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BREAKTHROUGHS AND VIEWS

The Importance of Being Dolichol

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Linear, long-chain isoprenoids, polyisoprenoids with chain lengths greater than thirty carbons, are ubiquitous membrane constituents now known to be involved in glycan synthesis. They are present in small amounts (0.1%) relative to glycerol-based phospholipids (1,2,3), and are thought not to form bilayers, but rather type II lamellar structures (4,5). There are two different types of polyisoprenoid lipids known - one with all the isoprene units in the polymer unsaturated and one with the isoprene unit of the polymer adjacent to the hydroxyl group (alpha isoprene) saturated. In this minireview, I will refer to the former as polyprenol and the latter as dolichol.

An interesting feature of these polyisoprenoid lipids is that they are not present as a species of a single chain length, but are found in cells as a family of four or five different chain lengths, with one or two chain lengths predominating. For example, in Chinese hamster ovary (CHO) cells the predominant polyisoprenol is dolichol with 95 carbons, although dolichols of 85, 90, and 100 carbons are detected (6,7). Most likely this feature is the result of the mechanism of action of the biosynthetic enzyme, *cis*-prenyl transferase, that condenses isoprenyl units of isoprenyl pyrophosphate onto farnesyl pyrophosphate to a distribution of chain lengths (8).

The chain length of the major form of prenol varies from 55 carbons in eubacteria (9,10), 55 and 60 in *Trypanosoma* (11), 80 and 85 in *Drosophila* (12), 75 and 80 in *Saccharomyces cerevisiaei* (13), 85 in *Schizosaccharomyces pombe* (14), 95 in hamsters (6,7) to 100 carbons in pigs and humans (15). Prenols in plants range from 40 to 110 carbons; some species of plants have two classes of polyisoprenols, those of 80 to 110 carbons and a second distribution of prenols up to 200 carbons in length (16). As yet, no mutant has been described that results in an alteration of the chain length of a polyisoprenol. Therefore, we have no understanding of the role or function of a particular chain length distribution in a given system.

Polyisoprenoids are found in cells and tissues in a number of different forms. They are present as the free alcohol or esterified with acetate or fatty acids. These forms are particularly abundant in mammalian tissues such as liver, brain, and kidney (17,18). In dividing cells and in *S. cerevisiae* the predominant polyisoprenoid is phosphorylated (1,2,3,19). In CHO cells, for example, oligosaccharide-P-P-dolichol is the most abundant form (40 - 50% of total dolichol pool), with dolichyl phosphate, monoglycosylated phosphorylated dolichols, free dolichol, and esterified dolichol being less abundant (1,2,19).

Glycosylated phosphopolyisoprenoid lipids serve as glycosyl donors, substrates for glycosyl transferases in glycan synthesis, as do sugar nucleotides. The obvious difference in these two types of sugar donors is their solubility and consequently their intracellular location. There are a variety of glycosylated phosphopolyisoprenoid lipids known, two examples being mannosylphosphoryldolichol (Man-P-dolichol) and N-acetylglucosaminylpyro-phosphorylundecaprenol (GlcNAc-P-P-undecaprenol). Sugar nucleotides and glycosylphosphoryllipids are similar in that the linkage broken during the transfer of a glycose moiety in both cases is the phosphoglycosidic bond, generating NDP (or CMP in the case of CMP-sialic acids) or phosphopolyisoprenoid lipid. During the transfer of a phosphoglycose moiety, the linkage broken is the pyrophosphoryl bond, generating NMP or phosphopolyisoprenoid lipid, since in this latter case these reactions involve glycosylated pyrophosphopolyisoprenoid lipids.

In addition to being a sugar donor, phosphorylated polyisoprenoid lipids can serve as carriers of oligosaccharide units as they are being assembled before transfer. For example, in eukaryotic systems a specific oligosaccharide is preassembled on dolichyl pyrophosphate

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before it is transferred *en bloc* to an asparagine residue in the Asn-X-Ser/Thr consensus sequence of N-linked glycoproteins (20).

These oligosaccharide-P-P-polyisoprenoid molecules are amphipathic, with the lipid portion present in the endoplasmic reticulum (ER) membrane, and the oligosaccharide portion specifically localized either to the cytosolic face of the ER membrane or to the lumenal face of the ER membrane. Membrane-bound glycosyltransferases with their active sites facing either the cytosol or the lumen of the ER transfer glycosyl moieties to the oligosaccharide-P-P-polyisoprenoid lipid using as substrates either sugar nucleotides (UDP-GlcNAc and GDP-man) at the cytosolic face of the ER or glycosylphosphorylpolyisoprenoids at the lumenal face of the ER (21). At a particular point in the assembly process, the oligosaccharide portion of the oligosaccharide-P-P-polyisoprenoid lipid is thought to be translocated from the cytosolic face to the lumenal face of the ER. $Man_5GlcNAc_2$-P-P-dolichol is thought to be the oligosaccharide-P-P-lipid translocated (22). Man-P-dolichol, glucosylphosphoryldolichol (Glc-P-dolichol), dolichyl phosphate, and pyrophosphoryl dolichol are also translocated in the currently proposed biosynthetic pathway of the glycan of N-linked glycoproteins (23). Proteins involved in these translocations have yet to be isolated, although candidates have been proposed (24,25). Finally, the oligosaccharide portion of the oligosaccharide-P-P-lipid is transferred to the asparagine of the Asn-X-Ser/Thr consensus sequence, generating a glycosylated protein and dolichyl pyrophosphate (20). The latter molecule is hydrolyzed to dolichyl phosphate and translocated back to the cytosolic face of the ER (23).

Organisms contain either polyprenyl or dolichyl derivatives. For example, mammalian sources such as bovine liver (26), pig and human liver (15) and CHO cells (6,7) contain dolichyl phosphate while eubacteria (9,10) contain polyprenyl phosphate. *Drosophila* (12), *Trypanosoma* (11), *S. cerevisiae* (13), and *S. pombe* (14) all appear to contain dolichyl phosphate. Different species of Archae (27,28) and plants (16) seem to have either dolichyl phosphate or polyprenyl phosphate.

The determination of the identity of the prenol in a certain organism is based on three types of experiments. In some cases, enough material has been isolated to do mass spectrometry and nmr analyses on the isolated lipid (15,17,26,27). Alternatively, chromatography and chemical stability studies have been done on material labeled by cells incubated with metabolic precursors (*in vivo* experiments, 6,7,12,14). Finally, in some cases the identification of the prenol, as polyprenol or dolichol, used by an organism has been inferred by determining whether dolichyl or polyprenyl derivatives are utilized preferentially as a substrate in assays of membrane fractions (*in vitro* experiments, 28).

Studies thus far have not provided clear insight as to the reason some organisms have dolichols and some have polyprenols. Two possible hypotheses have been suggested. Organisms with higher growth temperatures may have dolichol because of its greater chemical stability (29). Rush, Rick, and Waechter suggested an alternative hypothesis, that because of the acid lability of polyprenyl derivatives, eukaryotes having acidic compartments such as lysosomes and endosomes involved in their intracellular trafficking, may have evolved the ability to produce dolichyl derivatives (30). However, finding polyprenyl derivatives in plants, that also have acidic compartments, argues against this hypothesis. The finding of polyprenyl derivatives in eubacteria and some archae, organisms having extremely harsh growth conditions, argues against both hypotheses.

As mentioned above, *in vitro* studies using either polyprenyl or dolichyl derivatives in assays using membranes generally agree with other types of studies on the identity of the prenol. For example, the UDP-NAc-Glc:dolichyl phosphate N-acetylglucosaminyl phosphate transferase from CHO cells prefers dolichyl phosphate (31) while the comparable enzyme from *E. coli* prefers undecaprenyl phosphate (30). The preferences for the prenol in *in vitro* systems can be profound - ten to twenty-fold differences, with affects on both $K_{m,app}$ and $V_{max,app}$ (31, 32).

However, the differences in substrate preference are often less pronounced. For example, only two-fold differences in the utilization of Man-P-dolichol and Man-P-polyprenol by the glycosyltransferase that mannosylates the GPI anchor in CHO cells has been reported (33). Also, the Glc-P-dolichol synthase of CHO membranes only utilizes dolichyl phosphate two-times better than polyprenyl phosphate and there appears to be no substrate preference for either GlcNAc-P-P-dolichol or GlcNAc-P-P-polyprenol by the chitobiosyl transferase in CHO membranes (34). Finally, the "wrong" lipid was found to be a better substrate for the phosphatase in liver membranes, in that polyprenyl phosphate was hydrolyzed faster than dolichyl phosphate (35).

It is clear from an analysis of mutants that the saturation state of the polyisoprenoid lipid is important for the functioning of the cell. Mutants in the CHO Lec9 complementation group have been found to synthesize and utilize polyprenyl derivatives rather than dolichyl derivatives (6,7,29,36). Lec9 cells incubated with labeled mevalonolactone, the metabolic precursor of prenols, to steady state levels contain primarily polyprenol whereas parental CHO cells contain primarily dolichol, although some of the precursor polyprenol is found (6,7,29,36). The enzyme activity responsible for the conversion of polyprenol to dolichol, polyprenol reductase, is thought to be deficient in these cells, although polyprenol reductase activity has yet to be assayed directly in membrane preparations in CHO cells or Lec9 mutants (37).

Lec9 cells have been isolated from parental CHO cells in a variety of different ways, but all based on altered glycosylation of the cells. Two members of this complementation group were isolated based on lectin resistance. Lec9.4A cells were resistant to ricin (38) and B211 cells were resistant to concanavalin A (36). F2A8 cells were isolated due to their lower incorporation of labeled mannose into glycoprotein compared to parental cells (39). Finally, CHB11-1-3 cells were isolated based on their lowered amount of intracellular lysosomal enzyme activity (40). Interestingly, B211 cells had also been found previously to synthesize lowered amounts of intracellular lysosomal enzyme activity (41). It is unclear why the Lec9 complementation group is so easily isolated from CHO cells. An eventual analysis of the gene of polyprenol reductase may reveal a large mutational target size or a hot spot of mutation within the gene.

Parental and Lec9 CHO cells have the same steady-state level of prenol; parental cells have dolichol and Lec9 cells have polyprenol. However, the form of the prenol, whether it is neutral (prenol and esterified prenol) or activated (prenyl phosphate, monoglycosylated phosphorylated prenol, and oligosaccharide-P-P-prenol) differs significantly between parental and Lec9 cells (6,7,19). The level of oligosaccharide-P-P-polyprenol is reduced three- to four-fold in Lec9 cells compared to the level of oligosaccharide-P-P-dolichol in parental cells, and the level of neutral polyprenol in Lec9 cells is increased compared to the level of neutral dolichol in parental cells. In addition, Lec9 cells accumulate primarily $Man_5GlcNAc_2$-P-P-polyprenol in the oligosaccharide-lipid fraction, whereas parental cells accumulate primarily $Glc_3Man_9GlcNAc_2$-P-P-dolichol in the oligosaccharide-lipid fraction (7). These differences in the levels of the various prenol forms and the synthesis of $Man_5GlcNAc_2$-P-P-polyprenol rather than $Glc_3Man_9GlcNAc_2$-P-P-polyprenol can be explained by examining the substrate preferences of enzymes for polyprenol and dolichol (31,34,42) and by assuming that translocation occurs normally with both polyprenol and dolichol derivatives (19).

N-linked protein glycosylation is affected in Lec9 cells, although not to a level as quantitatively significant as one might expect. The amount of mannose incorporation into $Glc_3Man_9GlcNAc_2$-P-P-polyprenol is reduced twenty fold in Lec9 cells compared to the amount of mannose incorporation into $Glc_3Man_9GlcNAc_2$-P-P-dolichol in parental cells (43). However, the amount of mannose incorporation into N-linked proteins is only reduced two-fold in Lec9 cells compared to parental cells.

An analysis of the oligosaccharides initially transferred to protein in Lec9 cells demonstrated that both $GlcMan_5GlcNAc_2$ and $Glc_3Man_9GlcNAc_2$ are transferred to protein (36). As mentioned above, the steady-state level of $Man_5GlcNAc_2$-P-P-polyprenol is three- to four-fold lower in Lec9 cells than the level of $Glc_3Man_9GlcNAc_2$-P-P-dolichol in parental cells. Presumably any $Glc_3Man_9GlcNAc_2$-P-P-polyprenol synthesized by Lec9 cells does not accumulate, but is used immediately as a substrate for oligosaccharide transferase. The implication of these results is that the oligosaccharide transferase activity in Lec9 cells transfers oligosaccharides from available oligosaccharide-P-P-prenol rather than having a preference for dolichyl over polyprenyl derivatives.

Studies of the glycosylation of G protein of vesicular stomatitis virus (VSV) in the various infected Lec9 lines demonstrated that G protein can be normally glycosylated (two glycans in CHO cells), underglycosylated (one glycan), and non-glycosylated (19,36,39,43). In the case of CHB11-1-3, for example, G protein with two, one, or no glycans attached was observed and in the case of one glycan, no preferential usage of the two potential glycosylation sites was seen (19). The simplest explanation of the observations is that reduced levels of oligosaccharide-P-P-polyprenol in Lec9 cells leads to lower oligosaccharide transferase activity.

There are interesting biological effects of the Lec9 mutation, although the mechanisms responsible for these effects are as yet unclear. Lec9 cells have reduced tumorgenicity in mice (44). VSV virions produced by Lec9 cells have increased infectivity (43). These latter two phenotypes are correlated with increased processing of the glycans transferred in Lec9 cells, indicated by increased levels of β-1,6 branching of N-linked carbohydrates (43,44). One possible explanation for this increased processing, that the level of the β-1,6 branching enzyme (GlcNAc-transferase V) is increased in Lec9 cells, has been ruled out (43). This increased processing of G protein occurs on the G protein that contains two glycan chains. In contrast, when a single glycan moiety is transferred to G protein in Lec9 cells, it does not appear to be processed (19,36).

As mentioned above, other phenotypes of Lec9 cells are observed, many of which led to the isolation of the mutants. The final structures of surface glycoproteins seem to be different in Lec9 cells and parental cells, leading to altered lectin resistance. Intracellular concentrations of some lumenal lysosomal enzymes are lower due to altered targeting of these enzymes. Lec9 cells incorporate less labeled mannose into glycoprotein, two-fold less at the growth temperature of 34°C and even less at 39°C, a temperature at which many screens were carried out in order to allow for the isolation of temperature-sensitive mutants. In fact, Lec9 cells are temperature sensitive for growth at 39°C (unpublished results). Finally, Lec9 cells grow in culture at 34°C, although some isolates grow at a slower rate than parental cells (unpublished results).

The finding in Lec9 mutants that the saturation state of the polyisoprenoid lipid is important to a cell has recently been extended to two other systems. Mu-

tants with reduced N-linked glycosylation have recently been isolated in *Trypanosoma brucei brucei* by Hwa and Englund (45) by selecting cells resistant to concanavalin A. One of the two mutants isolated (ConA1-1) was found to accumulate reduced levels of dolichol and increased levels of polyprenol (45). The dolichol/polyprenol ratio in parental cells was 9 while it was 0.6 in ConA1-1. The finding that the mutant has half dolichol and half polyprenol indicates that the mutant has lowered polyprenol reductase activity, and is consistent with there being two active polyprenol reductase alleles in *Trypanosoma,* one of the alleles being inactivated in the lectin-resistant mutant. This finding also implies that polyprenol reductase activity is rate-limiting for dolichol synthesis, a hypothesis also supported by data from experiments with CHO cell fusions (46).

It is interesting that the presumed heterozygote for the polyprenol reductase gene is detectable as defective in N-linked glycosylation. Preliminary experiments indicate that there is less activated (phosphorylated) prenol and more neutral prenol in ConA1-1 relative to parental cells, a phenotype similar to that seen in Lec9 cells.

A similar situation has recently been described by Ohkura et al., 1997 (47) in human fibroblasts. It appears that Carbohydrate-deficient Glycoprotein Syndrome (CDGS) Type I is a heterogeneous disease with a number of different complementation groups. Some CDGS Type I fibroblasts appear to have defects in lipid-linked oligosaccharide biosynthesis (48,49), defective mannose uptake (50) or defective conversion of mannose 6-phosphate to mannose 1-phosphate (51), but not to have defects in polyprenol reductase (49). However, Ohkura and coworkers found that some CDGS Type I fibroblasts had reduced dolichol synthesis and accumulated more polyprenol than dolichol (47).

In the human patient fibroblasts, there appears to be a partial reduction of polyprenol reductase activity rather than a marked reduction of activity seen in mutant CHO cells, consistent with there being one active allele and one inactive allele of polyprenol reductase in the defective human fibroblasts. Also, the heterozygote human fibroblasts appeared to be defective in N-linked glycosylation, being classified as a CDGS Type I. Parental fibroblasts had a dolichol/polyprenol ratio of 3 to 10 while CDGS Type I fibroblasts had a dolichol/polyprenol ratio of 0.2 to 1 (47). The oligosaccharide-lipid produced is oligosaccharide-P-P-dolichol, but it is present in lesser amounts in the CDGS Type I fibroblasts (47). It is not yet clear in either the trypanosomes or the human fibroblasts why an increase in the level of polyprenol leads to less oligosaccharide-P-P-prenol. However, preliminary data in the trypanosome system suggests that neutral prenols accumulate at the expense of the activated prenols, a situation that was also seen in Lec9 CHO cells. This accumulation of neutral polyprenol may result from preferential cleavage of polyprenyl phosphate compared to dolichyl phosphate (35) and the specificity for dolichol over polyprenol of the dolichol kinase (52).

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