Lec9 CHO glycosylation mutants are defective in the synthesis of dolichol

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Abstract Lec9 Chinese hamster ovary cells were found previously to be defective in the synthesis of N-linked glycans. This appeared to be the result of a defect in the synthesis of oligosaccharide lipid and lipid phosphate (Rosenwald, Stanley, and Krag. 1989. *Mol. Cell. Biol.* **9:** 914–924). In this study we analyzed the steady state levels of long-chain polyisoprenyl lipids in Lec9 cells. We found that Lec9 cells are defective in the synthesis of dolichol. They accumulated a presumed precursor to dolichol, *cis*-a-unsaturated polyprenol and used this lipid in the synthesis of oligosaccharide lipid. Chain lengths of the activated polyprenols in Lec9 were the same lengths as dolichols in parental cells. Lec9 cells had increased levels of monosaccharylphosphoryl lipid and decreased levels of oligosaccharylpyrophosphoryl lipid compared to parental cells. The defect in Lec9 cells was specific for dolichol synthesis, since other aspects of [^{3}H]mevalonate metabolism in Lec9 cells were the same as in parental cells. We hypothesize that Lec9 cells are defective in polyprenol reductase activity. — **Rosenwald, A. G., and S. S. Krag.** Lec9 CHO glycosylation mutants are defective in the synthesis of dolichol. *J. Lipid Res.* 1990. **31:** 523–533.

Supplementary key words polyprenol • glycosylation mutants • mevalonate • N-linked glycans

Lec9 Chinese hamster ovary (CHO) cell glycosylation mutants have an increased proportion of β1,6-branched carbohydrates on proteins with complex asparagine-linked (N-linked) glycans (1, 2). This increased branching correlated with decreased tumorigenicity of Lec9 CHO cells in nude mice compared to parental CHO cells (1). In addition, proteins in Lec9 cells with high mannose N-linked glycans were also altered in structure and function, and in general, Lec9 cells underglycosylated proteins. The synthesis of dolichol-linked oligosaccharide intermediates was abnormal in Lec9 cells; incorporation of [2-^{3}H]mannose into $Glc_3Man_9GlcNAc_2$-P-P-lipid was decreased ~40-fold and incorporation into $Man_5GlcNAc_2$-P-P-lipid was decreased ~2-fold in Lec9 cells compared to parental cells. Lec9 cells also contained less polyisoprenyl lipid phosphate than parental cells (2).

Since long-chain polyisoprenyl lipid phosphate metabolism in Lec9 cells appeared to be altered, we examined long-chain polyisoprenoid synthesis in these cells. Dolichol is a poly-*cis* isoprenol in which the terminal (α) isoprene unit is saturated. The major long-chain lipid found in mammalian cells (3), including CHO cells (4), is dolichol. The concentration of dolichyl phosphate, the immediate precursor for synthesis of the lipid-linked oligosaccharides used for N-linked glycosylation, is believed to be an important factor in determining the amount of glycosylation that occurs (5–11).

The initial steps in the synthesis of dolichol phosphate are in common with the synthesis of cholesterol (12, 13). First, mevalonate, the product of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), is converted by a series of enzymatic steps to isopentenyl pyrophosphate. Then, three molecules of isopentenyl pyrophosphate are condensed to form farnesyl pyrophosphate. Long-chain polyprenols appear to be synthesized by sequential head-to-tail additions of isopentenyl pyrophosphate onto a farnesyl pyrophosphate core, forming polyprenyl pyrophosphates of increasing chain length. Such a long chain *cis*-prenyl transferase has been described in mammalian cells (14, 15) and in yeast (16).

The order of the final steps in the synthesis of dolichyl phosphate, if there is an obligate order, is as yet unclear (17). As mentioned above, dolichyl phosphate contains a saturated isoprene at its terminus. In order to convert polyisoprenyl pyrophosphate (the presumed end-product of the *cis*-prenyl transferase reaction) to dolichyl phosphate, reduction and dephosphorylation (and perhaps rephosphorylation) of the terminal isoprene unit are re-

Abbreviations: CHO, Chinese hamster ovary; FBS, fetal bovine serum; MEM, modified essential medium; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; CM 2:1, chloroform–methanol 2:1; Glc-P-lipid, glucosylphosphoryl lipid; Man-P-lipid, mannosylphosphoryl lipid; Glc-P-dol, glucosylphosphoryldolichol; Man-P-dol, mannosylphosphoryldolichol.

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quired. Further, dolichols exist not only as free alcohols and as phosphorylated and glycosylated derivatives but also as acyl esters (17). Interconversions of the various dolichol derivatives occur in cells and may play a role, in addition to de novo synthesis, in regulating the level of dolichyl phosphate (5).

In this study, we present evidence that Lec9 cells, as analyzed by incorporation of [^{3}H]mevalonate into polyisoprenoid lipids, have a specific defect in the synthesis of dolichol. Lec9 cells were found to synthesize prenols of the same lengths as those in parental cells. However, it appeared that Lec9 cells were unable to saturate the double bond in the terminal isoprene unit, so that Lec9 cells contained *cis*-α-unsaturated polyprenol rather than α-saturated dolichol. Further, Lec9 cells used primarily the phosphorylated derivatives of polyprenol to synthesize lipid-linked oligosaccharides. Use of the unsaturated polyprenol affected the steady state levels of all the glycosylated derivatives of prenols in Lec9 cells. We hypothesize that Lec9 cells are deficient in polyprenyl reductase activity.

MATERIALS AND METHODS

Enzymes, chromatography media, chemicals, and radiochemicals

Wheat germ acid phosphatase, n-octyl glucoside, mevalonate, and all chromatography standards, with the exception of polyprenol, were from Sigma Chemical Co. (St. Louis, MO). *Cis*-α-unsaturated polyprenol was extracted from cherry leaves as described (18). MnO_2 (activated and suitable for organic oxidations) was from Aldrich Chemical Co. (Milwaukee, WI). DEAE-cellulose was from Schleicher and Schuell (Keene, NH). Silica TLC plates were from J. T. Baker Chemical Co. (Phillipsburg, NJ). Fractogel HW.40F was from E.M. Science (Cherry Hill, NJ). [2-^{3}H]Mevalonate was from Amersham (Arlington Heights, IL). [5-^{3}H]Mevalonate was from DuPont/NEN (Wilmington, DE). En^{3}Hance spray was also from DuPont/NEN and was used according to manufacturer's directions. X-Omat AR film was from Eastman-Kodak (Rochester, NY). Liquiscint scintillation fluid was from National Diagnostics (Palmetto, FL). Mevinolin was a gift from Dr. April R. Robbins (NIH).

Cells and cell culture

The Chinese hamster ovary (CHO) cells used in these experiments were the glycosylation mutant Lec9.4A and its parent, wild type Pro$^-$5 (19). Many of the experiments shown here were also performed with two other members of the Lec9 complementation group, Lec9.4C and Lec9.12A. All three mutants gave similar results (data not shown). The cells were provided by Dr. Pamela Stanley (Albert Einstein College of Medicine). Cell culture was performed as previously described (2), except the cells were always passaged in α-modified essential medium (MEM) containing 10% fetal bovine serum (FBS) and were always grown attached to plates, rather than sometimes in suspension.

Incubation of cells with [^{3}H]mevalonate and isolation of labeled polyisoprenoid lipids

We have established conditions for steady state labeling of long-chain polyisoprenyl lipids in CHO cells (A. G. Rosenwald, J. Stoll, and S. S. Krag, unpublished results). Briefly, cells were plated on 100-mm dishes in α-MEM containing 10% fetal bovine serum (FBS). The following day, the media was replaced with 10 ml α-MEM containing 10% FBS with 20 μCi/ml [2-^{3}H] or [5-^{3}H]mevalonate (0.3 mM final concentration) and mevinolin (12 μg/ml final concentration), a hydroxymethyl glutaryl CoA reductase inhibitor. Both isotopes gave similar results. Cells were incubated with the radioactive mevalonate for 24-96 h. Steady state labeling was achieved by 24 h. Under these conditions, the doubling times of Pro$^-$5 and Lec 9.4A were 16–19 h, the same as under normal growth conditions.

The incubation was terminated by removing the radioactive medium, washing the cells quickly four times with chilled phosphate-buffered saline, and scraping the cells in three 1-ml aliquots of ice-cold methanol. Lipids and oligosaccharide-lipids were extracted as previously described (2). The chloroform–methanol 2:1 (CM 2:1) fraction was separated on DEAE-cellulose as described (4) to obtain a neutral lipid fraction and an anionic lipid fraction. All chromatographic separations were performed as described in the appropriate figure legends.

RESULTS

Steady state levels of phosphorylated prenols are altered in Lec9 cells

We previously found that the alterations seen in the glycosylation of proteins with either complex or high-mannose glycans in Lec9 cells could be explained as pleiotropic manifestations of a mutation early in the synthesis of lipid-linked oligosaccharides (2). In that study, the amount of polyisoprenyl lipid phosphate in the mutant was less than that found in parental cells. This decrease in lipid phosphate in Lec9 cells was always accompanied by an increase in the amount of monoglycosylated, phosphorylated lipids, glucosylphosphoryl lipid (Glc-P-lipid) and mannosylphosphoryl lipid (Man-P-lipid), so that the ratio of counts incorporated into these lipids, to incorporation into lipid phosphate was always higher in the mutant (average 4.7 $\pm$ 1.5) than in the pa-

rent (average 2.3 ± 0.6%). We found the decrease in lipid phosphate to be somewhat variable, however; the mutant contained 20–90% of the amount found in the parent, with the average being 65%.

The TLC system used to determine the ratios described above does not separate Man-P-lipid from Glc-P-lipid. To further explore the difference in ratios seen, we analyzed this fraction by a paper chromatography system that separates glucosylphosphoryldolichol (Glc-P-dol) and mannosylphosphoryldolichol (Man-P-dol) (**Fig. 1**). The results show that, in the mutant, there was a decrease in the amount of lipid phosphate and an increase in the amount of both monosaccharide-P-lipids, especially Man-P-lipid. As had been previously determined in wild type cells (A. G. Rosenwald, J. Stoll, and S. S. Krag, unpublished results), [^{3}H]mevalonate-labeled products with the mobility expected for lipid pyrophosphate or N-acetylglycosaminylpyrophosphoryl lipid were not found in Lec9 cells.

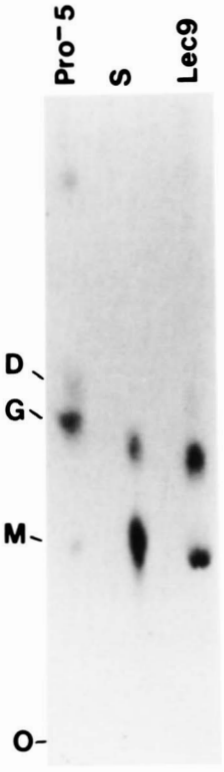

Fig. 1. Separation of [5-^{3}H]mevalonate-labeled Man-P-lipid, Glc-P-lipid, and Lipid-P on silica-impregnated paper. SG-81 paper was pretreated by dipping once in 2.5% Na_2EDTA and twice in 0.4 M boric acid. Samples of anionic lipids from a total of 14.0×10^6 parental cells and 9.2×10^6 Lec9 cells (two plates of each) that had been incubated for 72 h with [5-^{3}H]mevalonate were desalted by extraction, dried under a stream of N_2, resuspended in a small volume of CM 2:1, and spotted on the activated paper. Standards ([^{14}C]mannosylphosphoryldolichol, [^{3}H]glucosylphosphoryldolichol, and cold dolichyl phosphate) were also spotted. The chromatogram was developed in chloroform–methanol–ammonium hydroxide–water 60:25:2:2. The dolichyl phosphate lanes were stained with I_2 vapor. The rest of the chromatogram was sprayed with En^{3}Hance and exposed to film at −80°C for 5 days; lane 1: lipids from parental cells (Pro$^-$5); lane 2: radioactive standards; lane 3: lipids from Lec9 cells (clone Lec9.4A). Radioactivity incorporated into the various lipids was determined by cutting the chromatogram into 0.5-cm slices, eluting the counts from the paper with methanol–water 1:1 for 60 min at 60°C, adding 3 ml scintillation fluid, and counting. Counts per minute/million cells was determined. The counts shown have also been corrected for recoveries. Recoveries of counts from the chromatogram were 51% for parental cells and 61% for Lec9 cells. Parent: Man-P-lipid = 130, Glc-P-lipid = 320, and Lipid-P = 170 cpm/million cells. Lec9: Man-P-lipid = 510, Glc-P-lipid 450, and Lipid P = 150 cpm/million cells.

We found previously, using short pulses with ^{3}H-labeled sugars as tracer, that incorporation into the major oligosaccharide-lipid found in wild type cells, $Glc_3Man_9GlcNAc_2$-P-P-lipid, was severely reduced in Lec9 cells compared to wild type cells. The minor intermediate in parental cells, $Man_5GlcNAc_2$-P-P-lipid, was also reduced in the mutant, but not to as large an extent (2). To determine steady state levels of these intermediates, we analyzed the [^{3}H]mevalonate-labeled oligosaccharide lipids from wild type and Lec9 cells by silica TLC (**Fig. 2**, note the difference in scales). The data show that the amount of the large oligosaccharide-lipid in Lec9 was less (30-fold) than in wild type cells, and that the amount of the smaller oligosaccharide-lipid was also reduced (2-fold).

From the data presented in Figs. 1 and 2, we know that the steady state levels of *1*) Man-P-lipid and Glc-P-lipid were increased; *2*) polyisoprenyl lipid phosphate was decreased; and *3*) oligosaccharide-lipid intermediates, especially $Glc_3Man_9GlcNAc_2$-P-P-lipid, were decreased in the mutant compared to parental cells. This phenotype is not consistent with the notion that the mutation in Lec9 cells resides in a mannosyl transferase that uses Man-P-lipid as a substrate, for even though Man-P-lipid appeared to accumulate in the mutant, there was not a concomitant increase in an oligosaccharide-lipid acceptor (Fig. 2 and in previous data with [^{3}H]sugar-labeled oligosaccharide lipids [2]). Also, we found the same intermediates on proteins at early times after synthesis in Lec9 and wild type cells, although in different proportions (ref. 2 and data not shown). Thus it appeared that Lec9 and parental cells transferred the same oligosaccharides from lipid to protein.

Since we found that the mutant cells had twofold less total activated (i.e., phosphorylated and glycosylated) prenol than parental cells (**Table 1**), an alternative explanation for our results is that Lec9 cells are defective in maintaining the correct ratio of activated to neutral prenols (free and acylated). For example, Lec9 cells may have an inactive dolichol kinase or an over-active phosphatase. To examine these possibilities, we analyzed the neutral lipids found in wild type and Lec9 cells after incubation with [^{3}H]mevalonate.

Lec9 cells accumulate polyprenol rather than dolichol

Dolichol and dolichyl acyl esters are a small proportion (approximately 5%) of the labeled lipids found in the neu-

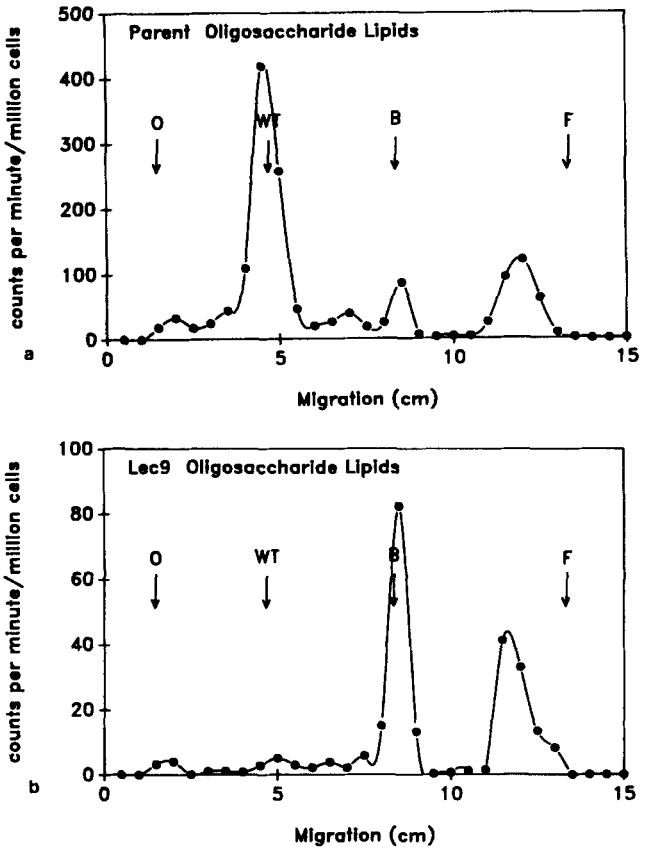

Fig. 2. Separation of [5-^{3}H]mevalonate-labeled oligosaccharide-lipids on silica TLC. Oligosaccharide-lipid samples from a total of 10.6 × 10^6 parental cells (a) and 10.2 × 10^6 Lec9 cells (b) (two plates of each) that had been incubated with [5-^{3}H]mevalonate for 72 h were dried under a stream of N_2, brought up in small volume of chloroform–methanol–water 10:10:3, and spotted onto a silica TLC plate (nonactivated). The chromatogram was developed in the same solvent mixture in a tank equilibrated with solvent for 1 h. Radioactivity in 0.5-cm slices of the chromatogram was determined as described in the legend to Fig. 1. The relative migration of peaks of radioactivity was compared to the migration of [2-^{3}H]mannose-labeled oligosaccharide lipids from wild type cells (WT) in which the primary species found is $Glc_3Man_9GlcNAc_2$-P-P-lipid and B4-2-1 cells (B) in which the only species found is $Man_5GlcNAc_2$-P-P-lipid (4). Recovery from the TLC was 50% for both cell types. The data shown have been corrected for recoveries and are normalized for cell number. The identity of the material at the front is unknown, but may be due to incomplete extraction of CM 2:1-soluble material, breakdown of the oligosaccharide-lipids during sample preparation, or the presence of oligosaccharide-lipids containing only a few sugars. Parent: $Glc_3Man_9GlcNAc_2$-P-P-lipid = 890, $Man_5GlcNAc_2$-P-P-lipid = 130 cpm/million cells. Lec9: $Glc_3Man_9GlcNAc_2$-P-P-lipid = 15, $Man_5GlcNAc_2$-P-P-lipid = 115 cpm/ million cells.

tral fraction in wild type cells (**Fig. 3**). However, in the mutant cells, a three- to fourfold increase in the amount of labeled material that comigrated with dolichol was seen when the neutral lipids were analyzed by gel filtration chromatography (compare the two panels in Fig. 3). This column does not separate dolichol from dolichyl esters, so this peak represented both compounds. Thus, in parental cells the ratio of activated to neutral prenols was approximately 3:1, while in the mutant cells the ratio was approximately 1:1.5 (Table 1).

To analyze the accumulated neutral material further, we took advantage of an HPLC system that separates long-chain isoprenoid compounds that differ in the oxidation state and configuration of the terminal (α) isoprene unit; the column will separate *cis*-α unsaturated from α-saturated from *trans*-α unsaturated prenols (4, 21). We found that the material that accumulated in Lec9 cells was not α-saturated dolichol, but instead comigrated with *cis*-α-unsaturated polyprenol (**Fig. 4**, Lec9 Neutral Prenols), whereas the material that was found in the parental cells was primarily dolichol (Fig. 4, Parental Neutral Prenols). The peak of labeled material that eluted at the breakthrough of the HPLC column appeared to be prenyl ester. When the pool of material that comigrated with cold dolichol by gel filtration was saponified prior to separation on silica HPLC, the material from wild type cells appeared to be mostly dolichol, suggesting that the esters were mostly dolichyl esters (Fig. 4, Parent Neutral Prenols [Saponified]), while the saponified material from Lec9 cells appeared to be polyprenol, suggesting the esters in the mutant cells were polyprenol esters (Fig. 4, Lec9 Neutral Prenols [Saponified]). Thus, the defect in the mutant cells appeared to be in the synthesis of dolichol itself. The aberrant ratio of activated to neutral prenols found in the mutant may, therefore, be secondary to the defect in dolichol synthesis.

Lec9 cells use polyprenol to synthesize saccharide-lipids

Since Lec9 cells appeared to have little or no neutral dolichol (Fig. 4), we next determined whether these cells were using polyprenyl phosphate or dolichyl phosphate to synthesize saccharide-P-P-lipids. Wild type and Lec9

TABLE 1. Comparison of amount of long-chain polyisoprenols in Pro$^-$5 and Lec9.4A cells

Prenols	Pro$^-$5 Cells	Lec9.4A Cells
	cpm/million cells	
Neutral prenols	690	1990
Anionic prenols (total)	990	1100
Prenyl phosphate	420	200
Man-P-lipid	200	470
Glc-P-lipid	370	440
Oligosaccharide prenols (total)	1270	260
$Glc_3Man_9GlcNAc_2$-P-P-lipid	580	20
$Man_5GlcNAc_2$-P-P-lipid	220	90
Total activated prenols	2260	1360
Total prenols	2950	3360

Parental cells (Pro$^-$5) and Lec9 cells (clone Lec9.4A) were incubated with [5-^{3}H]mevalonate for 72 h. Amounts of the different polyisoprenyl forms in each cell type were determined by separation of lipids into neutral, anionic (followed by separation on silica paper as in Fig. 1), and oligosaccharide lipid fractions (followed by separation on silica TLC as in Fig. 2). All the values shown are the average of at least three determinations and have been corrected for recoveries at the various steps in isolation. Standard deviations were not more than 30%.

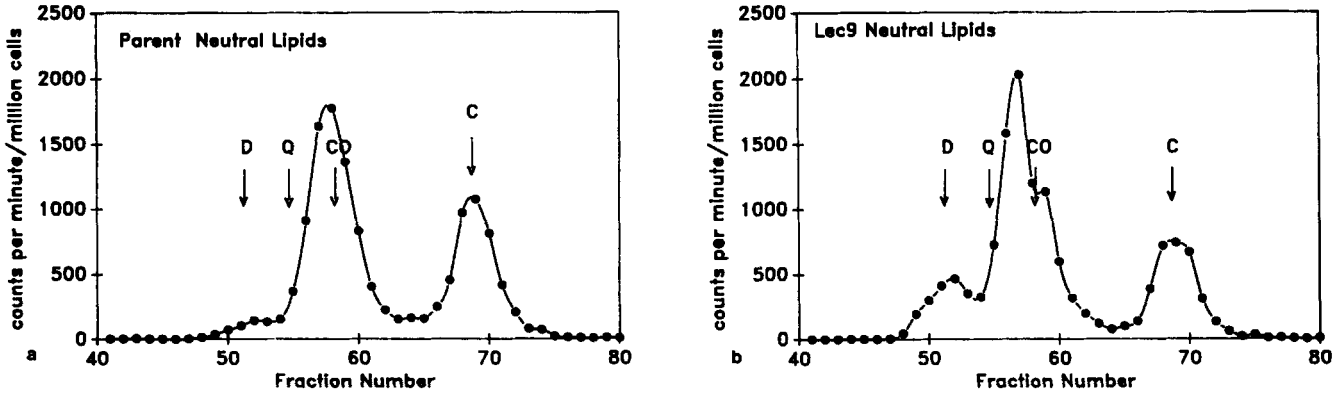

Fig. 3. Separation of [5-^{3}H]mevalonate labeled neutral lipids by gel filtration chromatography. Neutral lipids from a total of 19.4 × 10^6 parental cells (a) and 17.5 × 10^6 Lec9 cells (b) (three dishes of each) incubated with [5-^{3}H]mevalonate for 72 h were separated by gel filtration chromatography on a 1 × 45 cm Fractogel HW.40F column equilibrated in CM 1:1 plus 50 mM ammonium acetate. Two-hundred and fifty μg each of dolichol (D), coenzyme Q (Q), cholesteryl oleate (CO), and cholesterol (C) were added as internal standards. Fractions were resuspended in 1 ml CM 2:1 and 100-μl aliquots were removed to determine radioactivity. Ten-μl aliquots were spotted on silica TLC plates to determine elution position of the standards. The TLC plates were developed in hexane–diethyl ether–glacial acetic acid 7:3:0.15 and the standards were detected by anisaldehyde stain (20). The data shown have been normalized for cell number and corrected for recoveries. Recoveries from the column were 79% for Parent Neutral Lipids (top panel) and 87% for Lec9 Neutral Lipids (bottom panel).

cells were incubated with [^{3}H]mevalonate and the activated forms were extracted, converted to neutral species by treatment with mild acid and phosphatase, and analyzed by adsorptive HPLC. As shown in **Fig. 5**, the lipid used to synthesize both the monoglycosylated, phosphorylated species and the oligosaccharide-containing species in parental cells was primarily dolichyl phosphate (Fig. 5, Parent Anionic Lipids and Parent Oligosaccharide Lipids). The lipid used to synthesize these forms in the mutant, on the other hand, was primarily polyprenyl phosphate. Polyprenyl (pyro)phosphates are susceptible to hydroxyl migration in the presence of mild acid (3); the other forms seen on HPLC analysis of the Lec9 samples (eluting at 3 min and 6.5 min) are diagnostic of polyprenyl (pyro)phosphates. In both fractions, however, some dolichol was seen in the mutant (8% in the anionic lipid fraction (Fig. 5, Lec9 Anionic Lipids) and 15% in the oligosaccharide lipid fraction (Fig. 5, Lec9 Oligosaccharide Lipids).

The presence of dolichol in the fractions derived from Lec9 cells was not an artifact of the way the samples were treated. When labeled cells were immediately subjected to saponification, and the lipids were separated by DEAE-cellulose chromatography, the material that bound to the column (originating from all glycosylated, phosphorylated lipids except Man-P-lipid) after treatment with phosphatase also had approximately 12% dolichol (**Fig. 6**, bottom panel). Polyprenyl (pyro)phosphates are not susceptible to hydroxyl migration under conditions of strong base; in this case, all of the remaining material co-migrated with polyprenol. The material that did not bind to the anion-exchange column after saponification (originating from the neutral lipids and Man-P-lipid) did not contain significant amounts of dolichol (Fig. 6, top panel), consistent with the results found previously (see Fig. 4). The average amount of total prenols seen was similar (70% of the totals derived from Table 1 for both wild type and Lec9 cells) when cellular lipids were directly saponified, as shown here, compared to the analysis of separate fractions (Table 1).

As further proof that the long-chain polyisoprenyl lipid found in Lec9 cells was α-unsaturated polyprenol, we took advantage of the fact that allylic hydroxyl groups are susceptible to oxidation under mild conditions whereas hydroxyl groups adjacent to saturated bonds are not (23). Total labeled long-chain prenols were isolated from Lec9 and parental cells and treated with the mild oxidant, MnO_2. As can be seen in **Fig. 7**, prenols derived from parental cells are not altered by this treatment, while prenols derived from Lec9 cells are altered from the mobility of polyprenol to that for the oxidized form, polyprenal. It should be noted that all samples contained unlabeled polyprenol as carrier during the reaction. The extent of oxidation of the unlabeled material was similar in both samples.

Defect in [^{3}H]mevalonate metabolism in Lec9 cells is specific to long-chain polyisoprenoid synthesis

It appeared that Lec9 cells were unable to convert α-unsaturated polyprenol to dolichol, but were able to use polyprenol to synthesize saccharide-lipids, although the ratios of the various polyprenyl derivatives in Lec9 cells were different from the dolichyl derivatives seen in parental cells (Table 1). We then examined other aspects of mevalonate metabolism in parental and Lec9 cells to determine the specificity of the lesion.

The major products of [^{3}H]mevalonate incorporation in CHO cells are cholesterol, cholesteryl esters, and ubiquinone (4). As can be seen by gel filtration analysis of

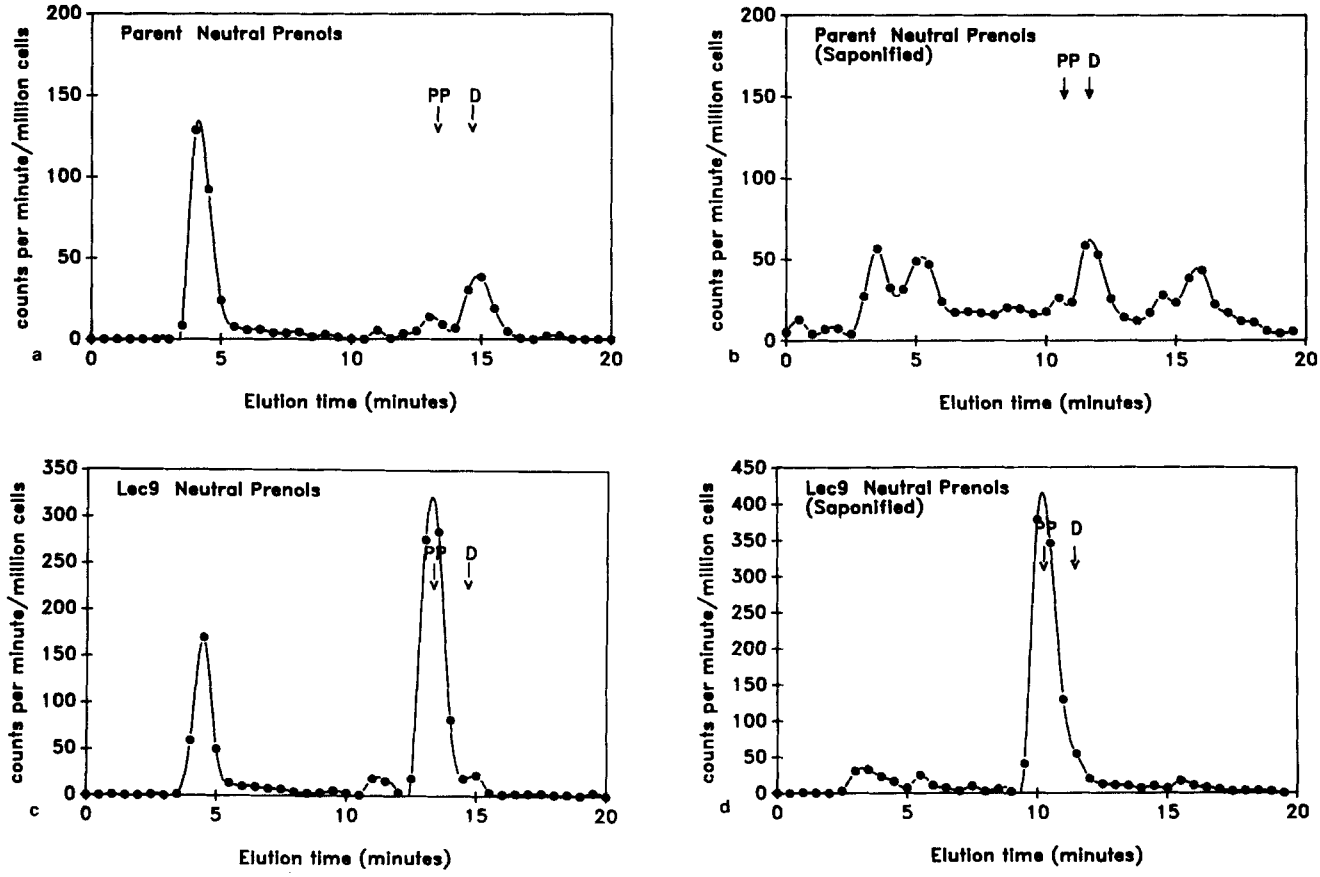

Fig. 4. Analysis of neutral prenols by adsorptive HPLC. Parental cells (4.24×10^6 for Neutral Prenols (a) and 8.33×10^6 for Neutral Lipids [Saponified] (b)) and Lec9 cells (3.77×10^6 for Neutral Lipids (c) and 7.7×10^6 for Neutral Lipids [Saponified] (d)) were labeled for 24 h with [2-^{3}H]mevalonate. Neutral lipids were isolated and separated by gel filtration (Fig. 3). The radioactive material that comigrated with the internal dolichol standard was pooled and desalted by extraction. The samples were separated on a 3.9 mm × 30 cm silica (straight-phase) column (Microporosil, Waters, Milford, MA) run isocratically in hexane–diethyl ether 97:3 on a Varian 5000 HPLC at a flow rate of 1 ml/min. Fractions (0.5 min) were collected and counted after evaporation of the solvent. Internal standards were detected by absorbance at 214 nm. The standards are dolichol (D) and *cis*-α-unsaturated polyprenol (PP). For the first two panels, the lipids were dried under a stream of N_2, brought up in hexane–diethyl ether 97:3, and injected onto the HPLC column to measure the amount of free alcohol. For the last two panels (denoted Saponified), the lipids were first saponified (see the legend to Fig. 6), then extracted again before preparation for injection, to measure the amount of total neutral prenols. The data are presented as counts incorporated/million cells and have been corrected for recoveries. Recoveries from the HPLC column were 30%, 36%, 53%, and 66% for panels Parent Neutral Prenols, Lec9 Neutral Prenols, Parent Neutral Prenols (Saponified) and Lec9 Neutral Prenols (Saponified), respectively. The small peaks of material eluting between 3 and 7 min in the saponified samples appear to be the result of saponification-induced breakdown products of coenzyme Q that contaminated this fraction due to generous pooling from the gel filtration column (see Fig. 3 for the relative elution positions of prenols [denoted dolichol] and coenzyme Q), rather than incomplete saponification. This was demonstrated by analyzing the silica HPLC elution profile of purified, labeled coenzyme Q after it had been subjected to the same saponification conditions as described above (data not shown).

neutral lipids (Fig. 3), the amount of label incorporated into these products was not significantly different between mutant and wild type cells. Further, we found that parental and mutant cells had approximately the same mass amounts of cholesterol and cholesteryl ester and these products had approximately the same specific activity as determined by the cholesterol oxidase method described (data not shown; A. G. Rosenwald, J. Stoll, and S. S. Krag, unpublished results).

Dolichols in cells are found as a distribution of isoprenyl chain lengths (3). The major form found in the neutral polyisoprenyl lipids of CHO cells contains 19 isoprene units (C_{95}) (4). We next determined chain lengths of the neutral and activated polyprenols found in Lec9 cells and compared these to chain lengths of neutral and activated dolichols found in parental cells. We found both neutral (data not shown) and activated (**Fig. 8**) forms to be primarily 19 isoprene units in length in parental cells and Lec9 cells.

Post-translation modification of proteins with mevalonate has been described (24). We found that [^{3}H]mevalonate was incorporated into cellular proteins of parental and Lec9 cells to approximately the same extent. When the labeled proteins were analyzed by SDS-PAGE, it ap-

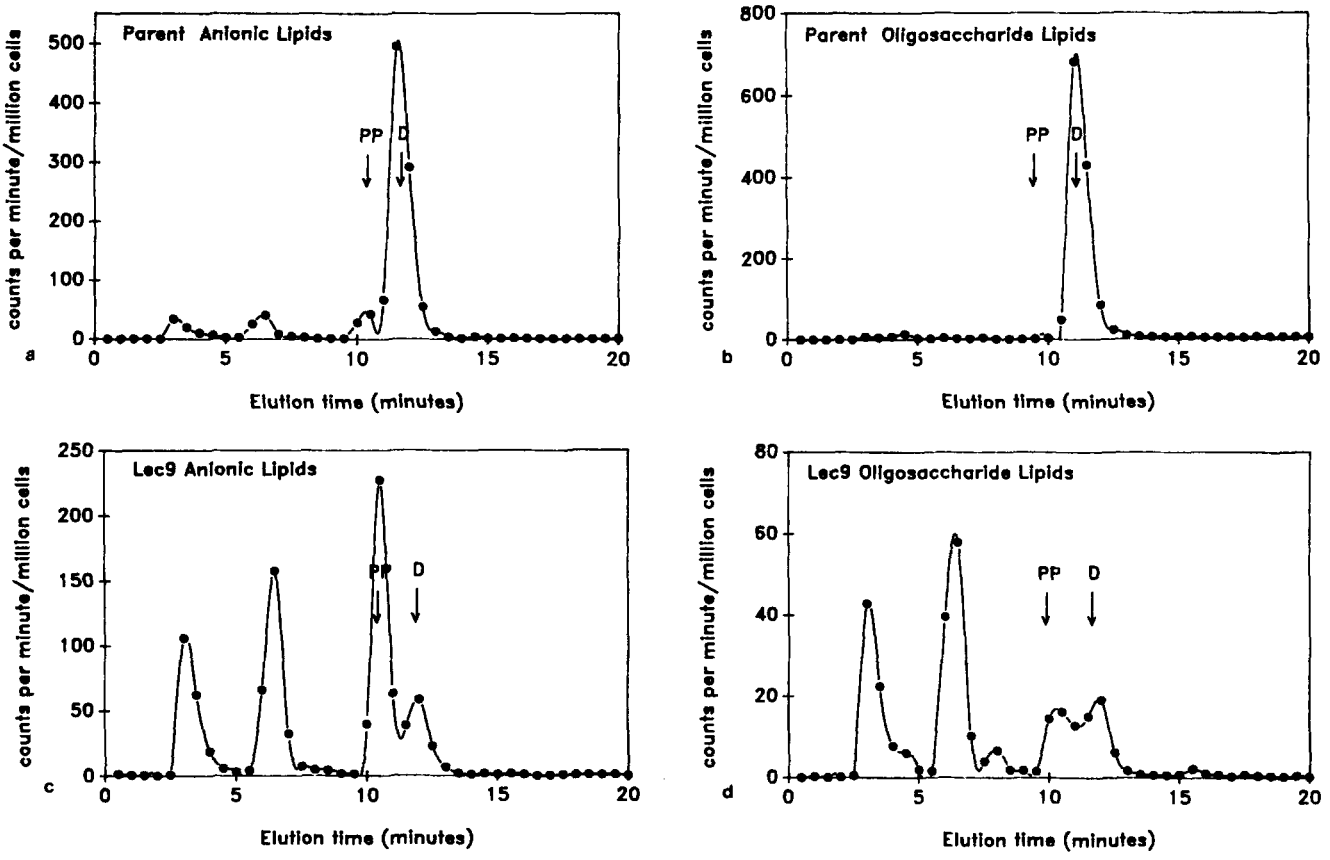

Fig. 5. Analysis of the lipid moiety used in the activated prenols. The anionic lipids and oligosaccharide lipids from a 72-h incubation of 18.3 × 10^6 parental cells (a, b) (three dishes) and 37.5 × 10^6 Lec9 cells (c, d) (five dishes) with [5-^{3}H]mevalonate were converted into neutral molecules and analyzed by adsorptive HPLC (Fig. 4). The glycosylated, phosphorylated molecules were first subjected to mild hydrolysis (2, 4, 22). The resulting phosphorylated lipids were dried, desalted, and then dephosphorylated by treatment with wheat germ acid phosphatase in n-octyl glucoside (4). The lipids were then purified by gel filtration chromatography (Fig. 3) to rid the samples of octyl glucoside. The data shown are normalized for cell number and have been corrected for recoveries. The recoveries from the HPLC column were 75%, 83%, 86%, and 41% for panels Parent Anionic Lipids, Lec9 Anionic Lipids, Parent Oligosaccharide Lipids, and Lec9 Oligosaccharide Lipids, respectively.

peared that parental and Lec9 cells contained similar molecular weight proteins (data not shown).

We found that wild type cells secreted [^{3}H]mevalonate-labeled, chloroform-extractable material, 20% of which appeared to be cholesterol (A. G. Rosenwald, J. Stoll, and S. S. Krag, unpublished results). Lec9 cells secreted approximately the same amount of material, and of this, about 20% appeared to be cholesterol. Thus, the mutation seen in Lec9 cells appeared to be confined to synthesis of long-chain prenols, specifically, reduction of the terminal isoprene unit.

DISCUSSION

Lec9 CHO cells aberrantly glycosylate a variety of proteins, including vesicular stomatitis virus glycoprotein (G protein) and the lysosomal enzyme, β-glucuronidase, as well as bulk [^{3}H]mannose-labeled proteins. In addition, the function of the mannose 6-phosphate receptor and the compartmentalization of β-glucuronidase and β-hexosaminidase are altered in Lec9 cells. All of the effects on glycosylation appear to stem from a deficit in the amount of lipid phosphate present in these cells from which oligosaccharide lipid intermediates are synthesized (2). In this study, we directly analyzed the synthesis of polyisoprenyl lipid phosphate by incorporation of [^{3}H]mevalonate into lipids of the Lec9 mutant and the parental cells.

Lec9 cells are defective in the synthesis of dolichol. *Cis*-α-unsaturated polyprenol was the major prenol found in the neutral fraction (Figs. 3 and 4) and was the major substrate used in the synthesis of the oligosaccharide lipid intermediates (Fig. 6). Lec9 cells were capable of synthesizing prenols of the same chain lengths as the parental cells (Fig. 8), but appear to be incapable of efficiently catalyzing the reduction of the double bond in the terminal isoprene unit of long-chain prenols.

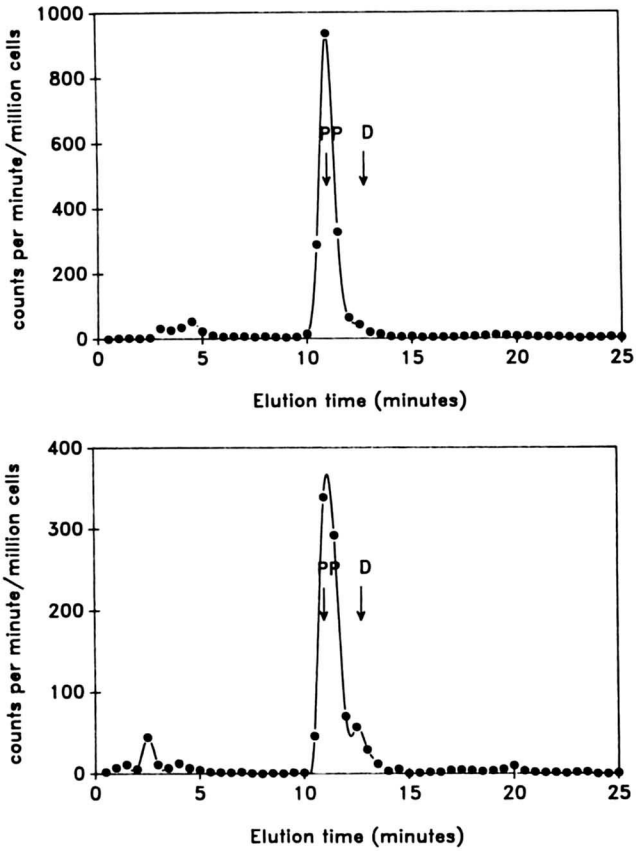

Fig. 6. Analysis of total prenols from Lec9 after saponification. A total of 9.1 × 10^6 Lec9 cells (two dishes) were incubated with [5-^{3}H]mevalonate for 72 h. The labeling reaction was terminated by scraping the cells into three 1-ml aliquots of ice-cold methanol. Sixty percent KOH (1.5 ml) and one crystal of butylated hydroxytoluene were added to the methanol and this mixture was heated for 1 h at 100°C. The reaction mixtures were cooled and the mixture was extracted with three 3-ml aliquots of diethyl ether. The ether was then extracted with 9 ml 5% acetic acid (23). The ether was dried under a stream of N_2. The lipids were resuspended in CM 2:1 and separated by DEAE-cellulose chromatography. The neutral lipids were directly separated by gel filtration chromatography (Fig. 3), while the bound lipids were first treated with phosphatase (Fig. 5). The material comigrating with the internal dolichol standard was pooled, desalted, and analyzed by adsorptive HPLC (Fig. 4). The top panel shows the neutral lipids from Lec9 and the bottom panel shows the phosphorylated lipids after treatment with phosphatase. The data shown have been normalized for cell number and corrected for recovery. Recoveries from the HPLC column were 56% and 72% for the top and bottom panels, respectively. The internal standards are D, dolichol and PP, polyprenol.

The most straightforward explanation of these results is that polyprenol reductase activity is deficient in Lec9 cells. The characteristics of this enzyme are presently unknown, including the subcellular location, the chemical nature of the substrate (i.e., polyprenol or a phosphorylated form), and the source of reducing equivalents, although it has been found that rat liver supernatants require NADH to synthesize dolichols from mevalonate (25). Another possibility is that polyprenol pyrophosphatase or phosphatase is defective in Lec9 cells rather than the reductase. In this case, the product of the prenyl transferase, polyprenyl pyrophosphate, would not be converted to polyprenol to be subsequently reduced. However, our finding that polyprenol rather than polyprenyl phosphate or polyprenyl pyrophosphate accumulated in Lec9 cells argues against this possibility.

The effect of the lack of polyprenol reductase activity in Lec9 cells was an alternation in the steady-state levels of the various polyisoprenyl derivatives in Lec9 cells compared to parental cells. We found a 2-fold reduction in the amount of lipid phosphate (Fig. 1), a 1.5- to 2-fold accumulation of monosaccharide-P-lipid (Fig. 1), a 1.5-fold re-

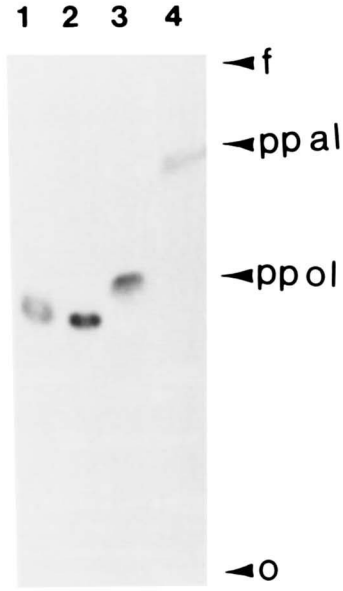

Fig. 7. Oxidation of polyprenol, but not dolichol, by treatment with MnO_2. Total long-chain prenols were isolated from parental cells (11.84 × 10^6 from two dishes) and Lec9 cells (5.76 × 10^6 from two dishes) after incubation of cells with [5-^{3}H]mevalonate for 72 h by directly saponifying cells as described (Fig. 6). The material extracted into the ether fraction was then treated with wheat germ acid phosphatase (Fig. 5). The long-chain prenols were then separated from the other nonsaponifiable lipids by gel filtration chromatography (Fig. 3) and thin-layer chromatography on silica plates developed in hexane–diethyl ether–glacial acetic acid 7:3:0.15. Each sample was divided into two aliquots and dried under a stream of N_2. Each aliquot received 400 μg unlabeled polyprenol in 200 μl hexane. One aliquot of prenols from each cell type also received 3.5 mg MnO_2. All aliquots were incubated at 4°C with shaking for 1 h. The MnO_2 was removed by centrifugation and the supernatant was transferred to a clean tube. The prenols were further extracted from the MnO_2 by incubating for 5 min at ambient temperature with 0.3 ml H_2O, then adding 2 ml of chloroform–methanol (1/1). The chloroform–methanol–H_2O 10:10:3 supernatant was then pooled with the hexane supernatant, then the entire mixture was dried under a stream of N_2, brought up in a small volume of chloroform–methanol 2:1, spotted onto a silica TLC plate, and developed in hexane–diethyl ether–glacial acetic acid 7:30:0.15. The internal unlabeled polyprenol was detected with I_2 vapor, and then the chromatogram was sprayed with En^{3}Hance and exposed to film for 7 days. Lanes 1 and 2 are prenols derived from parental cells, lanes 3 and 4 from Lec9 cells. The prenols in lanes 1 and 3 were untreated and the prenols in lanes 2 and 4 were treated with MnO_2. PPol refers to the migration of the *cis*-α-unsaturated polyprenol standard and PPal refers to the migration of the oxidized product, polyprenaldehyde. Note that the prenols found in the samples from parental cells have a slightly slower migration compared to the prenols found in the samples from the mutant cells. This is another indication that parental cells contain dolichol rather than polyprenol.

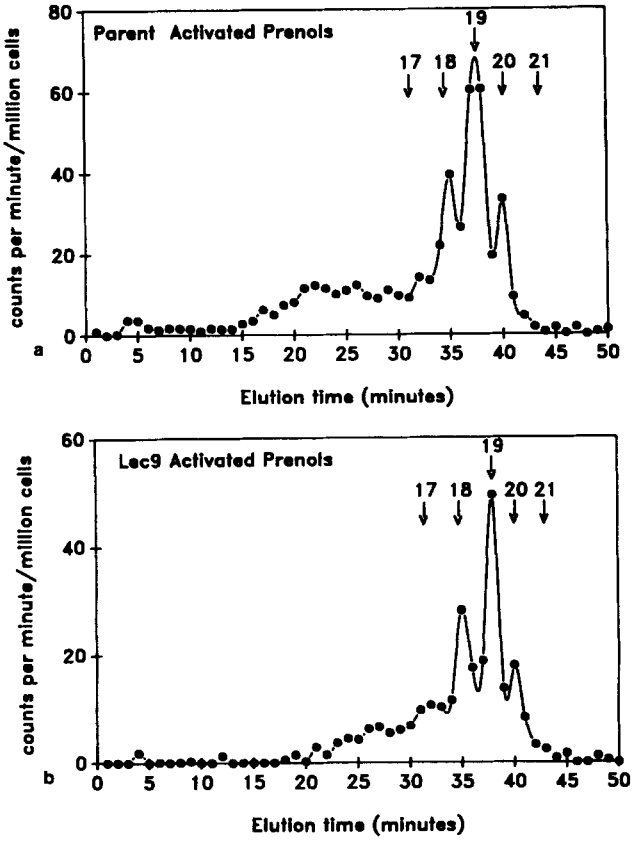

Fig. 8. Determination of chain length of activated long-chain polyisoprenoid lipids. Parental cells (9.0×10^6) and Lec9 cells (6.8×10^6) (two dishes of each cell type) were incubated with [5-^{3}H]mevalonate for 72 h. To obtain the activated prenol fraction, the anionic lipids and the oligosaccharide lipids were pooled, saponified, treated with phosphatase, and purified by gel filtration chromatography. The activated prenols were then analyzed by reverse-phase HPLC on a C_{18} column as previously described (4), except the column was mounted in a Dionex BioLC (Dionex, Sunnyvale, CA) with an internal UV detector. The standards were detected by absorbance at 214 nm. The chain lengths of the internal dolichol standard are given as 17–21. A single chain-length polyprenol (PP_{18}) was used to determine which peak of the standards was 18 isoprene units in length. The data shown have been corrected for recoveries and normalized for cell number. Recoveries from the HPLC column were 68% (Parent Activated Prenols) (a), and 66% (Lec9 Activated Prenols) (b), respectively.

duction in the amount of $Man_5GlcNAc_2$-P-P-lipid, and a 40-fold reduction in the amount of $Glc_3Man_9GlcNAc_2$P-P-lipid in Lec9 cells compared to parental cells (Fig. 2 and Table 1). We also found that neutral polyprenols (free alcohol and esters) accumulated in Lec9 cells 3- to 4-fold over the amounts of neutral dolichols found in parental cells. This accumulation is of interest since the presence of dolichol in bilayers has effects on permeability and fluidity of the bilayer (17). Polyprenol may have similar effects on aspects of membrane function.

It appears that in vivo, Man-P-dol synthase and Glc-P-dol synthase can use polyprenol phosphate effectively (Fig. 1 and Table 1). In vitro, it has been found that Man-P-dol synthase and Glc-P-dol synthase can use polyprenyl phosphate, but at reduced efficiency (26–29). Although mannosylphosphorylpolyprenol and glucosylphosphorylpolyprenol are made in Lec9 cells, the mannosyl and glycosyl transferases appear unable to efficiently transfer mannose and glucose from these aberrant lipids to the growing oligosaccharide. Alternatively, since it is believed that Man-P-dol and Glc-P-dol are synthesized on the cytoplasmic face and translocated to the lumen of the endoplasmic reticulum where they are used in the synthesis of the growing oligosaccharide chain (30) mannosylphosphorylpolyprenol and/or glucosylphosphorylpolyprenol may not be effective substrates for the "flippase(s)."

In contrast to the Man-P-dol synthase and Glc-P-dol synthase, it appears that GlcNAc-1-P transferase does not compete effectively in vivo for the limited amount of polyprenyl phosphate, since the steady state level of oligosaccharide lipid in Lec9 cells was 20% of the level found in parental cells. Spiro and Spiro (31) have suggested that all three enzymes that use dolichyl phosphate as a substrate (namely Man-P-dol synthase, Glc-P-dol synthase, and GlcNAc-1-P transferase) have access to the same pool of dolichyl phosphate. Presumably, they all have access to the same pool of polyprenyl in Lec9 cells. In vitro, GlcNAc-1-P transferase appears to be able use polyprenyl phosphate to only a limited extent (28, 29). This differential utilization of polyprenyl phosphate has a pronounced effect on oligosaccharide lipid levels and this appears to account for the aberrant glycosylation seen in Lec9 cells (1, 2).

We found a significant percentage of dolichol (about 12%) in both the anionic lipid fraction and the oligosaccharide-lipid fraction in Lec9 cells. Very little dolichol (<5%) was found in the neutral lipid fraction. This suggests that reductase activity is not totally abolished in these cells and that dolichyl phosphate is the preferred substrate in vivo. We also found a higher percentage of dolichol as the lipid moiety in the oligosaccharide lipid fraction than in the anionic lipid fraction (Fig. 5). This may reflect the preference of GlcNAc-1-P transferase for dolichyl phosphate in vivo and/or the presence of biosynthetic intermediates in long-chain prenyl synthesis (i.e., polyprenyl pyrophosphate) in the anionic lipid fraction.

It is not known whether Lec9 cells transfer glycans to asparagine residues exclusively from the limited amount of oligosaccharyl-P-P-dolichol available or whether oligosaccharyl-P-P-polyprenol can also act as a substrate, albeit a poor one, for oligosaccharyl transferase. It is known, however, in a mutant (F2A8) that contains only polyprenol (less than 1% of the long-chain prenols in this mutant are dolichol) (4) that glycosylation of proteins occurs at a level approximately 7-fold less than parental cells (22), implying that oligosaccharyl transferase can use polyprenol-containing substrates to some extent. F2A8 shares the same genetic defect as Lec9 cells (A. G. Rosenwald, P. Stanley, and S. S. Krag, unpublished results).

Finally, as mentioned above, it is not clear whether polyprenol or polyprenyl (pyro)phosphate is the immediate precursor to dolichol or dolichyl phosphate. Our finding that Lec9 cells accumulated *cis*-α-unsaturated polyprenol and polyprenyl esters (Figs. 3 and 4) rather than the phosphorylated forms may indicate that polyprenol is the substrate for reduction to dolichol. Alternatively, the substrate for reduction may be a phosphorylated form, but active phosphatases quickly convert the substrate to the neutral form. If polyprenol is a poor substrate in vivo for the CTP-dependent dolichol kinase as it is in vitro (32–34), this would explain the accumulation of polyprenol in the neutral fraction (Fig. 3), the decrease in lipid phosphate (Fig. 1), as well as the decrease in the overall amount of the activated forms in Lec9 cells (Table 1). These issues cannot be resolved until the reductase deficiency has been demonstrated directly in Lec9 cells and the nature of the substrate for this enzyme is determined. However, our results do suggest that addition of a presaturated C_5 unit, as proposed by Chojnacki and Dallner (17), is not occurring in CHO cells, since polyprenols in Lec9 cells had the same distribution of chain lengths as dolichols in parental cells (Fig. 8).

In summary, Lec9 cells have aberrant glycosylation as a direct result of a deficiency in the synthesis of dolichol, the polyisoprenoid lipid carrier for the synthesis of the oligosaccharide precursors to N-linked glycans. Lec9 cells appear to be defective in polyprenol reductase activity. The myriad effects on cellular functions, including changes seen in the branching of glycans on proteins (1, 2) in Lec9 cells resulting from this mutation provides evidence that the amount of dolichyl phosphate is an important determinant in regulation of glycosylation. Lec9 cells represent members of a unique class of glycosylation mutants: those that are defective in glycosylation as a result of a lesion in the synthesis of the long-chain polyisoprenyl carrier for oligosaccharide synthesis.

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