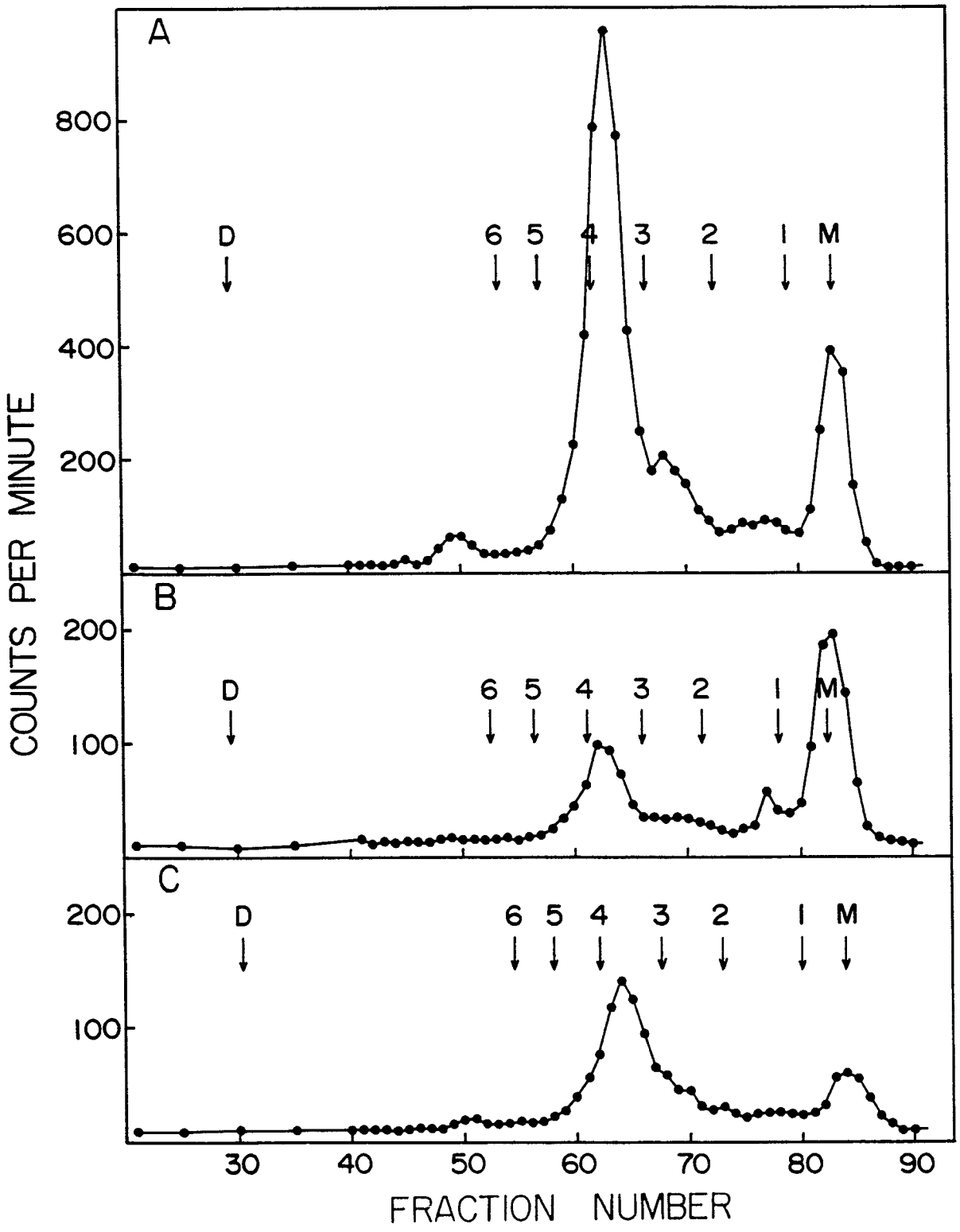


Fig. 2. Determining the structure of $Man_5GlcNAc_2$ released from prenols in parental and CHB11-1-3 cells. Cells were incubated with [2-^{3}H]mannose, and the labeled oligosaccharide-lipids were extracted with chloroform:methanol:water 10:10:3. Following release from the lipid by mild acid hydrolysis, the oligosaccharides were separated by gel filtration chromatography. The fractions eluting as $Man_5GlcNAc_2$ were pooled, desalted, and subjected to acetolysis. The products were separated by gel filtration chromatography on a Bio-Gel P6 column. Internal standards: D, dextran; M, mannose; 1–6, oligomers of N-acetylglucosamine. **A:** $Man_5GlcNAc_2$ from parental cells incubated for 5 min with labeled mannose. **B:** $Man_5GlcNAc_2$ from CHB11-1-3 cells incubated for 5 min with labeled mannose. **C:** $Man_5GlcNAc_2$ from CHB11-1-3 cells incubated for 5 min with labeled mannose followed by 3.5 min with unlabeled medium.

nucleocapsids were removed by centrifugation at 55,000 rpm (200,000*g*) for 1 h at 4°C in a Ti75 rotor. To the supernate, containing the G proteins, 1 mg/ml BSA was added prior to application to a 2.5 ml ricin-agarose (galactose-specific) affinity column, previously equilibrated with 1% NP40, 10 mM Tris-Cl, pH 7.4, and 1 mg/ml BSA. The column was washed with ~15 ml of the same buffer; 1 ml fractions were collected. The intermediate form of G protein from CHB 11-1-3 virions was collected from the exclusion volume, and the parental form was eluted from the column with the same buffer containing 0.2 M lactose and 0.15 M NaCl. Due to inability to strip and regenerate the ricin-agarose, a fresh column was used for each sample.

Separation of G Protein Glycopeptides Containing Different Glycosylation Sites

Purified G proteins were concentrated by TCA precipitation, treated with trypsin in 1 mM HCl adjusted to pH 7.5 with Na_2CO_3, and incubated overnight at 37°C. Glycopeptides were isolated by application to a 2.5 ml ricin-agarose affinity column as above, except the buffer lacked NP40; glycopeptides were eluted with 0.2 M lactose containing no NaCl. Tryptic glycopeptides from the intermediate form of G protein eluted in the void volume with the other peptides, so this step was not used routinely with the intermediate form. The isolated tryptic glycopeptides were fractionated on a 1 × 17 cm DE-52 anion exchange resin column; the column was washed with 45 ml 0.05 M Tris-Cl, pH 8.5 (1.8 ml fractions were collected), and subjected to a 200 ml linear gradient of 0–1.0 M NaCl in the same buffer. This procedure, although developed independently, is similar to one published previously [Stanley, 1982].

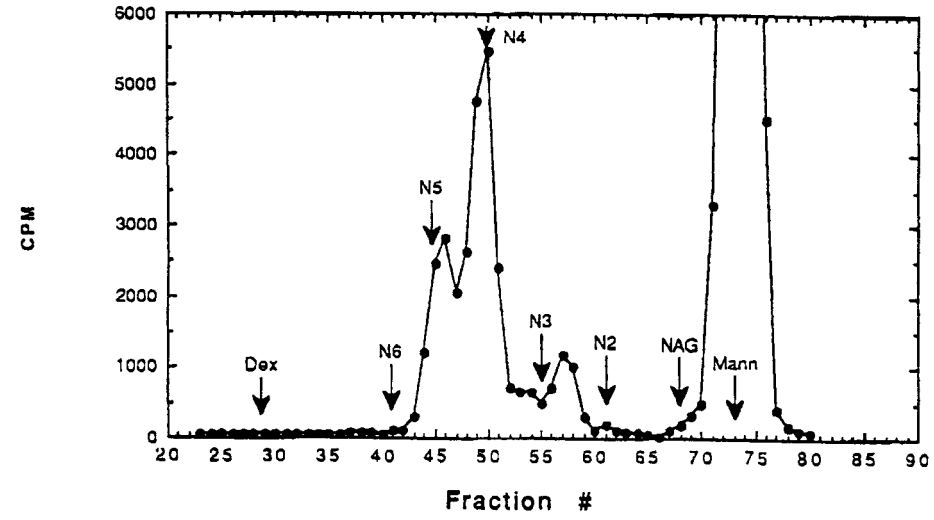
RESULTS

As described recently, CHB11-1-3 cells synthesize polyprenol rather than dolichol, the defining characteristic of Lec9 CHO cells [Quellhorst et al., 1997; Stoll et al., 1988; Rosenwald and Krag, 1990]. We determined the steady-state levels of various lipid intermediates involved in the glycosylation pathway by in vivo mevalonate labeling of CHB11-1-3 cells (see Methods). As shown in Table I, parental and mutant cells synthesize similar amounts of total prenol; levels of lipid phosphate, mannosylphosphoryllipid, and glucosylphosphoryllipid also were comparable. However, CHB11-1-3 had increased amounts of neutral prenols and lower amounts of oligosaccharide-lipid. Previous work had shown that the oligosaccharide-lipid made in CHB11-1-3 was primarily $Man_5GlcNAc_2$-P-P-lipid [Hall et al., 1986] rather than $Glc_3Man_9GlcNAc_2$-P-P-lipid, which is the major oligosaccharide-lipid in parental cells [Krag, 1979]. Levels of mannose-labeled $Man_5GlcNAc_2$-P-P-lipid were similar in parent and mutant [Hall et al., 1986].

CHB11-1-3 Shows Reduced Utilization of Lipid Intermediates

Reduced levels of oligosaccharide-lipid in CHB11-1-3 could reflect increased rates of oligosaccharide transfer to protein, increased degradation of lipid-linked oligosaccharide, and/or decreased synthesis. The first is unlikely, given that proteins are underglycosylated in this mutant [Hall et al., 1986; see "Protein Glycosylation in CHB 11-1-13 Cells"]. To distinguish between the latter two possibilities, we examined utilization of mannosylphosphoryllipid and $Man_5GlcNAc_2$-P-P-lipid during a pulse-chase experiment. WTB and CHB11-1-3 were incubated with [2-^{3}H]mannose for 5 min; then the label was chased for 3.5 min. Following the chase, parental cells showed a twofold reduction in labeled mannosylphosphoryldolichol and a tenfold reduction in $Man_5GlcNAc_2$-P-P-dolichol (Fig. 1A). In contrast, the amount of labeled mannosylphosphorylpolyprenol in CHB11-1-3 did not change during the chase, and $Man_5GlcNAc_2$-P-P-polyprenol decreased only twofold (Fig. 1B).

These results indicate that the level of $Glc_3Man_9GlcNAc_2$-P-P-lipid in CHB11-1-3 is reduced due to the decreased rate of elongation of the $Man_5GlcNAc_2$-P-P-lipid. Results in Figure 2 indicate that synthesis of $Man_5GlcNAc_2$-P-P-lipid also proceeds more slowly in the mutant. Following labeling of cells with [2-^{3}H]mannose, $Man_5GlcNAc_2$ isolated from WTB and CHB11-1-3 was subjected to acetolysis. This procedure preferentially cleaves 1,6 linkages, yielding $Man_4GlcNAc_2$ and mannose. Following a 5 min pulse, the ratio of radioactivity in $Man_4GlcNAc_2$ and mannose in $Man_5GlcNAc_2$ from WTB was 3:1 (Fig. 2A), and from CHB11-1-3 it was 1:3 (Fig. 2B). Labeled oligosaccharide from CHB11-1-3 following a 5 min pulse and a 3.5 min chase yielded 3:1, $Man_4GlcNAc_2$:Man (Fig. 2C). Taken together, the results in Figure 1 and 2 indicate that in CHB11-1-3 both synthesis of lipid-linked $Man_5GlcNAc_2$ and subsequent elonga-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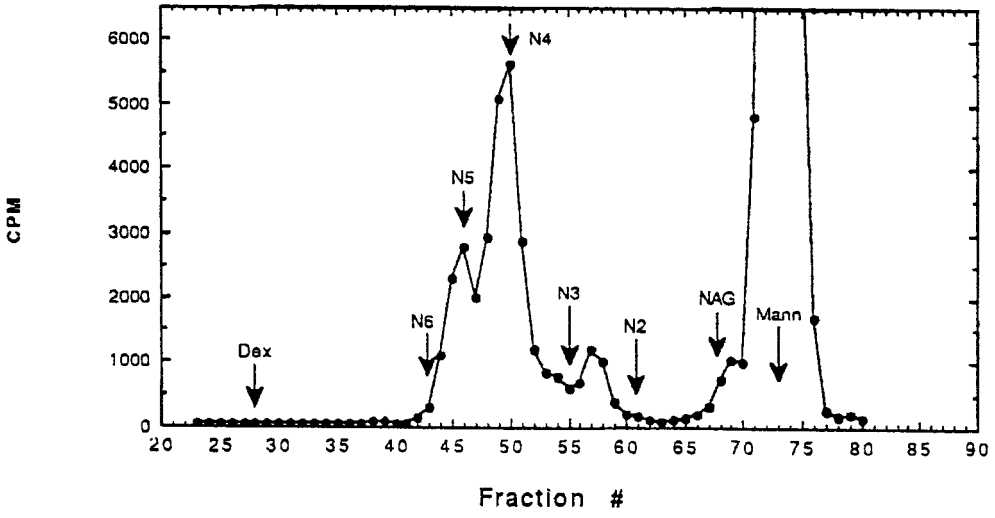
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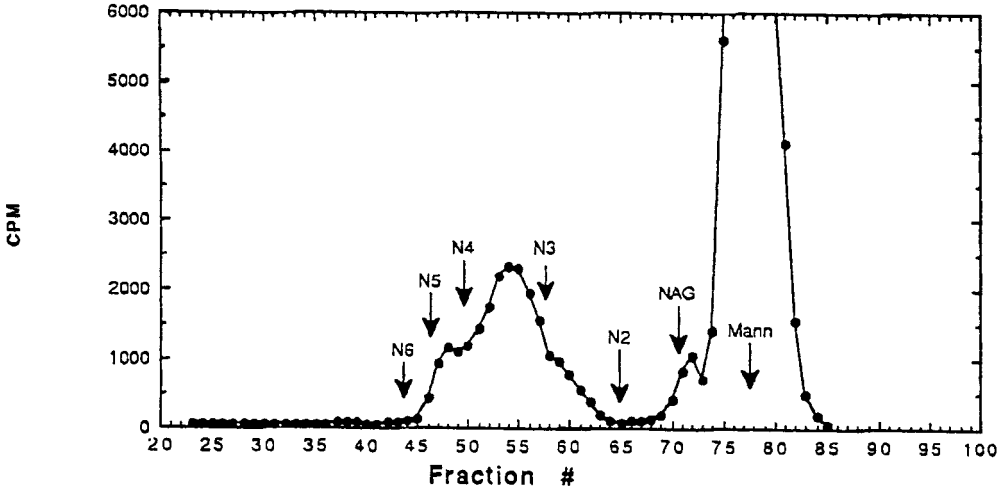
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NAG
Mann
20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Fraction #

Figure 3.

tion of this oligosaccharide proceed more slowly than in parental cells.

The products obtained on acetolysis of $Man_5GlcNAc_2$ from parent and CHB11-1-3 were the same, indicating that the structure of this oligosaccharide is not altered in the mutant. Additional support for this comes from the resistance of $Man_5GlcNAc_2$-P-P-polyprenol to cleavage by endo-β-N-acetylglucosaminidase H (data not shown), indicating that this oligosaccharide has an unblocked α1,6 mannose, as does $Man_5GlcNAc_2$-P-P-dolichol. Also, saccharides from $Man_5GlcNAc_2$-P-P-lipids isolated from WTB and CHB11-1-3 eluted identically on HPLC using an amino-bonded silica column eluted with acetonitrile/water (data not shown).

Translocation of $Man_5GlcNAc_2$-P-P-Polyprenol

It has been proposed that $Man_5GlcNAc_2$-P-P-dolichol is the intermediate that translocates from the cytoplasmic to the lumenal surface of the endoplasmic reticulum (ER) membrane [Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1990]. The slower elongation of $Man_5GlcNAc_2$-P-P-polyprenol in CHB11-1-3 could result from decreased translocation of this lipid into the lumen of the ER.

We initially attempted to examine translocation using dolichol- and polyprenol-linked oligosaccharides generated in vivo. Membrane vesicles were prepared from cells that had been incubated with [2-^{3}H]mannose. We found that after 2 h at 4°C, the time required for vesicle preparation, a significant fraction of labeled $Man_5GlcNAc_2$-P-P-polyprenol had been converted to $Glc_3Man_9NAcGlc_2$-P-P-polyprenol. In retrospect, this might have been expected: first, all of the enzymes required for oligosaccharide elongation are present in CHB11-1-3 membranes; second, at 4°C, elongation of $Man_5GlcNAc_2$-P-P-polyprenol is not competing with transfer of that oligosaccharide to newly synthesized proteins. Thus, given enough time, even poor substrates (polyprenyl derivatives) will be utilized.

To circumvent this problem, translocation was examined using in vitro–generated oligosaccharide-lipids. Intact membranes were incubated with labeled GDP-mannose in the presence of EDTA in order to accumulate $Man_5GlcNAc_2$-P-P-lipid; mannosyltransferases that elongate this oligosaccharide-lipid and mannosylphosphoryldolichol synthase that produces the mannosyl donor used in elongation both require Mg^{++} ions. Labeled sugar nucleotide was removed by centrifugation of the membranes through sucrose. The membranes were then incubated with purified α-mannosidase in order to determine if $Man_5GlcNAc_2$-P-P-polyprenol and $Man_5GlcNAc_2$-P-P-dolichol were accessible to this enzyme.

As shown in Figure 3, labeled $Man_5GlcNAc_2$-P-P-dolichol was resistant to α-mannosidase unless Triton X-100 was present. As shown in Figure 4, $Man_5GlcNAc_2$-P-P-polyprenol was also resistant to α-mannosidase in the absence of detergent. These results suggest that during the incubation and subsequent centrifugation $Man_5GlcNAc_2$-P-P-dolichol and $Man_5GlcNAc_2$-P-P-polyprenol are synthesized on the cytoplasmic face and translocated into the lumenal space, inaccessible to the added α-mannosidase. Because no intermediate with an oligosaccharide smaller than $Man_5GlcNAc_2$ was detected, these results lack a positive control for the accessibility to α-mannosidase of oligosaccharide-lipids on the cytoplasmic face. On the other hand, elongation of some of the $Man_5GlcNAc_2$-P-P-dolichol to larger oligosaccharides (Fig. 3) provides independent evidence that the oligosaccharide had translocated to the lumenal side in parental cells. Consistent with the Lec9 phenotype, no elongation was detected in CHB11-1-3 membranes (see Fig. 4).

Protein Glycosylation in CHB11-1-3 Cells

We had previously observed that the defect in CHB11-1-3 had varying effects on N-linked glycosylation of proteins depending on the nature of the protein acceptor [Hall et al., 1986]. Thus, we turned to a model system in which only one glycoprotein, vesicular stomatitis virus G protein, is synthesized. G protein has two potential glycosyation sites; in parental CHO cells both

Fig. 3. Localization of $Man_5GlcNAc_2$-P-P-dolichol labeled in in vitro reactions using parental membranes. Membranes prepared from parental cells as described in Methods were incubated with labeled GDP-mannose in the presence of EDTA in order to accumulate labeled $Man_5GlcNAc_2$-P-P-dolichol. Following this in vitro reaction, membranes were collected on a sucrose cushion which removed most but not all of the labeled GDP-mannose. Aliquots of membranes were incubated for 30 min at 30°C in buffer (**top**), in buffer containing purified α-mannosidase (**middle**), or in buffer containing purified α-mannosidase and Triton X-100 (**bottom**). These incubations were terminated by mild acid hydrolysis. The samples were neutralized and extracted, and the resultant oligosaccharides were separated by Bio-Gel P4 chromatography. Internal standards: Dex, dextran; Mann, mannose; N_{2-6}: oligomers of N-acetylglucosamine; NAG, N-acetylglucosamine.

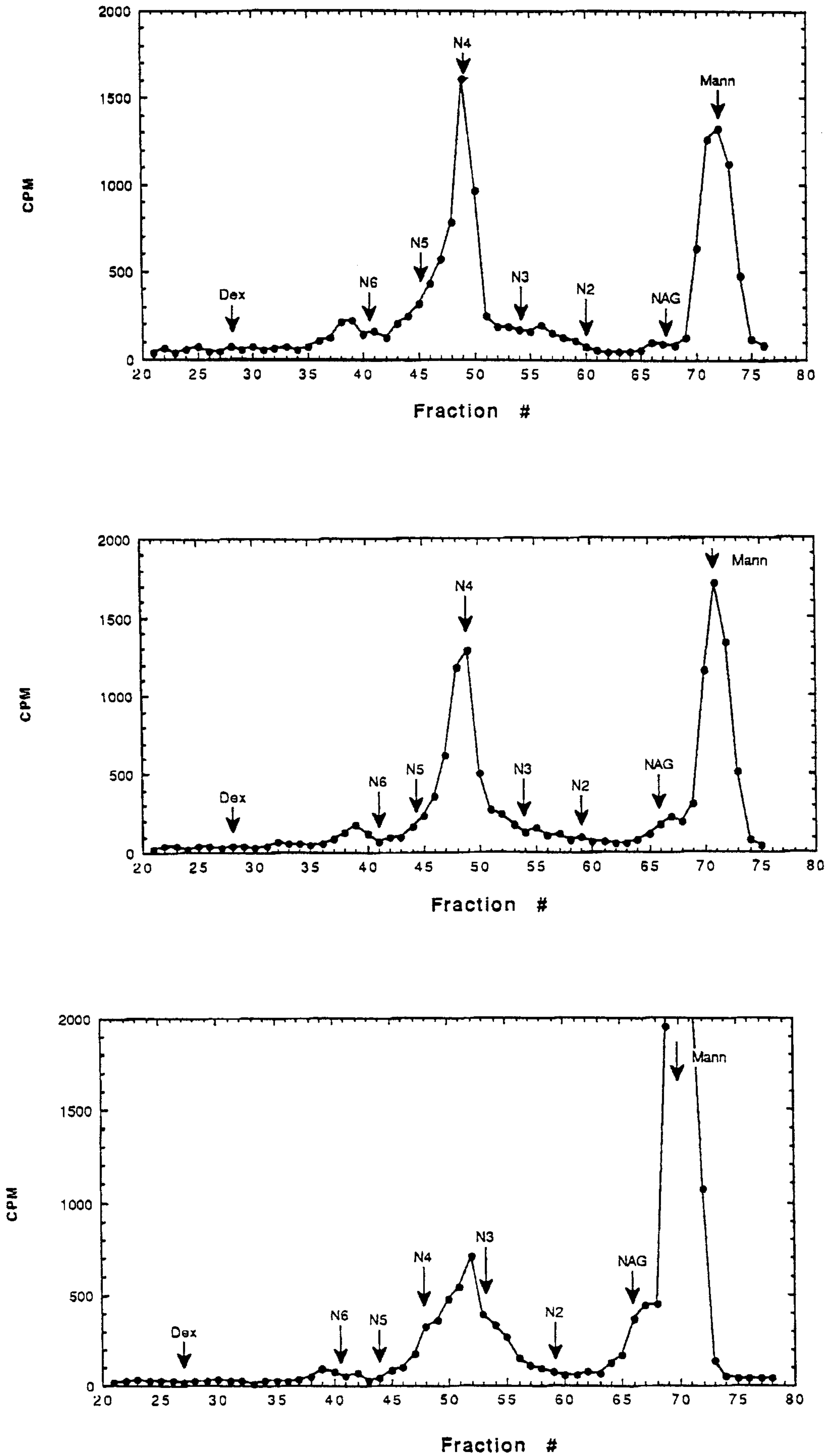

Fig. 4. Localization of $Man_5GlcNAc_2$-P-P-polyprenol labeled in in vitro reactions using CHB11-1-3 membranes. Experimental details are identical to those described in the legend of Figure 3, except that membranes from CHB11-1-3 cells were used.

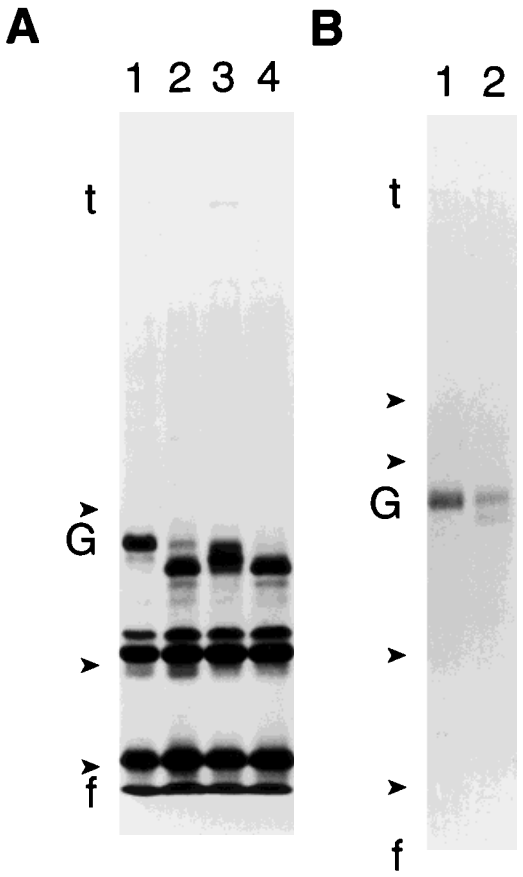

Fig. 5. Synthesis of G protein of VSV in parental and CHB11-1-3 cells. Arrowheads designate mobilities of [^{14}C]methylated protein standard. f, dye front; *t*, top of gel. **A:** VSV-infected parental and CHB11-1-3 cells were incubated with [^{35}S]methionine for 3 min in the absence or presence of tunicamycin. **Lanes 1,2:** Parental cells. **Lanes 3,4:** CHB11-1-3 cells. **Lanes 2,4:** In the presence of tunicamycin. **B:** VSV-infected parental and CHB11-1-3 cells were incubated with tritiated mannose for 2 h. **Lane 1:** Parental cells. **Lane 2:** CHB11-1-3 cells.

sites are used and both oligosaccharides are processed at maturity to biantennary, complex structures [Stanley, 1982].

Newly synthesized G protein from parental and CHB11-1-3 cells labeled with [^{35}S]methionine was examined by SDS-PAGE (Fig. 5A). Whereas G protein from WTB electrophoresed as one band, three bands were obtained from CHB11-1-3. The least mobile form corresponded to G protein from parental cells, as judged by electrophoretic mobility and by incorporation of radioactivity following incubation of the mutant with labeled mannose, fucose, galactose, or glucosamine (Fig. 5B and data not shown). The most mobile form appeared to be unglycosylated; it electrophoresed with G protein synthesized in the presence of tunicamycin (Fig. 5A) and did not incorporate radioactivity during incubation of the cells with any of the four labeled sugars (Fig. 5B and data not shown). The form electrophoresing between these two was predominant, as judged by [^{35}S]methionine labeling, but incorporated noticeably less mannose than the "parental" form of G protein made by CHB11-1-3 (Fig. 5B). Although mannosylated, this form of G protein did not incorporate fucose or galactose, nor did the mobility of methionine-labeled intermediate form increase during chase, in contrast to the shifts observed with "parental" forms synthesized by WTB and CHB11-1-3 (data not shown).

Comparison of mannose and methionine labeling suggested that CHB11-1-3 synthesized nonglycosylated, monoglycosylated, and diglycosylated forms of G protein. A similar situation was previously observed with cathepsin L in this mutant [Hall et al., 1986]. Both the "parental" and monoglycosylated forms of G protein were incorporated into virions, facilitating determination of whether monoglycosylation reflected preferential transfer of oligosaccharide to one of the two sites on the protein. Previous studies had shown that the two glycopeptides generated by trypsin digestion were separable by chromatography on DEAE cellulose [Stanley, 1982]. To identify these sites tryptic peptides were prepared from parental cells infected with VSV and incubated with radiolabeled mannose (Fig. 6A), cysteine (Fig. 6B) or phenylalanine (Fig. 6C); based on the deduced amino acid sequence [Rose and Gallione, 1981], cysteine identifies the tryptic peptide containing the glycosylation site located at Asn_{178} and phenylalanine the peptide with the glycosylation site at Asn_{335}. Comparison of the three panels in Figure 6 reveals that the glycopeptide eluting first from DEAE-cellulose contains Asn_{335}, while the glycopeptide eluting second contains Asn_{178}. As shown in Figure 7, the intermediate form of VSV G protein synthesized by CHB11-1-3 is glycosylated at both sites. Thus, monoglycosylation results not from preferential transfer of oligosaccharide to one site on the polypeptide but from decreased glycosylation at both sites.

DISCUSSION

The levels of saccharide lipid intermediates in the Lec9 mutant CHB11-1-3, measured by both mevalonate labeling of the lipid (Table I) and mannose labeling of the oligosaccharide

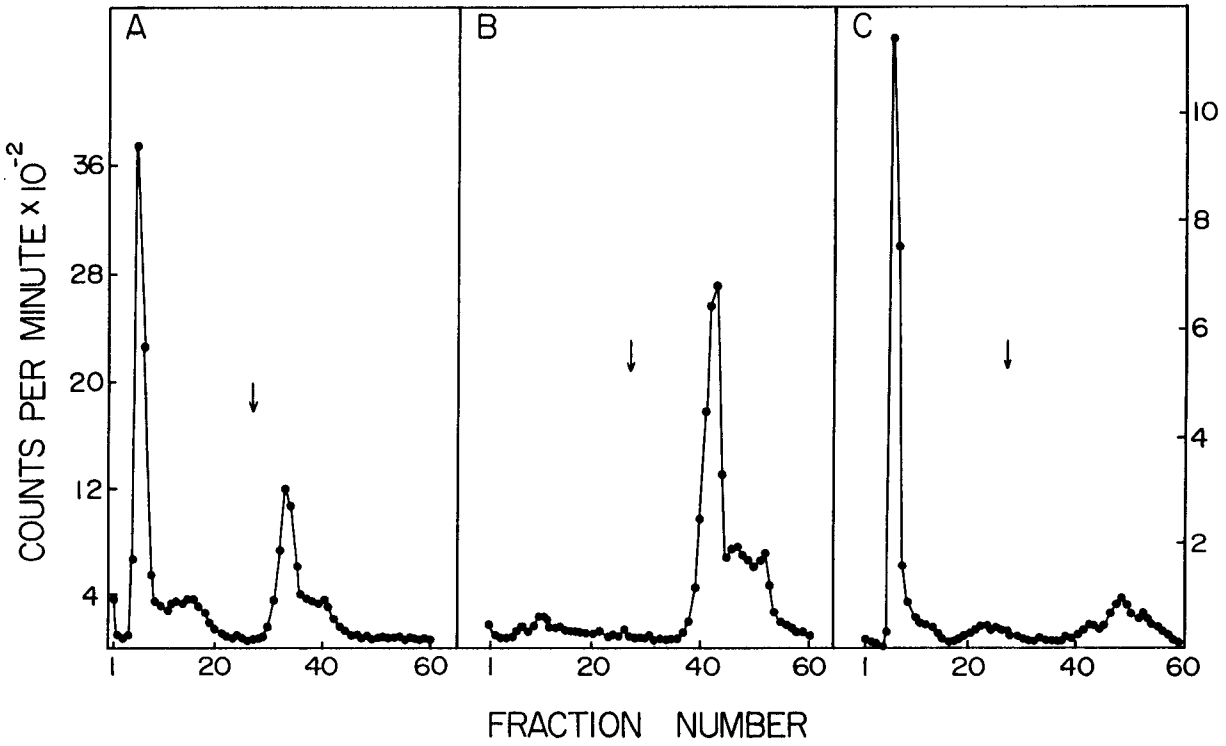

Fig. 6. Separation of tryptic glycopeptides of G protein of VSV. Tryptic peptides of G protein from virions labeled by parental cells were prepared, and glycopeptides were isolated by ricin-agarose affinity chromatography. The tryptic glycopeptides were fractionated on a DE-52 anion exchange column as described in Methods. The arrows denote the beginning of the linear salt gradient. **A:** Mannose-labeled peptides. **B:** Cysteine-labeled glycopeptides. **C:** Phenylalanine-labeled glycopeptides.

(Fig. 1), are greater than would be predicted from the in vitro activities of the relevant synthetic enzymes with polyprenol versus dolichol substrates (summarized in Table II). For example, the mutant contains normal levels of $Man_5GlcNAc_2$-P-P-lipid, although the V_{max}/K_m of GlcNAc 1-P transferase, the enzyme responsible for addition of the first saccharide to what will become oligosaccharide lipid, is reduced eightyfold with polyprenol as substrate. Similarly, mannosylphosphoryllipid is present in normal amounts in CHB11-1-3, although the Man-P-Dol synthase responsible for its formation is six times less efficient when utilizing polyprenol vs. dolichol.

This seeming disparity between levels of lipid intermediates in CHB11-1-3 and the activities of the enzymes involved in their synthesis is reconciled by decreased synthesis of the intermediates being balanced by decreased utilization. $Man_5GlcNAc_2$-P-P-polyprenol is synthesized more slowly than $Man_5GlcNAc_2$-P-P-dolichol (Fig. 2), but it is also elongated more slowly (Fig. 1), due to the decreased activity of mannosyltransferase with mannosylphosphoryl-polyprenol as compared to mannosylphosphoryl-dolichol (Table II). In turn, reduced transfer of mannose from mannosylphosphorylpolyprenol results in normal levels of that intermediate, despite its reduced rate of synthesis.

The largest difference (about tenfold) between mutant and parent is observed in the levels of $Glc_3Man_9GlcNAc_2$-P-P-lipid, the final intermediate in the pathway. This is consistent with the reduced activities of GlcNAc 1-P-transferase and mannosyltransferase with polyprenol as acceptor and donor, respectively. We explored the possibility that other factors may contribute to decreased elongation of $Man_5GlcNAc_2$-P-P-polyprenol. Analysis of the oligosaccharide structure revealed no abnormality (Fig. 2). Addition of radiolabeled mannosylphorphoryldolichol to cell-free extracts of CHB11-1-3 followed by analysis of lipid-linked oligosaccharides demonstrated incorporation of label into oligosaccharides larger than $Man_5GlcNAc_2$ [unpublished data]. This indicates both that the mannosyltransferase of the mutant is functional and that the enzyme can use the polyprenol-linked oligosaccharide as acceptor.

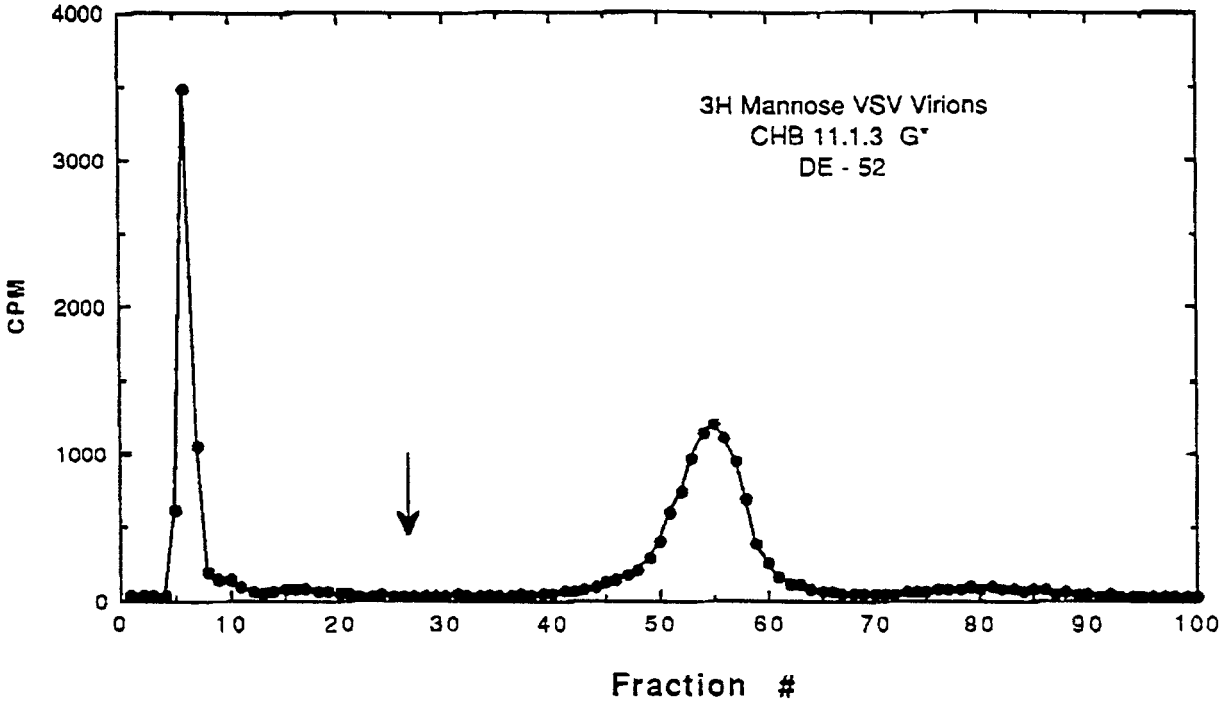

Fig. 7. Separation of mannose-labeled glycopeptides prepared from the underglycosylated form of G protein made by CHB11-1-3 cells. The underglycosylated form of G protein was purified from virions produced by CHB11-1-3 cells incubated with [2-^{3}H]mannose as described in Methods. Tryptic peptides were prepared, and tryptic glycopeptides were fractionated on a DE-52 anion exchange column. The arrow indicates the beginning of the linear salt gradient.

TABLE II. Summary of In Vitro Data Comparing the Effectiveness of Dolichyl and Polyprenyl Derivatives*

Enzyme	Substrate	K_m, app μM	V_{max}, app pmol/min	V_{max}/K_m	Ratio
GlcNAc 1-P transferase	Dol-P	0.13	3.1	24	83
	Pol-P	1.2	0.35	0.29	
Man-P-Dol synthase	Dol-P	64	29	0.45	6
	Pol-P	41	2.9	0.07	
Glc-P-Dol synthase	Dol-P	18	7.6	0.42	1.3
	Pol-P	14	4.6	0.32	
Chitobiose synthase	GlcNAc-PP-Dol	0.2	0.79	3.95	1.6
	GlcNAc-PP-Pol	0.23	0.58	2.5	
Mannosyl transferase	Man-P-Dol	1.0	6.4	6.4	17
	Man-P-Pol	6.8	2.5	0.37	
Glucosyl transferase	Glc-P-Dol	2.7	0.24	0.09	4.5
	Glc-P-Pol	6.0	0.1	0.02	

*The data used to prepare this table was published in McLachlan and Krag [1992, 1994] and D'Souza-Schorey et al. [1994]. app = apparent.

Mannosylphosphoryldolichol, glucosylphosphoryldolichol, and $Man_5GlcNAc_2$-P-P-dolichol each appear to be synthesized on the cytoplasmic face of the endoplasmic reticulum and then are translocated to the lumenal face [Hirschberg and Snider, 1987; Abeijon and Hirschberg, 1990] where $Glc_3Man_9GlcNAc_2$-P-P-dolichol is formed. Given the reduced utilization of both mannosylphosphorylpolyprenol and $Man_5GlcNAc_2$-P-P-polyprenol, an intriguing possibility is that polyprenol-linked saccharides are poorer substrates for translocation than dolichol-linked saccharides. Direct measurement of this translocation has not been possible as yet, although a model system for studying the translocation of mannosylphosphoryldolichol has recently

been developed utilizing citronellyl compounds [Rush and Waechter, 1995].

In this paper, we have analyzed the location of in vitro generated $Man_5GlcNAc_2$-P-P-dolichol and $Man_5GlcNAc_2$-P-P-polyprenol and found them both to be in the lumen (Figs. 3, 4). Thus, although we were unable to examine translocation itself, our results clearly indicate that $Man_5GlcNAc_2$-P-P-polyprenol can be translocated.

In CHB11-1-3, mannose-labeled $Glc_3Man_9GlcNAc_2$-P-P-lipid is reduced to a greater extent than mannose-labeled protein, tenfold and twofold, respectively, as compared to parental cells. This suggests that the oligosaccharide transferase is able to effectively utilize polyprenyl-linked oligosaccharides. Using VSV-infected cells as a model system, we have shown that a portion of the G protein synthesized in the mutant appears normally glycosylated with Endo H–sensitive oligosaccharides that are subsequently processed to the complex form. In addition, Endo H–resistant oligosaccharides are also transferred to protein in CHB11-1-3, generating a monoglycosylated form of G protein (Figs. 5, 7). Transfer can occur at either of the two glycosylation sites. We have been unable to detect VSV G containing either two Endo H–resistant oligosaccharides or one Endo H–sensitive and one Endo H–resistant oligosaccharide. Although both mono- and diglycosylated proteins are transported to the cell surface and packaged into virions, oligosaccharides on the former do not appear to undergo processing. Due to a defect in mannosylphosphoryldolichol synthase, only Endo H–resistant oligosaccharides are synthesized and transferred to protein in Lec15 mutants [Stoll et al., 1982]. In contrast to CHB11-1-3, VSV G in Lec15 mutants is diglycosylated, and the oligosaccharides are processed normally, albeit at a slightly slower rate [Stoll et al., 1992]. It remains to be determined why, in CHB11-1-3, Endo H–resistant oligosaccharides on G protein do not undergo processing.

Finally, Lec9 mutants contain higher levels of neutral prenol than do parental cells (Table I). Dolichyl pyrophosphate phosphatase and dolichyl phosphate phosphatase have been shown to utilize polyprenyl derivatives more efficiently than dolichyl derivatives [Keller et al., 1986; Adair and Cafmeyer, 1989; Wolf et al., 1991], whereas dolichol kinase utilizes dolichol better than polyprenol [Keller et al., 1982]. In Lec9 mutants, as polyprenyl phosphate or polyprenyl pyrophosphate are synthesized and/or recycled during glycosylation, they may be subjected to enhanced dephosphorylation, yielding polyprenol, which is inefficiently phosphorylated by the kinase.

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REFERENCES

Abeijon C, Hirschberg CB (1990): Topography of initiation of N-glycosylation reactions. J Biol Chem 265:14691–14695.

Adair WL Jr, Cafmeyer N (1989): Characterization of dolichyl diphosphate phosphatase from rat liver. Chem Phys Lipids 51:279–284.

Cummings RD (1992): Synthesis of asparagine-linked oligosaccharides: Pathways, genetics, and metabolic regulation. In: Allen HJ, Kisailus EC (eds): "Glycoconjugates: Composition, Structure, and Function." New York: Marcel Dekker, Inc., pp 333–360.

D'Souza-Schorey C, McLachlan KR, Krag SS, Elbein AD (1994): Mammalian glycosyltransferases prefer glycosyl phosphoryl dolichols rather than glycosyl phosphoryl polyprenols as substrates for oligosaccharyl synthesis. Arch Biochem Biophys 308:497–503.

Guthrie RD, McCarthy JF (1967): Acetolysis. Adv Carbohyd Chem 22:11–23.

Hall CW, Robbins AR, Krag SS (1986): Preliminary characterization of a Chinese hamster ovary cell glycosylation mutant isolated by screening for low intracellular lysosomal enzyme activity. Mol Cell Biochem 72:35–45.

Hirschberg CB, Snider MD (1987): Topography of glycosylation in the rough endoplasmic reticulum and Golgi apparatus. Annu Rev Biochem 56:64–87.

Kaiden A, Krag SS (1991): Regulation of asparagine-linked glycoproteins. Trends Glycosci Glycotech 3:275–287.

Keller RK, Rottler GD, Cafmeyer N, Adair WL Jr (1982): Subcellular localization and substrate specificity of dolichol kinase from rat liver. Biochim Biophys Acta 719:118–125.

Keller RK, Adair WL Jr, Cafmeyer N, Simon FA, Fleischer B, Fleischer S (1986): Characterization of polyisoprenyl phosphate phosphatase activity in rat liver. Arch Biochem Biophys 249:207–214.

Kornfeld R, Kornfeld S (1985): Assembly of asparagine-linked oligosaccharides. Annu Rev Biochem 54:631–664.

Krag SS (1979): A concanavalin A–resistant Chinese hamster ovary cell line lacks the ability to synthesize [^{3}H]glucosyl oligosaccharide-lipid. J Biol Chem 254:9167–9177.

Krag SS, Robbins PW (1977): Sindbis envelope proteins as endogenous acceptors in reactions of guanosine diphosphate [^{14}C]mannose with preparations of infected chicken embryo fibroblasts. J Biol Chem 252:3561–3564.

Krag SS, Robbins AR (1982): A Chinese hamster ovary cell mutant deficient in glucosylation of lipid-linked oligosaccharide synthesizes lysosomal enzymes of altered structure and function. J Biol Chem 257:8424–8431.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275.

McLachlan KR, Krag SS (1992): Substrate specificity of N-acetylglucosamine 1-phosphate transferase activity in Chinese hamster ovary cells. Glycobiology 2:313–319.

McLachlan KR, Krag SS (1994): Three enzymes involved in oligosaccharide-lipid assembly in Chinese hamster ovary cells differ in lipid substrate preference. J Lipid Res 35:1861–1868.

Quellhorst GJ Jr, Hall CW, Robbins AR, Krag SS (1997): Synthesis of dolichol in a polyprenol reductase mutant is restored by elevation of *cis*-prenyl transferase activity. Arch Biochem Biophys. 343:19–26.

Rome LH, Garvin AJ, Allieta MM, Neufeld EF (1979): Two species of lysosomal organelles in cultured human fibroblasts. Cell 17:143–153.

Rose JK, Gallione C (1981): Nucleotide sequences of the mRNA's encoding the vesicular stomatitis virus G and M proteins determined from cDNA clones containing the complete coding regions. J Virol 39:519–528.

Rosenwald AG, Krag SS (1990): Lec9 CHO glycosylation mutants are defective in the synthesis of dolichol. J Lipid Res 31:512–533.

Rosenwald AG, Stoll J, Krag SS (1990): Regulation of glycosylation. Three enzymes compete for the same pool of dolichyl phosphate in vivo. J Biol Chem 265:14544–14553.

Rush JS, Waechter CJ (1995): Transmembrane movement of a water-soluble analogue of mannosylphosphoryldolichol is mediated by an endoplasmic reticulum protein. J Cell Biol 130:529–536.

Sagami H, Kurisake A, Ogura K (1993): Formation of dolichol from dehydrodolichol is catalyzed by NADPH-dependent reductase localized in microsomes of rat liver. J Biol Chem 268:10109–19113.

Schleif RF, Wensink PC (1981): "Practical Methods in Molecular Biology." Berlin: Springer-Verlag.

Stanley P (1982): Carbohydrate heterogeneity of vesicular stomatitis virus G glycoprotein allows localization of the defect in a glycosylation mutant of CHO cells. Arch Biochem Biophys 219:128–139.

Stoll J, Robbins AR, Krag SS (1982): A mutant of Chinese hamster ovary cells with altered mannose 6-phosphate receptor activity is unable to synthesize mannosylphosphoryl dolichol. Proc Natl Acad Sci U S A 79:2296–2300.

Stoll J, Rosenwald AG, Krag SS (1988): A Chinese hamster ovary cell mutant F2A8 utilizes polyprenol rather than dolichol for its lipid-dependent asparagine-linked glycosylation reactions. J Biol Chem 263:10774–10782.

Stoll J, Cacan R, Verbert A, Krag SS (1992): Lec15 cells transfer glucosylated oligosaccharides to protein. Arch Biochem Biophys 299:225–231.

Wolf MJ, Rush JS, Waechter CJ (1991): Golgi-enriched membrane fractions from rat brain and liver contain long-chain polyisoprenyl pyrophosphate phosphatase activity. Glycobiology 1:405–410.

Zilberstein A, Snider MD, Porter M, Lodish HF (1980): Mutants of vesicular stomatitis virus blocked at different stages in maturation of the viral glycoprotein. Cell 21:417–427.