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At the membrane frontier: A prospectus on the remarkable evolutionary conservation of polyprenols and polyprenyl-phosphates

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Abstract

Long-chain polyprenols and polyprenyl-phosphates are ubiquitous and essential components of cellular membranes throughout all domains of life. Polyprenyl-phosphates, which include undecaprenyl-phosphate in bacteria and the dolichyl-phosphates in archaea and eukaryotes, serve as specific membrane-bound carriers in glycan biosynthetic pathways responsible for the production of cellular structures such as N-linked protein glycans and bacterial peptidoglycan. Polyprenyl-phosphates are the only form of polyprenols with a biochemically-defined role; however, unmodified or esterified polyprenols often comprise significant percentages of the cellular polyprenol pool. The strong evolutionary conservation of unmodified polyprenols as membrane constituents and polyprenyl-phosphates as preferred glycan carriers in biosynthetic pathways is poorly understood. This review surveys the available research to explore why unmodified polyprenols have been conserved in evolution and why polyprenyl-phosphates are universally and specifically utilized for membrane-bound glycan assembly.

Keywords

polyprenol; polyprenyl-phosphate; dolichol; dolichyl-phosphate; N-linked glycosylation

Introduction

The long-chain polyisoprenoid alcohols, including polyprenols and dolichols, are a unique class of secondary metabolites within the isoprenoid natural product family (Fig. 1). These polyprenols and the phosphorylated derivatives comprise a small percentage of the total glycerophospholipid content in cellular membranes of bacteria (~1%) [1, 2] and eukaryotes (~0.1%) [3–5]. Polyprenyl-phosphates act as oligosaccharide carriers during glycan biosynthesis, which is essential to many conserved cellular processes including N-linked protein glycosylation, C- and O-protein mannosylation, and bacterial cell wall biosynthesis. Despite extensive research on the enzymes that utilize polyprenols in glycan assembly pathways, relatively little is known about why unmodified polyprenols populate cellular membranes and why polyprenyl-phosphates prevail as the most common membrane-bound glycan carriers in nature. This review begins with an overview of polyprenol structure and

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polyprenyl-phosphate dependent processes in biology. While providing essential background on the “what” and “where” of polyprenols, we will also address the more interesting question of “why polyprenols in the first place?”

Structural features of long-chain polyprenols

The linear polyprenols (or polyisoprenols) contain 7 to 24 isoprene units in either the *trans* or *cis* configuration and can broadly be separated into two subclasses [5–8]. The first class includes undecaprenol and related homologs and contains only unsaturated isoprene units, whereas the second class, known as dolichols, is distinguished by the presence of a single saturated isoprene unit in the α -position (Fig. 1). In both classes, α refers to the unit closest to the hydroxyl moiety and ω is used to designate the terminal isoprene unit in the linear structure. A typical polyprenol structure contains an ω isoprene unit followed by two or three *trans* isoprene units, and anywhere from eight to seventeen *cis* isoprene units (Fig. 1). Very recently, a third class of linear polyprenols termed ‘alloprenols’ has been identified in several plant species. Alloprenols possess an unsaturated structure as in the first class of polyprenols, but contain an unusual *trans* isoprene unit at the α -position [9, 10].

Many studies have focused on elucidating the process of polyprenol biosynthesis, which involves the enzyme-catalyzed condensation of dimethylallylpyrophosphate (DMAPP)¹ with multiple units of isoprenylpyrophosphate (IPP). The biosynthesis of isoprenoid alcohols depicted in Fig. 2 has been reviewed recently [7, 8, 11–13]. This general pathway is responsible for the production of a wide range of polyprenol structures, which vary in length across organisms, ranging from C55 polyprenols in bacteria and C95 dolichols in mammals to C200 polyprenols in plants (Table 1). Bacteria typically exploit undecaprenol, a C55 unsaturated polyprenol [14], while archaeal organisms typically contain C55–C65 dolichols [15], *Saccharomyces cerevisiae* (yeast) contain C80 dolichols [16] and mammalian tissues contain C90–C100 dolichols (Table 1) [17]. In addition, unusual polyprenols that include additional saturated isoprene units at the ω -terminus have been identified in *Mycobacterium* [18, 19] as well as the archaeal species, *Haloflex volcanii* [20] and *Sulfolobus acidocaldarius* [21].

Plants exhibit the widest range of polyprenol diversity, as both unsaturated polyprenols and dolichols with a wide range of lengths have been characterized [22–24]. The lipid bilayers of most plants contain C55 polyprenols, although in contrast to undecaprenol, these polyprenols contain three, rather than two, *trans* isoprene units, due to small differences at the beginning of the biosynthetic pathway [11]. Plants in the gymnosperm family contain C80–C100 polyprenols [23], while plants in the genus *Potentilla* contain mixtures of polyprenols with the longest possessing up to 200 carbons [25]. It is interesting to note the wide diversity of linear polyprenol architectures that has evolved amongst the three kingdoms of life, as the known functions of these molecules are very similar in all organisms.

To date, the question of why different organisms utilize polyprenols of different sizes and degrees of unsaturation has principally included studies on the biophysical effects of selected polyprenols in model lipid bilayers and the analysis of enzyme specificity for polyprenyl-phosphate substrates. However, neither of these methods can explain the

¹Abbreviations used: CDG, congenital glycosylation disorder; CHO, Chinese hamster ovary; diNAcBac, di-N-acetyl bacillosamine; Dol-P, dolichyl-phosphate; dolichyl-phosphate mannose; Dol-PP, dolichyl-diphosphate; DMAP, dimethylallylpyrophosphate; ER, endoplasmic reticulum; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; IPP, isopentenylpyrophosphate; Lipid I, Und-PP-MurNAc(pentapeptide); Lipid II, Und-PP-MurNAc(pentapeptide)GlcNAc; Man, mannose; MurNAc, N-acetylmuramic acid; NDP, nucleotide diphosphate; UDP, uridine diphosphate; Und-P, undecaprenyl-phosphate; Und-PP, undecaprenyl-diphosphate.

evolutionary driving force behind the varied lengths of polyprenols and it remains an important unanswered question in the field. Some speculation has focused on the origin of the α -isoprene subunit saturation. It has been suggested that α -saturation lends greater stability to dolichol relative to an unsaturated polyprenol, which might be an advantage for archaeal organisms that live in extreme conditions. However, many bacteria, which almost exclusively feature fully unsaturated polyprenols, survive in harsh conditions, and thus this argument is not convincing. As will be discussed later, it is well established that reduction of the α -isoprene subunit in dolichol biosynthesis (Fig. 2) is an important regulatory point in the eukaryotic pathway, and it may be that this element of structural diversity engenders greater biosynthetic complexity and allows for more precise regulatory control of the many processes reliant on dolichyl-phosphate.

Polyprenyl-phosphate as glycan carriers in biosynthetic pathways

Polyprenyl-phosphates are essential substrates for critical cellular functions in both eukaryotes and prokaryotes. These roles include N- and O-linked protein glycosylation in eukaryotes, archaea and bacteria and the biosynthesis of central structural components in bacteria such as peptidoglycan and O-antigen. Of these biosynthetic processes, N-linked protein glycosylation may be the most well known and the role of polyprenols in this process has been recently reviewed by Krag and coworkers [8]. It involves the stepwise assembly of an oligosaccharide on polyprenyl-diphosphate or polyprenyl-phosphate and occurs in eukaryotes, archaea, and several bacterial species (Fig. 3) [8, 26, 27]. In eukaryotes, a conserved dolichyl-diphosphate (Dol-PP) heptasaccharide (GlcNAc₂Man₅) is assembled on the cytoplasmic face of the endoplasmic reticulum (ER) membrane (Fig. 4) [28–30]. The Dol-PP-glycan intermediate is then translocated across the lipid bilayer to the ER lumen, where it is further glycosylated to form a GlcNAc₂Man₉Glc₃ tetradecasaccharide that is transferred to the nitrogen in the primary amide side chain of asparagine residues in nascent proteins (Fig. 4).

To date, characterization of N-linked glycosylation in archaea is limited, but these pathways are known to involve assembly of small, highly modified glycans on dolichyl-phosphate (Dol-P) carriers, such as the glycan depicted in Fig. 4 from *Methanococcus voltae* [31, 32]. Dolichyl-phosphate, as opposed to dolichyl-diphosphate, may act as the glycan carrier in certain archaeal species. Glycans linked to dolichol via mono- and diphosphate bridges have been identified [15, 20, 33–35], but Dol-P-glycans are used exclusively by archaea such as *Haloarcula marismortui* [36] and *Haloferax volcanii* [20]. It is intriguing that some archaeal species have evolved to use a monophosphate linkage exclusively in glycan assembly for N-linked glycosylation, and it may have to do with the greater stability of Dol-P-glycans. For instance, the diphosphate linkage is susceptible to hydrolysis under milder conditions than the monophosphate [15]. In addition, the β -glycosidic linkages of Dol-P-glycans, as opposed to the α -glycosidic linkages in Dol-PP-glycans, endow these molecules with greater stability, since the oxygen lone pair is not positioned to facilitate the C–O bond cleavage. Although the stereochemistry of Dol-P-glycans in archaea has not yet been determined, Dol-P-glycans found elsewhere in nature contain a β -glycosidic linkage. If monophosphate β -linkages are common in archaeal species, it could represent a way that these organisms have adapted to the extreme conditions of archaeal habitats.

Over ten years ago, the first bacterial N-linked glycosylation pathway was identified in the Gram-negative pathogen *Campylobacter jejuni* [37] and was later validated by the transfer of the operon into *Escherichia coli* [38]. More recently, genomic analysis suggests that homologous N-linked glycosylation pathways may be found in at least 84 different prokaryotic species [39]. The *C. jejuni* pathway represents the most well-characterized bacterial glycosylation system [27, 40–46], and involves the biosynthesis of an undecaprenyl-diphosphate (Und-PP) heptasaccharide prior to glycan transfer to asparagine

in proteins in the periplasmic space (Figs. 3 and 5). The similarities of the *C. jejuni* N-linked pathway to the eukaryotic pathway have been recently reviewed [26, 27], but it is important for the following discussion to note that both pathways rely on the stepwise addition of glycans to a membrane-anchored polyprenyl-phosphate carrier (Fig. 3). An interesting side note to bacterial N-linked glycosylation is that an O-linked protein glycosylation pathway dependent on undecaprenyl-phosphate has been discovered in *Neisseria* species [47–50]. O-linked glycosylation is a ubiquitous process in eukaryotes and prokaryotes, which relies on sequential transfer of monosaccharide units to assemble the glycan on the protein. Uniquely, this is the first example in which a glycan destined for a serine or threonine residue is assembled on undecaprenyl-diphosphate (Fig. 5) and then transferred to protein [47, 48, 51, 52], although genomic studies indicate that homologous pathways exist in many other bacterial species [39].

In addition to playing a role in bacterial protein glycosylation, undecaprenyl-phosphate acts as a membrane-associated carrier in the biosynthesis of many extracellular, oligosaccharide-based structures in bacteria (Fig. 5). Importantly, peptidoglycan intermediates are biosynthesized on an undecaprenyl-phosphate carrier; these intermediates include Und-PP-MurNAc(pentapeptide) (Lipid I) and Und-PP-MurNAc(pentapeptide)GlcNAc (Lipid II). After Lipid II is translocated across the membrane, peptidoglycan is assembled through disaccharide polymerization and peptide cross-linking to form a rigid cell wall barrier essential for bacterial viability. Because peptidoglycan biosynthesis has provided excellent targets for antibiotic development, much effort has focused on understanding how Lipid II precursors are biosynthesized and incorporated into the cell wall as described in the following references [12, 53, 54]. Several other essential bacterial components are synthesized via undecaprenyl-diphosphate intermediates including O-antigen polymers [55, 56], teichoic acids [57–60], and capsular polysaccharides [61–63]. These extracellular glycans are involved in bacterial defense mechanisms, mediate crucial microbial-host interactions and may also represent potential antibacterial target pathways.

Polyprenyl-phosphates as glycan donors

In addition to acting as glycan carriers, polyprenyl-phosphates play a second, essential biosynthetic role by acting as activated glycan donors; these molecules are membrane-bound alternatives [64] to nucleotide-diphosphate (NDP) activated donors (Fig. 6). Polyprenyl-phosphate monosaccharides are assembled on the cytoplasmic surface of cellular membranes from an NDP-sugar and polyprenyl-phosphate; these substrates are then translocated across the ER membrane in eukaryotes or the plasma membrane in prokaryotes (Figs. 4 and 5). Once translocated, they serve as glycan donors in the ER lumen or periplasm, where NDP-sugars are absent. In this way, cellular systems exploit polyprenyl translocation to transport activated sugar donors to regions that require glycan biosynthesis, but lack NDP-sugars.

More specifically, dolichyl-phosphate mannose (Dol-P-Man) and dolichyl-phosphate glucose (Dol-P-Glc) act as membrane-bound monosaccharide donors for a variety of biosynthetic purposes (Fig. 4). Importantly, Dol-P-Glc and Dol-P-Man participate in eukaryotic N-linked glycosylation by serving as monosaccharide donors for the assembly of Dol-PP-GlcNAc₂Man₉Glc₃ on the ER luminal interface [3]. Dol-P-Man is also the mannose donor for glycosylphosphatidylinositol (GPI)-anchor biosynthesis in the ER lumen, which is an essential process responsible for attaching eukaryotic proteins to the plasma membrane [65]. Dol-P-Man can also serve as a glycan donor for direct glycosylation of protein substrates. For instance, protein O-mannosylation relies on direct transfer of mannose from a Dol-P-Man donor in eukaryotes and archaea, or from an Und-P-Man donor in bacteria [64]. In addition, C-mannosylation of tryptophan residues in proteins via Dol-P-Man is a rare modification that has been identified in a handful of eukaryotic proteins [66].

Evolutionary conservation of polyprenols and polyprenyl-phosphates

In contrast to the well-established responsibilities of polyprenyl-phosphates in biology, free polyisoprenols have no clearly identified functions in biological systems, despite accumulating at high levels within certain organisms [3, 17, 23, 67–71]. As a result of this observation, it is believed that these compounds may play a distinct role in cellular physiology, rather than simply providing a substrate pool for polyprenyl-phosphate dependent processes. In the remainder of this review, the evolutionary conservation of polyprenols and polyprenyl-phosphates will be examined. In the next section we explore the potential roles of unmodified polyprenols in light of the distribution and abundance of polyprenols in eukaryotic and bacterial membranes. Then we will evaluate evidence for three hypotheses to explain why polyprenyl-phosphates may be the preferred glycan carriers in biology. (1) Polyprenyl-phosphates exert regulatory roles controlling glycan biosynthesis and ultimately cell growth. (2) Glycan translocation across lipid bilayers is a ubiquitous feature of all polyprenyl-phosphate-dependent pathways, suggesting that polyprenol structures may be involved in the enzyme-mediated mechanism. (3) Glycan assembly pathways reliant on polyprenol derivatives often contain many membrane-associated enzymes, which potentially form macromolecular complexes at the lipid bilayer interface dependent on the presence of polyprenyl-phosphate substrates.

Distribution and physical effects of polyprenols and polyprenol derivatives in membranes

Role of unmodified dolichols in eukaryotes

Polyprenyl-phosphates are important molecules in many biochemical processes, but unmodified polyprenols and esterified polyprenols are also present in biological membranes, and the functions for these molecules have not been well defined [3, 5, 11, 72, 73]. The concentrations of dolichol and dolichyl-phosphate have been determined for a variety of eukaryotic cell types including mammalian cell culture lines and tissue extracts from rats and humans using several quantification techniques (Table 2). These studies establish that a significant portion of the dolichol pool is in the unmodified alcohol form.

Close examination of the values in Table 2 reveals discrepancies in both the absolute and relative amounts of dolichol and dolichyl-phosphate. Some inconsistency is likely due to differences in the extraction, purification and quantification protocols. A recent review highlights the latest liquid chromatography/tandem mass spectrometry methods used for polyprenol isolation [74]. In general, Table 2 indicates that unmodified polyprenols constitute from as little as 9% to as much as 90% of the polyprenol pool depending on the organism and tissue type. One study suggests polyprenols tend to accumulate over the lifetime of the organism in a variety of tissues types [69], which is a phenomenon that has been observed in plants as well [23, 75] and may also account for other inconsistencies observed in Table 2. Importantly, the increases in the total dolichol level can be attributed to increases in the amount of unmodified dolichol with dolichyl-phosphate levels showing little change [69]. This finding has also been verified in human brain tissue, where free dolichols appear to accumulate (3 to 20-fold) over a lifespan, but dolichyl-phosphate levels remain relatively constant [76–78]. The cause of this increase is unknown, but it is suggestive that accumulation of polyprenols such as dolichol could be a factor in the side effects of aging [11, 73].

In addition to the potential role in aging, altered free dolichol levels have been measured in a variety of disease states suggesting that an increased dolichol concentration could serve as a biomarker for various diseases [11, 73] including liver cancer and cirrhosis, cataract disease, lysosomal storage disorders, and neurodegenerative diseases including Alzheimer's and

neuronal ceroid lipofuscinosis [11, 17, 73, 79–85]. Several studies in plants and mammals have indicated that polyisoprenols may have antioxidant properties and participate in scavenging of free radical oxygen species [86–88]. If this result is further validated, then it could provide some explanation for the elevated dolichols levels observed in disease states [11].

Studies on the cellular localization of dolichols have also suggested alternative functions for native and esterified dolichols. As expected, the endoplasmic reticulum membrane, which is the site of N-linked glycosylation, contains a significant amount of dolichol [72]. More surprisingly, several studies identified lysosomes as possessing high levels of dolichol derivatives esterified with a variety of fatty acids [72, 89]. This observation is interesting in light of the finding that altered dolichol levels were observed in lysosomal storage disorders [80]. It has been suggested that dolichols could play a role in the intracellular trafficking of fatty acids, or alternatively, that fatty acid derivatives of dolichols may act as transport vehicles for dolichols [72]. In addition, a key enzyme in dolichol biosynthesis, the *cis*-prenyltransferase, has been implicated in protein trafficking and retention within the dynamic ER and Golgi membrane network, suggesting that dolichol and its derivatives may play a role in protein localization [90, 91].

Role of unmodified undecaprenol in bacteria

Most studies on polyprenols have focused on mammalian tissues and plants, as it is more facile to extract significant amounts of material from these sources. However, the bacterial polyprenol, undecaprenol (also termed ‘bactoprenol’) is a key mediator in the biosynthesis of many essential bacterial structures including peptidoglycan, and a few studies have examined the relative populations of various undecaprenol derivatives in bacterial membranes (Table 3). Unlike eukaryotes, the Gram-negative bacterium, *Escherichia coli*, was found to contain only undecaprenyl-phosphate and undecaprenyl-diphosphate with no reserve pool of free undecaprenol [2]. In contrast, several studies revealed significant amounts of free undecaprenol in *Staphylococcus aureus* and other Gram-positive bacteria like *Streptococcus faecalis* (Table 3) [92]. It should also be noted that no undecaprenol kinases have been identified in Gram-negative bacteria, whereas several have been identified in Gram-positive bacteria [93–96]. In contrast, undecaprenyl-diphosphate phosphatases have been identified in both Gram-negative and Gram-positive bacterial species [12, 61, 97–99] and have been shown to be important for the generation of undecaprenyl-phosphate from undecaprenyl-diphosphate in *E. coli* [100]. Taken together, this evidence may suggest that *E. coli* uniquely produce undecaprenyl-phosphate from undecaprenyl-diphosphate via the action of a phosphatase, and therefore do not require unmodified undecaprenol. Further studies are imperative to determine if low levels of undecaprenol is a shared characteristic of all Gram-negative bacteria.

Biophysical effects exerted by polyprenols on membranes

The potential roles of polyprenols and dolichols in membranes have also been examined with biophysical approaches focused on elucidating the effects of these molecules in model membrane vesicles. A variety of techniques have been used to address this question including nuclear magnetic resonance (NMR) spectroscopy, differential scanning calorimetry, small angle X-ray diffraction, electrophysiology, and freeze-fracture electron microscopy [101–112]. From these studies, there is considerable evidence that polyisoprenols increase membrane fluidity, ion permeability and the propensity of membranes to adopt a non-bilayer, hexagonal II conformation (Fig. 7). Interestingly, a recent study determined that alloprenols, a newly discovered class of polyprenols in plants containing an α -isoprene unit in the *trans* configuration, increase membrane permeability to

a greater extent than the more common *cis* configuration [6, 9]. The authors postulated that *trans/cis* isomerization may be one way that plant cells regulate membrane permeability [6].

Additional structural information is gleaned from these experiments. Studies have suggested that dolichol and dolichyl esters are oriented parallel to the plane of the membrane and form aggregates near the center of the bilayer, whereas dolichyl-phosphates are monodispersed and oriented perpendicular to the membrane with the charged headgroup at the hydrophilic surface (Fig. 7) [103, 106]. Small-angle X-ray scattering and NMR studies [104, 109, 110, 113] suggest that the long-chain polyprenols do not adopt a linear conformation (Table 1) and instead collapse into three separate domains with a coiled center region, such that they can easily reside within the width of the lipid bilayer (Fig. 8). Biophysical studies comparing polyprenols with dolichols shows that the saturation at the α -subunit has little effect on the conformation of the molecules [104, 109, 110], although, some evidence suggests that increased dolichol length can slightly increase membrane destabilization [103]. However, in general, C95 dolichol and C55 undecaprenol have remarkably similar conformations and effects on model membranes (Fig. 8), despite significantly different lengths [104, 109, 110]. Currently, there is no clear evidence that the evolutionary conservation of these molecules within each species can be attributed to the biophysical effects exerted on the membranes. However, since the studies have to date been rather limited, it is possible that interesting paradigms may emerge in the future.

One caveat with many of the reported studies is that biophysical experiments on polyisoprenols are performed in model membranes containing pure phospholipids. These model membranes may be inadequate substitutes for native phospholipid bilayers, which contain a diverse array of phospholipids and membrane-associated proteins. Indeed, in a recent study, the inclusion of a small hydrophobic peptide into dolichol-containing membrane vesicles reversed the bilayer destabilization caused by dolichols [109, 110]. A second consideration is that biophysical studies are typically carried out at non-physiological concentrations with polyprenol comprising 1–20% of the phospholipids present [101–106, 109–112]. These concentrations are certainly higher than the overall 0.1% polyprenol present in eukaryotic membranes [3–5]. However, the local concentration of dolichol in membrane regions performing polyprenol-dependent biosynthesis is unknown and the higher concentrations may be a reasonable approximation. Clearly, further studies are needed to understand the effects of polyisoprenols in the context of physiological membranes, but it is apparent from the biophysical studies that have been reported to date that dolichol and dolichyl-phosphate have a profound effect on membrane properties. In the future it will be interesting to learn whether the biophysical effects of dolichol accumulation in the phospholipid bilayer could contribute to aging or other cellular processes. In addition, these biophysical studies may suggest that the absence of undecaprenol in *E. coli* has a structural role in maintaining the unique dual membrane architecture of Gram-negative bacteria, as it would minimize membrane fluidization. However, the undecaprenol levels have not been examined in other Gram-negative bacteria, and more work is needed in this area. In both eukaryotes and prokaryotes, much remains to be understood about the presence or absence of unmodified polyprenols, however, the quantification and biophysical studies presented here are important first steps towards understanding how these molecules may be exerting their influence on biology.

Pathway regulation by polyprenyl-phosphates

Our understanding of the roles and functions of unmodified polyprenols is still at an early stage. In contrast, significant research has focused on the ways in which polyprenyl-phosphates are utilized in membranes. This section will explore the role of polyprenyl-phosphates as regulators of essential cellular processes as a potential explanation for why

polyprenyl-phosphates are ubiquitous glycan carriers in both eukaryotes and prokaryotes. The role of polyprenyl-phosphates as regulatory molecules is well understood and the current status of that research will be summarized here.

Dolichyl-phosphate as a regulatory molecule in eukaryotes

The importance of dolichyl-phosphate in eukaryotes is well established [5, 13, 73, 114] and was first demonstrated in several early studies, which suggested that the absolute amount of dolichyl-phosphate modulated the levels of glycoprotein biosynthesis in eukaryotes [4, 115–118]. For instance, it was observed that estrogen-induced differentiation of hen oviducts resulted in increased production of Dol-P- ^{14}C Man when incubated with GDP- ^{14}C Man [116]. Further experimentation determined that the elevated amounts of Dol-P- ^{14}C Man could be attributed to higher levels of dolichyl-phosphate instead of increased enzymatic activity, thus implying that production of dolichyl-phosphate is an important regulator of cell differentiation [116]. In a related study, incubation of sea urchin embryos with an inhibitor of polyisoprenol biosynthesis resulted in abnormal gastrulation, which was correlated with the inability of the cell to produce glycoproteins. Furthermore, addition of exogenous dolichol allowed for normal gastrulation, suggesting that dolichyl-phosphate is a limiting reagent for N-linked glycoprotein biosynthesis and subsequent cellular transformations [119]. The rate of dolichyl-phosphate and glycoprotein synthesis has also been linked to the growth rate of CHO cells and cell division [4]. Other studies have confirmed that dolichyl-phosphate is a rate limiting substrate in N-linked glycosylation and is thereby a key factor in cellular development [3, 115]. It is clear from these studies that dolichyl-phosphate levels regulate the amount of glycoprotein biosynthesis, thus affecting important transitions during cellular development and growth. Because dolichol biosynthesis operates independently from phospholipid biosynthesis, it can be differentially regulated such that production of glycoproteins is unrelated to other membrane-bound processes.

An interesting facet of dolichyl-phosphate regulation is that it is as a substrate for several competing enzymes. Three glycosyltransferases utilize dolichyl-phosphate to produce the three dolichol derivatives required for N-linked glycosylation: Dol-P-Man, Dol-P-Glc and Dol-PP-N-acetylglucosamine (Dol-PP-GlcNAc) [3]. It has been shown that the activities of these three transformations are linked *in vivo* such that if one enzyme is inhibited, the activities of the other two increase with the availability of dolichyl-phosphate substrate [3, 120]. These results imply that the enzymes share a common substrate pool and further confirm that dolichyl-phosphate concentrations can limit production of glycoproteins.

Since dolichyl-phosphate levels control the rate of cellular development, it is known that dolichyl-phosphate biosynthesis must be regulated during growth. Dolichyl-phosphate biosynthesis, outlined in Fig. 2, is a complex process that is regulated at several important steps. Three of these are responsible for the direct production of dolichyl-phosphate; it is synthesized *de novo* by phosphorylation of dolichol, regenerated by hydrolysis of dolichyl-diphosphate, or released after monosaccharide transfer from Dol-P-Glc or Dol-P-Man (Fig. 9). For the first route, a CTP-dependent dolichol kinase has been identified, which has been shown to be essential during cellular growth and development [121]. However, *de novo* phosphorylation of dolichol is not considered to be the most important source of dolichyl-phosphate. Instead, the recycling route is considered more central to the generation of dolichyl-phosphate, in which pyrophosphatases hydrolyze dolichyl-diphosphate formed after glycan transfer reactions in the N-linked protein glycosylation pathway (Fig. 9). A third source of cellular dolichyl-phosphate arises from release of the molecule after transfer of Man or Glc from dolichyl-phosphate activated donors (Figs. 4, 6, and 9) [13]. The regenerated or released dolichyl-phosphate is then flipped by an unknown protein catalyst to the cytoplasmic face of the ER membrane to begin another cycle of glycan assembly. A recent study has established that recycling of dolichyl-phosphate, rather than

phosphorylation of dolichol, is the principal source of dolichyl-phosphate in ER membranes (Fig. 8) [119, 120]. In general, production of dolichyl-phosphate by *de novo* phosphorylation of dolichol may be important during periods of rapid cellular growth, but recycling of dolichyl-diphosphate by a pyrophosphatase may play a more significant regulatory role than the kinase in the maintenance of dolichyl-phosphate levels in non-dividing cells.

Two other crucial steps in dolichol biosynthesis are the elongation of the polyprenol by *cis*-prenyltransferase [122–124] and the reduction of the α -isoprene unit by polyprenol reductase [71]. Importantly, *cis*-prenyltransferase action, which involves the addition of 14–17 isoprene units onto geranyl-diphosphate, has been shown to regulate dolichol production [124]. A recent study has identified the Nogo-B receptor as important for regulation of this enzyme [125]. The Nogo-B receptor was found to be essential for the stability of the enzyme and provides some of the first experimental evidence into how dolichol biosynthesis might be regulated [125]. It will be important to learn how this regulation correlates to the requirement for dolichyl-phosphate in developing cells.

The second key step involves the conversion of the polyprenol into dolichol (Fig. 2) and occurs after the long-chain polyprenol has been assembled from IPP and DMAPP by prenyltransferases. It is believed that phosphatases convert polyprenyl-diphosphate to polyprenol prior to the reduction step, which acts as an additional point of regulation for glycoprotein production (Fig. 2). This additional reduction step provides eukaryotes an opportunity for regulation not observed in bacteria, which contain fully unsaturated polyprenols. Viewed from a regulatory perspective, this hypothesis would imply that the evolutionary conservation of α -saturated dolichols in eukaryotes and archaea is driven by the additional biosynthetic opportunity for regulation, instead of any inherent benefits in polyprenol structure. As little evidence has yet been provided to support a specific functional advantage for the use of the dolichol family of polyprenols, this is a reasonable and potentially testable hypothesis.

The essentiality of the enzymes involved in dolichyl-phosphate production is underscored by the class of diseases known as congenital disorders of glycosylation (CDGs) [126–128]. CDGs are characterized by aberrant glycosylation, which can arise from mutations in the biosynthesis of both dolichols [129] and N- and O-linked glycoproteins [130], and to date 45 different CDGs have been identified [126]. Originally these diseases were divided into two classes based on the origin of the glycosylation defect; disorders of CDG type I resulted from mutations in genes encoding for the production of Dol-PP-GlcNAc₂Man₉Glc₃ (Fig. 4), whereas CDG type II contained defects in genes whose products are involved in glycan processing in the Golgi. However, due to the increasing numbers of identified diseases, a new nomenclature was proposed recently, which utilizes the name of the defective gene to identify the particular disorder [127]. Importantly, CDGs have been identified that result from mutations in dolichol kinase and polyprenyl-diphosphate reductase (Figs. 2 and 9) [130, 131], two enzymes that are essential for production of dolichyl-phosphate. From the standpoint of CDGs, it is imperative to understand the regulation of dolichyl-phosphate in order to determine how best to treat patients with defects in dolichyl-phosphate biosynthesis. In general, enzymes involved in biosynthesis of dolichyl-phosphate are important regulatory points, which control cell growth and overall function by determining production of glycoproteins.

Potential regulatory roles of undecaprenyl-phosphate and Lipid I/II in bacteria

In bacteria, undecaprenyl-phosphate serves as the glycan assembly carrier of choice, however, there is little evidence that it plays a regulatory role similar to that of dolichyl-phosphate. Limiting amounts of undecaprenyl-phosphate or Lipid II, the undecaprenyl-diphosphate linked monomer of peptidoglycan, (Fig. 5) could substantially decrease cell

wall production and thereby cellular growth. However, as described in the previous section, only a few studies have examined the amount of undecaprenol derivatives in bacteria (Table 3). Undecaprenyl-phosphate appears to comprise a substantial portion of the available polyprenol pool in both *E. coli* and *S. aureus* suggesting that it may not be a rate-limiting substrate under the analyzed conditions [2]. However, the undecaprenol kinase from *Streptococcus mutans* has been identified as a virulence factor, which suggests that undecaprenyl-phosphate production may play a role in bacterial adaptation to stress [94]. Further studies are needed to discern if production of undecaprenyl-phosphate is an important regulator in bacteria.

Only a handful of studies have attempted to quantify the amount of Lipid II in bacteria, which could also regulate cell wall biosynthesis. Metabolic labeling has been used in *E. coli* to quantify the pools of Lipid I/II relative to other substrates required for peptidoglycan biosynthesis resulting in estimation of ~3000 copies per cell [132]. Based on the work described in Table 3 [2], this would represent <2% of the undecaprenyl-phosphate supply in *E. coli*. It is important to note that the amount of Lipid I/II in *E. coli* has not been measured directly, and further quantitative studies could provide greater insight [132]. In contrast, the amount of Lipid I/II has been estimated in a variety of Gram-positive bacteria and in every case the amount is much higher than in *E. coli*. Metabolic labeling was used to determine that each cell of *Bacillus megaterium* contained approximately 34,000 Lipid I/II molecules [133]. Other studies estimated the amount by determining the number of binding sites for various Lipid II targeting antibiotics such as ramoplanin and mersacidin [134]. These reports suggest that there are 50,000 peptidoglycan precursors in *S. aureus* [135], 70,000 in *Micrococcus luteus* [136], and 200,000 in *Staphylococcus simulans* [136]. In *S. aureus*, this amount of Lipid I/II would represent over 40% of the total pool of undecaprenyl derivatives, a considerably larger percentage than in *E. coli*.

Similar to the studies on undecaprenyl-phosphate, further work is needed to more accurately quantify the amount of Lipid I/II present in different bacterial species. However, the available data implies that *E. coli* and *S. aureus* contain significantly different levels of undecaprenol undecaprenyl-phosphate, and Lipid I/II. Since Gram-positive bacteria have much thicker peptidoglycan layers (20–80 nm) than Gram-negative (7–8 nm), it is not surprising that Gram-positive bacteria may require larger pools of Lipid I/II. In addition, the differences may imply that Gram-negative and Gram-positive bacteria rely on different mechanisms for the regulation of cell wall biosynthesis and other undecaprenyl-phosphate dependent processes. Further studies are needed to determine how Gram-negative and Gram-positive bacteria regulate the production of undecaprenyl-phosphate and Lipid I/II and if this represents a general physiological distinction.

Enzyme specificity for polyprenols may serve a regulatory purpose

Most eukaryotic enzymes that rely on dolichol derivatives as substrates are specific for dolichol [137–142] and typically prefer S(–) dolichol to either R(+) dolichol or the corresponding unsaturated polyprenol of a similar length indicating clear specificity for the configuration of the α -isoprene subunit [137]. Correspondingly, several studies have indicated that in bacteria, polyprenol-binding enzymes are specific for undecaprenol, and many of these results are summarized in the following reference [143]. It is important to note that specificity is not uniform and that within both eukaryotes and bacteria, enzymes have been identified with relaxed specificity, and less specificity is observed in general for polyprenol length [137–143]. A recent study examined three glycosyltransferases in the bacterial N-linked glycosylation pathway in *C. jejuni* and found that all were specific for both the degree of saturation and *cis/trans* configurations of the isoprene units [42]. Interestingly, this was observed even in the case of a soluble glycosyltransferase that is not predicted to contain any transmembrane domains. Enzyme specificity for polyisoprenols in

prokaryotes and eukaryotes is an important component of polyisoprenol regulation because it allows these molecules to specifically interact with cognate enzymes in the milieu of the phospholipid bilayer. However, if we again consider the question of polyprenol structural diversity, it seems unlikely that enzyme specificity is a driving force for the differences observed across species given that not all enzymes are uniquely specific for a particular polyprenol saturation or length.

Current hypotheses on the role of polyprenyl-phosphates in glycan translocation

Polyprenyl-phosphate dependent pathways in bacteria, archaea, and eukaryotes rely universally on translocation or “flipping” of polyprenol derivatives across lipid bilayers [120, 139, 144–149]. However, very little is known about how proteins mediate glycan translocation and if polyprenols play a role in the mechanism. Studies using a variety of biophysical and biochemical methods have established that polyprenyl-phosphates and glycan-modified polyprenols have very slow trans-bilayer diffusion rates ($t_{1/2} = >2$ h) in model membranes when unassisted by protein catalysis [107, 108, 150]. Biosynthetic rates for the production of N-linked glycans in eukaryotes and peptidoglycan in bacteria are such that relatively fast rates of glycan translocation are required ($t_{1/2} = <1$ s) [132, 151]. Furthermore, *in vitro* studies have implied that the majority of dolichyl-phosphate is regenerated through translocation-dependent recycling (Fig. 9) [120], which requires much faster flipping rates than those observed in the uncatalyzed diffusion models. The difference between the observed and required flipping rates has led the long-standing hypothesis that proteins known as flippases catalyze the translocation of polyprenyl-phosphate derivatives. This section will summarize our current understanding of flippases with a description of the proposed protein-based mechanisms for the translocation reaction. Importantly, current evidence for the putative role of the polyprenol moiety in glycan translocation will also be reported and assessed.

Identified eukaryotic and bacterial flippases

A variety of dolichyl-phosphate derivatives, including Dol-PP-GlcNAc₂-Man₅, Dol-P-Man, Dol-P-Glc, and Dol-P, are flipped across the ER membrane to facilitate N-linked glycosylation and related processes (Fig. 4) [151]. However, until the last decade, the proteins involved in the process had not been identified or characterized biochemically. The development of *in vitro* flippase assays has allowed for the isolation of distinct protein-based activities for the translocation of Dol-PP-GlcNAc₂-Man₅ [148, 152, 153] and Dol-P-Man [146], and research is ongoing to identify the protein catalysts responsible for the observed flippase functionalities.

Similar to eukaryotic flippases, it is thought that bacteria contain different flippases for each of the unique undecaprenyl-phosphate-linked substrates present in the plasma membrane. The genes encoding bacterial enzymes are frequently found in clustered operons, which have enabled relatively facile identification of flippases, in contrast to the identification of eukaryotic flippases. Interestingly, it has been shown that two distinct protein families perform this function in bacteria [144]. The first class of flippases, wzx-type translocases, shares homology with eukaryotic flippases and shows no ATP dependence. Wzx-flippases are responsible for translocation of the individual subunits of peptidoglycan, heteropolymeric O-antigen, capsular polysaccharides and teichoic acids (Fig. 5). All of these substrates are polymerized in the periplasm, subsequent to translocation, and it has been proposed that polymerization may be a driving force for uni-directional translocation, which may explain why ATP is not required [144]. The second class of flippases is the ABC-type transporters, which have an associated ATPase activity and are implicated in N- and O-

linked protein glycosylation as well as the biosynthesis of homopolymeric O-antigen, which is polymerized in the cytoplasm before it is flipped to the periplasm (Fig. 5). The ATP-dependence is in contrast to eukaryotic flippases in the N-linked glycosylation pathway, which have no parallel ATP requirement. Unlike the *wzx*-flippases, the biosynthesis of homopolymeric O-antigen and N- and O-linked protein glycans does not involve polymerization after translocation.

All undecaprenyl-phosphate pathways rely on transfer of the glycan substrates from undecaprenol carrier after translocation and the reactions are chemically similar, so this does not explain why two flippase types have persisted in bacterial species. One potential reason is that the periplasmic polymerization of *wzx*-dependent undecaprenol derivatives requires higher concentrations of periplasmic-oriented precursors, and thus an equilibrative-based mechanism is efficient for facile transport of these substrates. This implies that the concentration of undecaprenyl-phosphate glycans in bacteria may be too low for an equilibrative mechanism to be effective, and thus ATP is needed to drive the translocation. Interestingly, this also suggests that eukaryotes possess higher concentrations of dolichyl-phosphate substrates in the ER membrane, or that they have an alternative way of driving translocation. Further work is required to validate this hypothesis and to better explain the biological necessity for two distinct mechanistic classes of polyprenyl-phosphate flippases.

While the identification of most bacterial flippases has been simplified due to gene clustering in bacterial genomes, one major exception has been discovery of the protein responsible for Lipid II translocation. Lipid II is synthesized in the cytoplasm and flipped to the periplasm for peptidoglycan biosynthesis, and identification of the Lipid II flippase has been a significant challenge, despite the large amount of research focused on this pathway due to its potential for antibacterial targets. Studies have implicated both MviN and FtsW in Lipid II translocation [145, 154–157], but recent biochemical evidence demonstrated that the presence of purified FtsW induced movement of Lipid II across the bilayer in model membranes, suggesting that FtsW is the Lipid II flippase [145].

Proposed mechanisms of glycan translocation

Limited information is available on the mechanism of flippase-mediated translocation, because, until recently, no biochemical methods for assaying translocation had been reported. Developing a biochemical assay for monitoring flippase activity has been challenging, as it requires a model membrane system and an assay that can specifically detect movement of the polyprenol derivative. In addition, polyprenols are hydrophobic molecules and pose significant challenges for purification and quantification due to weak UV absorbance. Similarly, flippases possess 10–12 transmembrane domains rendering them relatively intractable to biochemical purification. However, in recent years, several important studies have exploited a variety of techniques to quantify the localization of polyprenyl-phosphate derivatives within membrane vesicles including lectin binding, chemical-reporter and FRET-based methods [145, 146, 148, 153]. With the exception of the fluorescence-based method, which requires a non-native glycan substrate, these methods are limited by the timescale of visualization since flipping occurs faster than the time resolution of the assays, thereby complicating mechanistic studies. In addition, there are no X-ray crystal structures of flippases specific for polyprenol substrates, although the structure of a lipid flippase with homology to the ATP-binding cassette flippases has been recently published [158].

Several studies have suggested mechanisms for flippase-mediated translocation. For example, in a recent study that identified a protein fraction associated with Dol-P-Man translocation activity, the authors found that citronellyl-phosphate, a short C10 dolichyl-phosphate analog, inhibited translocation [146]. This led to the suggestion that the flippase

might be also involved in translocation of dolichyl-phosphate as well as Dol-P-Man [146]. In this model, the flippase acts as a pore through which the populations of Dol-P-Man and dolichyl-phosphate equilibrate, with Dol-P-Man moving to the ER lumen to act as a glycan donor and dolichyl-phosphate returning to the cytoplasmic leaflet to begin a new cycle of glycan biosynthesis. This model predicts that the cytoplasmic loops of the flippase enzyme might preferentially recognize Dol-P-Man, whereas the periplasmic loops may prefer dolichyl-phosphate, which would help facilitate the desired flow of molecules, but this model is highly speculative.

Another recent study utilized an unbiased truncation strategy to map the membrane topology of the Wzx flippase involved in O-antigen biosynthesis in *Pseudomonas aeruginosa* [147]. This study established that the flippase contained 12 transmembrane domains, similar to flippases from eukaryotic pathways, and had several sizeable cytoplasmic loops, which may be involved in recognition of the O-antigen glycan portion of the substrate. In addition, the transmembrane helices included more charged residues than are typical for transmembrane domains. This observation prompted the authors to propose that these mostly cationic residues could line a channel formed by the transmembrane domains to facilitate movement of the O-antigen glycan across the membrane [147]. This study provides the basis for future mutational and structural studies focused on understanding how flippases mediate translocation.

Potential role of polyprenol moieties in glycan translocation

In the studies presented above, mechanistic analysis of flippase reactions focused on the protein catalysis, but it is also important to consider the specific role of the polyprenol substrates. In particular, polyisoprenol recognition sequences (PIRS) are amino acid sequences present in predicted transmembrane domains that have been shown to selectively bind polyprenyl-phosphate-linked substrates in model membranes [109, 110]. PIRS were first identified as dolichol recognition sequences (DRS) in the predicted transmembrane domains of three essential glycosyltransferases in the yeast N-linked glycosylation pathway and were postulated to interact with dolichol [159]. Subsequent studies identified PIRS in other essential eukaryotic glycosyltransferases and *E. coli* enzymes involved in capsular polysaccharide biosynthesis [160, 161]. The importance of these sequences is unclear as PIRS was found to be essential for GlcNAc-1-phosphate transferase activity, but not for Dol-P-Man synthase activity [162, 163].

Recent studies have utilized NMR and molecular modeling to study the binding of short PIRS peptides to undecaprenol, C95 dolichol and the related phosphorylated derivatives [109, 110]. These studies have proposed that polyprenols and polyprenyl-phosphates adopt a coiled structure composed of three separate domains such that the head to tail length is shorter than the width of the membrane (32–33 Å for C95 dolichol and 22 Å for undecaprenol) [110] (Fig. 8), which is consistent with the overall architecture determined in a prior study on the conformation of dolichol [113]. Furthermore, conformational analysis of how polyprenols may interact with PIRS-based peptides suggested that multiple (4–5) peptides might be able to bind each polyprenol molecule (Fig. 10). The authors propose that polyprenyl-phosphate may promote recruitment of PIRS in nearby protein transmembrane domains and hence, the transient formation of a hydrophilic channel appropriate for glycan translocation [109].

The flippase and PIRS hypotheses for glycan translocation are not mutually exclusive. The PIR-sequence is not well defined, but a key element of the consensus sequence is the presence of a proline residue [109, 110]. Prolines are rarely found in transmembrane domains, but are frequently found in the transmembrane domains of transporter-related enzymes [164]. Indeed, many of the flippase enzymes discussed above contain at least one

transmembrane domain with a proline residue. This could suggest that the proposed PIRS-polyisoprenol interaction may have a role in the flippase mechanism, but further biochemical and biophysical studies are necessary to elucidate the potential roles of these domains. Glycan translocation across membranes and the use of polyprenyl-phosphate as membrane-bound glycan carriers are universal features of the pathways discussed here, and validation of a link between these two components of glycan biosynthesis would provide great insight into how these pathways operate in the membrane.

Potential roles of polyprenyl-phosphates in macromolecular complex assembly

Here we consider a third hypothesis, which centers on the potential role of polyprenyl-phosphates in promoting the association of glycan biosynthetic enzymes. Several important multi-enzyme complexes that rely on translocated polyprenyl-phosphate-linked substrates have been characterized, including the eukaryotic oligosaccharyltransferase (OTase) and peptidoglycan biosynthetic machinery in bacteria, although the role of polyprenols in the activities of these complexes remains unclear. The formation of experimentally established complexes and more hypothetical enzyme complexes will be evaluated in light of the required polyprenyl-phosphate substrates.

Multi-enzyme complexes with polyprenyl-phosphate-linked substrates

The enzyme complex that has been studied in most detail is the eukaryotic OTase, which is a multimeric membrane-bound enzyme [29, 30, 165, 166], which comprises eight to nine separate polypeptides and catalyzes transfer of the GlcNAc₂Man₉Glc₃ tetradecasaccharide from dolichyl-diphosphate to protein. While the OTase complex has been biochemically validated, very little is known about how the dolichol moiety interacts with the OTase complex, although the Wbp1 subunit has been suggested to bind a dolichol derivative [167]. *In vitro* reconstitution of the OTase complex within a membrane-mimetic system would be an effective means to determine if dolichol derivatives are important for complex formation and stability, however, this is extremely challenging due to the complexity of the membrane-bound subunits in OTase.

In many ways, the eukaryotic OTase complex is unique among polyprenyl-phosphate dependent reactions, because it possesses multiple protein subunits acting in concert to perform one reaction. Most other pathways dependent on polyprenols involve multiple biosynthetic steps carried out by separate enzymes. For example, 15–20 different proteins have been identified in the bacterial enzymatic machinery required for peptidoglycan biosynthesis [12, 53]. Recent studies have identified essential protein-protein interactions that suggest the formation of both an elongase complex, responsible for lateral growth of rod-like bacteria, and a distinct divisome complex, which mediates peptidoglycan synthesis at the site of cell division [53]. Interestingly, both of these complexes are associated with a cytoskeletal protein (MreB or FtsZ), which polymerizes at the cytoplasmic face of the cell surface and appears to recruit the required protein components. The elongase and divisome complexes require Lipid II to build peptidoglycan, but it is not known if Lipid II itself is involved in organizing and recruiting the elongase and divisome molecular machines. As discussed above, the relatively low pools of Lipid I/II found in Gram-negative bacteria suggests that cells must have a way to localize these substrates to sites of synthesis, but little is known about how this may occur in Gram-negative or Gram-positive bacteria.

A role for polyprenyl-phosphates in enzyme complex assembly?

Although studies have elucidated models of the eukaryotic OT complex and peptidoglycan biosynthesis, essentially nothing is known about enzyme complex formation in other

polyprenyl-phosphate dependent glycan assembly pathways. Identification of multi-enzyme biosynthetic complexes has been difficult; protein-protein interactions may be weaker than in the OTase complex and are not associated with a cytoskeletal protein as in peptidoglycan biosynthesis. Glycan biosynthesis in eukaryotes and prokaryotes involves sequential addition of individual glycan units to an undecaprenyl-phosphate carrier by distinct enzymes (Fig. 3). Thus, macromolecular enzyme association is an attractive model for how pathways may efficiently assemble polyprenyl-phosphate-linked glycans (Fig. 11). Furthermore, polyprenyl-phosphate-linked substrates may act as more than passive glycan carriers by altering membrane fluidity and specifically promoting both enzyme complex formation and substrate flux through the pathway.

The hypothesis of macromolecular complexes is not new, but previous evidence is scant. For instance, almost all bacteria produce peptidoglycan, which is an undecaprenyl-phosphate dependent process, and many use undecaprenyl-phosphate for a second biosynthetic end, including O-antigen and teichoic acid biosynthesis as well as N- and O-linked protein glycosylation as depicted in Fig. 5. The dynamics of undecaprenyl-phosphate usage amongst competing pathways are virtually unknown, however they have been examined in a handful of studies. Recently, it was shown that an unusual periplasmic enzyme in *E. coli* can phosphorylate lipid A, the subunit of lipopolysaccharide, using undecaprenyl-diphosphate exclusively as the phosphoryl donor [168]. This study provides an exquisite example of how bacteria have adapted to be both economical and efficient, and also suggests the presence of protein-protein interactions between the phosphoryltransferase enzyme and the enzymes involved in O-antigen biosynthesis [168].

Another informative study focused on the interplay between peptidoglycan and teichoic acid biosynthesis using *Bacillus licheniformis* as a model organism [169]. The authors treated bacterial membrane preparations with bacitracin and then examined the inhibition of peptidoglycan or teichoic acid biosynthesis. Bacitracin is an antibiotic that sequesters undecaprenyl-diphosphate, which is both the end-product of undecaprenol biosynthesis (Fig. 2) and also the product released after the completion of teichoic acid or peptidoglycan biosynthesis (Fig. 8) [170]. Interestingly, while bacitracin inhibited peptidoglycan biosynthesis significantly (55%), much less inhibition (0–4%) was observed for teichoic acid biosynthesis, suggesting that the teichoic acid pathway is not reliant on a common pool of the undecaprenyl-phosphate carrier [169]. Inhibition of peptidoglycan biosynthesis by bacitracin was expected since both the donor (Lipid II) and the growing peptidoglycan polymer are linked to undecaprenyl-phosphate, whereas teichoic acid biosynthesis only utilizes undecaprenyl-phosphate as a membrane carrier. This finding in combination with further inhibition studies led the authors to posit the presence of multi-enzyme complexes capable of internally recycling undecaprenyl-phosphate after each biosynthetic cycle, rather than releasing it into the substrate pool after each round of synthesis [169].

One final piece of evidence for the presence of macromolecular enzyme complexes dependent on polyprenols comes from the identification of specific interactions between PIRS peptides and polyprenol molecules discussed previously. The PIRS-polyprenol interaction was evaluated in terms of polyprenyl-phosphate translocation [109, 110], but a second interpretation, relevant here, is that the proposed ability of polyprenols to bind multiple PIRS domains is involved in facilitating protein-protein interactions within a biosynthetic pathway. This interpretation suggests that polyprenols may play an important role in recruitment of biosynthetic enzymes into a macromolecular complex (Figs. 10 and 11), which could also have important implications for how the polyprenyl-phosphate-linked glycan intermediates are shuttled through the pathway. Further experimental evaluation of multi-enzyme complexes in bacterial and eukaryotic is challenging and may require *in vitro*

reconstitution of a polyprenol-based pathway in a membrane-mimetic system, and to our knowledge, no such experiments have been performed to date.

Conclusion

Polyprenols and polyprenyl-phosphates are ubiquitous constituents of cellular membranes and play roles as substrates in essential biosynthetic pathways in both eukaryotes and prokaryotes. Much less is known about unmodified polyprenols, which act as precursors to polyprenyl-phosphates in developing cells, but have no other established function in biological systems and may be unnecessary in Gram-negative bacteria. Furthermore, polyprenols can accumulate at relatively high levels within many eukaryotic organisms as well as Gram-positive bacteria; biosynthesis of these molecules is a complex, energy-dependent process and it seems unlikely that polyprenol production would have been conserved in evolution if it did not serve an important role. Further work is necessary to establish the physical effects that polyprenols may exert on complex biological membranes and how polyprenol accumulation affects aging, disease, and intracellular transport processes. Research of this type could also provide illumination on the intriguing structural difference of polyprenols that are observed across the domains of life, which remains one of the most puzzling aspects of polyprenol biochemistry.

In contrast, the role of polyprenyl-phosphates as facilitators of glycan assembly in essential bioprocesses such as N-linked protein glycosylation and peptidoglycan biosynthesis has been extensively studied. It is our suggestion here that dolichyl-phosphates may have more significant purposes, in which these molecules influence enzyme and pathway regulation, flippase-mediated glycan translocation across membranes, and macromolecular enzyme complex formation. From these three hypotheses, it is interesting to consider which better explains the evolutionary conservation of polyprenols and dolichols. The regulatory role of dolichyl-phosphates is well established in eukaryotes, and it is certainly important that dolichyl-phosphates have independent biosynthetic and recycling pathways from other membrane components. However, it is not clear that the specific structure of polyprenols is necessary for regulatory function in most cases. In contrast, the ubiquitous use of polyprenols for translocation of glycans and their potential role in facilitating enzyme complex formation offer much more tantalizing possibilities. Both of these processes involve localization and movement within the lipid bilayer, processes that may be facilitated by the known biophysical effects exerted by polyprenols and by the specific interactions that occur between polyisoprenols and certain peptide (PIR) sequences. Recent biochemical characterization of several polyprenyl-phosphate dependent pathways [45, 52, 59, 60] coupled with exciting new membrane mimetic technologies [171, 172] provide the tools for future biochemical and biophysical-based elucidation of how polyprenyl-phosphates participate in glycan translocation and biosynthetic complex assembly.

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Fig. 1. Structures of fully unsaturated polyprenols and dolichols. The length of the polyprenol and the number of *cis* and *trans* isoprene units (*m* and *n*, respectively) is species dependent. (Left) Fully unsaturated polyprenols contain all *trans* isoprene units or a mixture of *trans* and *cis* units as shown. (Right) Dolichols are distinguished by a single saturated α -isoprene unit.

Fig. 2.

Polyprenol biosynthesis from DMAPP and IPP. Prenyltransferases catalyze the condensation of DMAPP with IPP to form long polyprenyl-diphosphate molecules and direct which stereoisomer (*cis* or *trans*) is produced in each reaction. In eukaryotes, the final steps of dolichol biosynthesis involve enzyme-catalyzed reduction of the α -isoprene subunit, which is thought to occur on the unmodified polyprenol, followed by phosphorylation. Specific phosphatases responsible for the hydrolysis of dolichyl-diphosphate have not yet been identified. Bacteria do not require reduction at the α -subunit, and thus their biosynthetic pathways terminate with a single hydrolysis reaction to generate undecaprenyl-phosphate.

Fig. 3. Schematic of N-linked protein glycosylation pathways

(Left) The eukaryotic pathway assembles dolichyl-diphosphate (Dol-PP) tetradecasaccharide (GlcNAc₂Man₉Glc₃) on both the cytoplasmic and luminal faces of the ER membrane prior to glycan transfer. Dol-P-Man and Dol-P-Glc act as glycan donors on the luminal face.

(Right) The pathway in the bacterium *Campylobacter jejuni* assembles an undecaprenyl-diphosphate (Und-PP) heptasaccharide (diNAcBacGalNAc₅Glc) prior to translocation and glycan transfer in the periplasm.

Fig. 4.

Dolichyl-phosphate-linked glycan substrates in eukaryotes and archaea. The glycans are depicted in the cellular region where they are biosynthesized and arrows indicate that the glycans undergo protein-catalyzed flipping across the membrane prior to subsequent reactions. (Top) From left to right, the eukaryotic ER membrane contains multiple species, including Dol-P-Man, Dol-P-Glc, Dol-PP-GlcNAc₂Man₅ and Dol-PP-GlcNAc₂Man₉Glc₃. (Lower right) The archaeal membrane contains Dol-P-Man and dolichyl-phosphate-linked glycan precursors to N-linked protein glycans. The glycan produced in *Methanococcus voltae* is shown, but it is unknown whether this intermediate contains a diphosphate or phosphate linkage, since both have been observed in archaeal species.

Fig. 5.

Undecaprenyl-phosphate-linked glycans in bacterial plasma membranes. The glycans are depicted in the cellular region where they are biosynthesized and arrows indicate that the glycans undergo protein-catalyzed flipping across the membrane prior to subsequent reactions. (Top) From left to right, all bacteria contain Und-P-Man and Lipid II, while teichoic acids are only present in Gram-positive bacteria such as the example shown from *Staphylococcus aureus*. (Bottom) From left to right, representative Und-PP-linked glycans from Gram-negative bacteria are shown including N-linked glycans from *C. jejuni*, O-linked glycans from *N. gonorrhoeae*, capsular polysaccharide from *E. coli* strain K27, heteropolymeric O-antigen subunit from *E. coli* strain O113, and homopolymeric O-antigen from *Klebsiella pneumoniae*.

Fig. 6. Activated sugar donors. (Left) Dol-P-Glc is the membrane-bound sugar donor in the ER lumen of eukaryotes. (Right) Uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) is a soluble sugar donor localized in the cytoplasm.

Fig. 7. Phospholipid structures in presence of polyprenols

(A) Lipids form a typical lipid bilayer in the absence of polyprenol. (B) Some evidence suggests that the hexagonal II conformation (shown here) is induced in the presence of polyprenols. (C) Polyphenol derivatives have different orientations in the membrane. From left to right, a lipid bilayer is shown with polyphenyl-phosphate glycan and polyphenyl-phosphate perpendicular to the membrane, and polyphenol and esterified polyphenol parallel to the membrane surface.

Fig. 8. Proposed structures of polyprenols. From left to right, the structures of undecaprenyl-phosphate, C95 dolichyl-phosphate, and C95 dolichol were determined by NMR and molecular modeling. The red group represents the phosphate. Image reproduced with permission from [110].

Fig. 9. Cellular sources of dolichyl-phosphate. Dolichyl-phosphate has three cellular sources. In the first, dolichol is phosphorylated *de novo* by a kinase (K) to generate dolichyl-phosphate. In the second and third, a salvage pathway occurs, in which the dolichyl-diphosphate released after glycan transfer is hydrolyzed by a pyrophosphatase (P) or dolichyl-phosphate is released by a glycosyltransferase (G) from Dol-P-sugar donors. In both cases, the dolichyl-phosphate is then translocated from the ER lumen to the cytoplasm by a flippase (F) to begin another round of glycan biosynthesis.

Fig. 10.

Proposed structure of PIRS peptide bound to dolichyl-phosphate. Top and side views are shown of dolichyl-phosphate interacting with five PIRS-containing peptides as determined by computational modeling studies and NMR structural data of the peptide (green) and dolichyl-phosphates (yellow). The red, pink and turquoise groups on the peptide denote the two leucines and one isoleucine that contact dolichyl-phosphate. Image reproduced with permission from [109].

Fig. 11.

Independent and sequential models of polyprenol-dependent glycan biosynthesis. (A) In the independent model, glycan intermediates are released after every glycosyltransferase reaction. This may result in significant pauses before the intermediate encounters the next enzyme in the pathway. (B) In the sequential model, the enzymes cluster together to produce the glycan efficiently without release of the intermediates to the lipid bilayer.

Table 1

Structures of polyprenols in representative species.

Species	n	m	α -saturation	Extended length ^a	Ref
Bacteria	2	8	No	50 Å	[14]
<i>Mycobacterium smegmatis</i>	3 ^b	3	No	34 Å	[18,19]
	1	8	No	46 Å	
<i>Halobacterium halobium</i> (archaea)	2	9	Yes	60 Å	[15]
<i>Saccharomyces cerevisiae</i>	2	12–13	Yes	73–77 Å	[16]
<i>Rattus norvegicus</i> (rat)	2	13–16	Yes	76–89 Å	[17]
<i>Homo sapiens</i>	2	14–17	Yes	81–94 Å	[17]
<i>Ficus elastica</i> (plant)	3	6–8	No	47–56 Å	[24]
<i>Aspergillus fumigatus</i> (plant)	2	14–20	Yes	86–107 Å	[22]
<i>Potentilla crinita</i> (plant)	3	15–17	No	86–94 Å	[23]
	3	25–27		129–137 Å	
	3	35–37		172–180 Å	

^aExtended length refers to the predicted length of the polyprenol in an extended conformation.

^bThe four isoprene units at the ω -terminal end are fully saturated in this short polyprenol from *Mycobacterium smegmatis*. Two types of polyprenols are found in this bacterium.

Table 2

Intracellular quantification of dolichol and dolichyl-phosphate in mammalian sources. Unless otherwise indicated, methods based on high performance liquid chromatography were used to quantify the molecules (given in μg dolichol per g of cells).

Species	Dolichol	Dolichyl-phosphate	Ref
CHO (Chinese hamster ovary) cells	74 cpm ^a	740 cpm ^a	[69]
	690 cpm ^a	270 cpm ^a	[1]
Rat liver	270 cpm ^a	59 cpm ^a	[66]
	43000 cpm ^b	890 cpm ^b	[66]
	43.0 $\mu\text{g/g}$	8.75 $\mu\text{g/g}$	[15]
	41 $\mu\text{g/g}$	9.38 $\mu\text{g/g}$	[65]
	17.1 $\mu\text{g/g}$	14.7 $\mu\text{g/g}$	[68]
	21.1 – 52.2 $\mu\text{g/g}^c$	22.0 – 16.7 $\mu\text{g/g}^c$	[67]
Rat testes	4.3 $\mu\text{g/g}$	16.0 $\mu\text{g/g}$	[68]
	9.36 – 27.9 $\mu\text{g/g}^c$	15.3 – 19.8 $\mu\text{g/g}^c$	[67]
Rat spleen	39.3 $\mu\text{g/g}$	18.7 $\mu\text{g/g}$	[68]
	58.9 – 157 $\mu\text{g/g}^c$	11.6 – 24.8 $\mu\text{g/g}^c$	[67]
Rat brain	11.3 $\mu\text{g/g}$	16.2 $\mu\text{g/g}$	[68]
	9.36 – 27.9 $\mu\text{g/g}^c$	15.3 – 19.8 $\mu\text{g/g}^c$	[67]
Rat kidney	7.8 $\mu\text{g/g}$	20.0 $\mu\text{g/g}$	[68]
	12.3 – 24.1 $\mu\text{g/g}^c$	22.0 – 20.0 $\mu\text{g/g}^c$	[67]
Human liver	465 $\mu\text{g/g}$	10.8 $\mu\text{g/g}$	[15]
	486 $\mu\text{g/g}$	11.9 $\mu\text{g/g}$	[65]
Human testis	1493 $\mu\text{g/g}$	169 $\mu\text{g/g}$	[15]

^a Incorporation of radiolabeled mevalonate was used for quantification.

^b Incorporation of radiolabeled acetate was used for quantification.

^c In this study, the dolichol and dolichyl-phosphate amounts were determined at 4, 6, 8, 10 and 14 weeks; the values for 4 and 14 weeks are shown.

Table 3

The percentages of undecaprenol, undecaprenyl-phosphate (Und-P), and undecaprenyl-diphosphate (Und-PP) in various bacterial species.

Species	Undecaprenol	Und-P	Und-PP	Reference
<i>E. coli</i>	nd ^a	22% (75 nmol/g) ^b	78% (260 nmol/g) ^b	[2]
<i>S. aureus</i>	31% (70 nmol/g)	19% (43 nmol/g) ^b	50% (113 nmol/g) ^b	[2]
	90%	<10%	n/a ^c	[14]
<i>S. faecalis</i>	82%	18%	n/a ^c	[92]

^aNo significant amounts of undecaprenol were detected.

^bThe amount is given in nmoles polyprenol per gram cell pellet.

^cFor the latter two studies, no attempt was made to quantify the amount of Und-PP.