Published in final edited form as:

Biochim Biophys Acta. 2009 June ; 1790(6): 485–494. doi:10.1016/j.bbagen.2009.03.030.

Structure and synthesis of polyisoprenoids used in N-glycosylation across the three domains of life

Meredith B. Jones¹, Julian N. Rosenberg¹, Michael J. Betenbaugh¹, and Sharon S. Krag²

¹Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD 21218

²Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, MD 21205

Abstract

N-linked protein glycosylation was originally thought to be specific to eukaryotes, but evidence of this post-translational modification has now been discovered across all domains of life: Eucarya, Bacteria, and Archaea. In all cases, the glycans are first assembled in a step-wise manner on a polyisoprenoid carrier lipid. At some stage of lipid-linked oligosaccharide synthesis, the glycan is flipped across a membrane. Subsequently, the completed glycan is transferred to specific asparagine residues on the protein of interest. Interestingly, though the N-glycosylation pathway seems to be conserved, the biosynthetic pathways of the polyisoprenoid carriers, the specific structures of the carriers, and the glycan residues added to the carriers vary widely. In this review we will elucidate how organisms in each basic domain of life synthesize the polyisoprenoids that they utilize for N-linked glycosylation and briefly discuss the subsequent modifications of the lipid to generate a lipid-linked oligosaccharide.

Keywords

isoprenoid; N-glycosylation; DOXP; MVA; dolichol; lipid-linked oligosaccharide

1. Introduction

Historically, it was thought that N-glycosylation was unique to eukaryotic organisms and that a single, defined pathway led to the biosynthesis of the polyisoprenoids that act as the lipid-linked oligosaccharide (LLO) donors in N-glycosylation. In the last few decades, however, both of these ideas have been disproven, evoking questions about how N-glycosylation occurs in different organisms, including what type of carrier lipids are used and how they are synthesized.

In 1976, Mescher and Strominger reported a protein from the cell envelope in *Halobacterium salinarum* that contained glycans covalently linked to asparagine residues [1]. This marked the first observation of N-glycosylation in what would later become the domain Archaea. With

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Corresponding Author: Sharon S. Krag 615 North Wolfe Street Department of Biochemistry and Molecular Biology Bloomberg School of Public Health Baltimore MD 21205 Phone: 410-955-3869 Fax: 410-625-0059 skrag@jhsph.edu.

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this discovery, the idea that N-linked glycosylation occurred only in eukaryotes was dispelled. More recently, evidence of N-glycosylation in bacteria was discovered by Szymanski et al. in the Gram-negative bacteria *Campylobacter jejuni* [2]. N-glycosylation is now thought to be a protein modification that is conserved across all three major domains of life: Eucarya, Bacteria, and Archaea [2,3].

The N-glycosylation pathways in each domain are similar in that they include the step-wise assembly of sugars, donated by nucleotide activated sugars or activated lipids, onto a polyisoprenoid carrier by specific glycosyltransferases to form a LLO [4,5]. At some point during synthesis, the LLO is flipped across a membrane, and the oligosaccharide is transferred from the carrier lipid onto the protein by an oligosaccharide transferase (OST) enzyme or enzyme complex [4,5]. The glycan is ultimately bound to an asparagine residue in the protein via a β -glycosylamide linkage [4].

Although eukaryotes, bacteria, and archaea all seem to have certain characteristics of the N-glycosylation pathway in common, the identity of the lipid oligosaccharide carrier, its method of biosynthesis, and the structure of the attached glycans are sometimes strikingly different. This review will focus primarily on the synthesis of the polyisoprenoid alcohols that provide the carrier lipid portion of the LLO. Polyisoprenoid alcohols are hydrophobic polymers generated by the condensation of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), the universal 5-carbon precursors of all isoprenoids [6-8]. A schematic diagram of how polyisoprenoid alcohols are formed and utilized in N-glycosylation is shown in Fig. 1. The review will begin with an overview of the two possible pathways for generating the IPP and DMAPP building blocks. It will then discuss the formation of the polyisoprenoid alcohols, dolichol and polyprenol, from IPP and DMAPP. Next, the review will identify the polyisoprenoid alcohols known to be used in N-glycosylation for organisms in each of the three domains of life and will determine which biosynthetic pathways are responsible for providing the IPP and DMAPP building blocks in each case. Finally, the sugar modifications that result in generation of a completed LLO will be described briefly for each domain.

2. Isoprenoid biosynthesis

Originally, there was thought to be a single pathway responsible for the biosynthesis of IPP and DMAPP, the universal 5-carbon precursors of all isoprenoids [6-8]. This was called the mevalonate (MVA) pathway and it involves the formation of IPP from acetyl-CoA with mevalonate as an intermediate (Fig. 2). It was widely accepted that all organisms form DMAPP and IPP only through this pathway [7]. However, in 1993, Rohmer et al. provided evidence for a new pathway for DMAPP and IPP synthesis that did not involve mevalonate (Fig. 3) [9, 10]. This work began with the observation of unexpected labeling patterns in bacterial hopanoids using ^{13}C -labeled acetate [11]. Independently, Arigoni et al. discovered the existence of a mevalonate-independent pathway in the plant, *Ginkgo biloba*, as well as in *E. coli* [12-14]. Since then, this pathway, called the 1-deoxy-D-xylulose-5 phosphate (DOXP) pathway, has been further elucidated and has proven to be an alternative to the MVA pathway for IPP and DMAPP synthesis [8,15,16].

2.1 The MVA pathway

The MVA pathway (Fig. 2) begins with the conversion of two molecules of acetyl-CoA to acetoacetyl-CoA by the enzyme acetyl-CoA acetyltransferase (also called thiolase) [6,17-19]. A third molecule of acetyl-CoA and the acetoacetyl-CoA are then condensed to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) via HMG-CoA synthase (HMGS) [6,17,20]. HMG-CoA is reduced to mevalonate by HMG-CoA reductase (HMGR), in a NADPH-dependent reaction. HMGR is the key regulatory enzyme in the MVA pathway [21-23]. In the

next step, MVA is phosphorylated by MVA kinase to form MVA-5-phosphate, which is further phosphorylated by phospho-MVA kinase to form MVA-5-pyrophosphate [24,25]. Both of these phosphorylation reactions are ATP dependent [26,27]. MVA-5-pyrophosphate undergoes a decarboxylation reaction via MVA-5-pyrophosphate decarboxylase (MPPD) to produce IPP [6,28]. In the final step of this pathway, some of the IPP is converted to DMAPP by IPP isomerase [27,29]. IPP isomerase is essential for the production of DMAPP via the MVA pathway [8].

2.2 The DOXP pathway

The DOXP pathway (Fig. 3, for review see [6]) does not require acetyl-CoA, but rather begins with the condensation of pyruvate and glyceraldehyde 3-phosphate into DOXP by DOXP synthase (Dxs) [30,31]. This reaction requires thiamine pyrophosphate as a cofactor [6]. DOXP reductase (IspC) converts DOXP to 2-C-methyl-D-erythritol 4-phosphate (MEP) [32] which then reacts with cytidine triphosphate (CTP) to form 4-pyrophosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) with the help of CDP-ME synthase (IspD) [8,33]. Next, 4-pyrophosphocytidyl-2C-methyl-D-erythritol kinase (IspE) catalyzes the phosphorylation of CDP-ME to CDP-ME-2-phosphate (CDP-MEP) [34]. CDP-MEP is then converted to 2C-methyl-D-erythritol-2,4-cyclopyrophosphate (MECPP) by MECPP synthase (IspF) in a reaction that also involves the elimination of cytidine monophosphate (CMP) [8,35,36]. The final stages in the DOXP synthesis pathway are the least understood. In the last two steps, MECPP undergoes reduction and elimination reactions catalyzed by two enzymes, designated IspG and IspH, with 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-pyrophosphate (HMBPP) as an intermediate [15,37,38]. Both IPP and DMAPP are formed by these reactions, so IPP isomerase is not essential for production of DMAPP via the DOXP pathway. Nonetheless, as in the MVA pathway, IPP isomerase can interconvert DMAPP and IPP, allowing for adjustments in the ratio of IPP to DMAPP [8]. Not much is known about regulation of the DOXP pathway, however, IspF has been shown to interact with IPP, DMAPP, and other small isoprenoids that are downstream products of the DOXP pathway [39]. This data raises the possibility that these small isoprenoids could be involved in regulation of the DOXP pathway by a feedback mechanism with IspF as the point of control [6,39]. There is also evidence that in plants, some of the enzymes in the DOXP pathway are regulated by light [40,41]. This will be discussed further in section 3.3.

2.3 Polyisoprenoid Alcohols – Dolichol and Polyprenol

Polyisoprenoid alcohols are hydrophobic polymers that consist of a number of 5-carbon isoprene units linked head to tail. The polyisoprenoid alcohols involved in N-glycosylation can be from several up to about 25 isoprene units in length and have a hydroxyl group at one end (α) and a hydrogen atom at the other (ω) (Fig. 4) [21,42]. Polyisoprenoid alcohols are formed via a condensation reaction between DMAPP and several molecules of IPP [21,29, 43]. This reinforces the importance of the IPP isomerase in the MVA pathway, since in this pathway, DMAPP cannot be formed without IPP isomerase, and both IPP and DMAPP are required for polyisoprenoid synthesis. DMAPP acts as an acceptor for molecules of IPP, which are added sequentially via specific prenyl transferases to form chains of various lengths, such as geranyl pyrophosphate (GPP, C₁₀), farnesyl pyrophosphate (FPP, C₁₅), geranylgeranyl pyrophosphate (GGPP, C₂₀), and farnesylgeranyl pyrophosphate (FGPP, C₂₅) [21,44-46]. Longer polyprenyl pyrophosphates (Poly-PP) are typically synthesized from FPP and IPP (Fig. 5) [46], and the overall chain length is determined by the specific prenyl transferases, most likely through a restriction in the size of the enzyme's active site [21,47,48]. The prenyl transferases also control lipid stereochemistry, as both *cis*- and *trans*-prenyl transferases exist in nature [46]. GPP and FPP are synthesized by a *trans*-prenyl transferase called FPP synthase [45,49], however, *cis*-prenyl transferase is the more important enzyme in the context of N-glycosylation lipid donors (Fig. 5) [21,48,50].

Dolichol and polyprenol are the polyisoprenoid alcohols used as sugar carriers for N-glycosylation in all domains of life. Polyprenol synthesis begins with all-*trans*-FPP or GGPP, which is elongated by *cis*-prenyl transferase, using IPP as a substrate and resulting in a *trans/cis*-Poly-PP (Fig. 5) [21,46]. This Poly-PP is hydrophobic and, thus, localized within the membrane structure [51]. Although the order of the final steps in this pathway have not been completely elucidated, it is currently believed that Poly-PP is dephosphorylated into polyprenyl phosphate (Poly-P), and further dephosphorylated into polyprenol [52-54]. Polyprenol can then undergo a reduction reaction, catalyzed by polyprenol reductase, in order to form dolichol (Fig. 4) [51,52,55,56]. Dolichol differs from polyprenol in that its α -isoprene unit is saturated. Dolichol can then be phosphorylated by dolichol kinase to form dolichol phosphate (Dol-P) [51,52,57,58]. Sanderman and Strominger discovered a similar kinase for the re-phosphorylation of polyprenol. They report an undecaprenol phosphokinase from *Staphylococcus aureus* that phosphorylates the 55-carbon polyprenol found in bacteria [21, 59,60].

These kinases are extremely important because they control the level of phosphorylation of dolichol and polyprenol. Since many of the enzymes involved in the N-glycosylation pathway can only utilize isoprenoids with specific phosphorylation states as substrates, these kinases have the ability to affect the size of the dolichol or polyprenol pool available for use in N-glycosylation [61]. For example, many glycosyltransferases can use Dol-P but not dolichol or Dol-PP as a substrate [62,63]. The saturation state and length of these polyisoprenoid alcohols are also important for their recognition by the enzymes involved in the N-glycosylation pathway [52,64-66]. Enzymes such as glycosyltransferases and oligosaccharide transferases in all domains of life have substrate specificity such that they preferentially utilize polyisoprenoids of particular lengths and saturation states [52,67].

3. Eukaryota

The N-glycosylation pathway in eukaryotes has been extensively researched and characterized (see references [63,68] and [69] for recent reviews). First described based on experiments done in higher eukaryotes, including plants and animals [63,70], the basic principles of this pathway were later confirmed to be conserved in lower eukaryotes, such as yeast [63,71,72]. Eukaryotes use dolichol pyrophosphate, which has a saturated α -position isoprene unit, as the oligosaccharide carrier in N-linked glycosylation [52,63,70,73]. Dolichol pyrophosphate is biosynthesized on the cytoplasmic face of the endoplasmic reticulum (ER) from FPP and IPP [4,46,52], but the biosynthetic pathways that supply the isoprene subunits and the overall length of the polyisoprenoid are species-specific (see subsections 3.1-3.3) [63]. The early steps in the N-glycosylation process, involving the synthesis of a tetradecasaccharide on the Dol-PP carrier seem to be highly conserved across Eucarya and are discussed below. One known exception is in trypanosomatid protozoa, which cannot glucosylate their LLO precursors and therefore do not form the completed tetradecasaccharide [74,75]. The final processing steps, which occur after the oligosaccharide is transferred onto the nascent polypeptide, vary between organisms, as do the final oligosaccharide structures.

In eukaryotes, N-glycosylation begins on the cytoplasmic face of the ER [76] with the transfer of sugar residues from nucleotide-activated sugar donors, such as uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) and guanosine diphosphate mannose (GDP-Man), onto a dolichol phosphate carrier [62,77]. In the first reaction, GlcNAc-P is transferred onto Dol-P giving rise to GlcNAc-PP-Dol. Another GlcNAc residue is subsequently added to the structure, followed by 5 mannose groups. These reactions occur with the aid of specific glycosyltransferases, which use sugar nucleotides as substrates and add sugars according to a pre-defined reaction sequence, generating the heptasaccharide Man₅GlcNAc₂-PP-Dol [4, 77-79]. This heptasaccharide is then flipped across the ER membrane to the luminal face

[76]. This translocation is thought to occur with the help of an enzyme “flippase,” [4,80] which was recently identified as Rft1 in *Saccharomyces cerevisiae* by Helenius et al. [81]. However, Frank et al. failed to correlate the presence of Rft-1 with flippase activity in fractions from a CM-Sepharose purification [82]. They also saw identical flippase activity in wildtype and Rft-1-depleted yeast ER membrane extracts, leading them to conclude that Rft-1 may not contribute to flippase activity.

Once on the luminal face of the ER, four additional Man residues, and three glucose (Glc) residues are added to the dolichol-linked oligosaccharide chain. These reactions require glycosyltransferases that utilize lipid activated monosaccharides, such as Dol-P-Man and Dol-P-Glc, as sugar donors [4,62]. The synthesis of these lipid-linked monosaccharide sugar donors also requires dolichol phosphate, which reacts with UDP-Glc or GDP-Man via additional glycosyltransferases on the cytoplasmic face of the ER to form Dol-P-Man and Dol-P-Glc. These lipid-linked monosaccharides are also translocated across the ER membrane to the lumen through the action of flippases [4,62]. The final result is a dolichol pyrophosphate-linked tetradecasaccharide with the structure $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ (Fig. 6A) [78,83,84]. As was mentioned earlier, trypanosomatid protozoa cannot synthesize this structure because they are unable to synthesize Dol-P-Glc [75]. The genes for certain glycosyltransferases also seem to be absent in the genome of *Trypanosoma brucei* [74]. Consequently, trypanosomatid protozoa produce LLO with the structure $\text{Man}_9\text{GlcNAc}_2\text{-PP-Dol}$ [74,75].

The completed oligosaccharides can then be transferred from the dolichol carrier onto specific asparagine residues on a nascent polypeptide located in the ER. This occurs cotranslationally with the help of an oligosaccharide transferrase (OST) enzyme or enzyme complex [73,84]. After the oligosaccharide is transferred onto the protein, the Dol-PP carrier is de-phosphorylated into Dol-P then diffuses or is flipped back to the cytoplasmic face of the ER [85]. In this way, the dolichol carrier is recycled and can be reutilized for additional rounds of N-glycosylation [52,85]. For information about the specific enzymes involved in each step of LLO synthesis and oligosaccharide transfer in *S. cerevisiae*, we refer readers to reviews by Burda and Aebi [63] and Kelleher and Gilmore [69].

3.1 Animalia

Animals require isoprenoids for a wide variety of biological functions. Cholesterol, for example, is involved in maintaining membrane structure [86] and coenzyme Q plays a crucial role in energy synthesis via oxidative phosphorylation [87]. Isoprenoid-derived molecules are also involved in coagulation (vitamin K) and vision (retinol), and can act as signaling hormones (steroids) [7,86]. Animals utilize only the MVA pathway for isoprenoid synthesis, and therefore make all of their dolichol derivatives from mevalonate via this pathway [35,88]. Interestingly, animals do not synthesize polyisoprenoid lipids of a single chain length; rather they produce homologous mixtures. These mixtures, or “families”, consist of six to eight different chain lengths with one or two lengths predominating [21,42]. Most likely, the chain length is determined by the *cis*-prenyl transferase, which condenses IPP subunits onto FPP and produces dolichols with a distribution of similar lengths [42,47]. In mammalian cells, dolichols are predominantly 18 – 21 isoprene units in length [52]. Human cells contain dolichol that is primarily 19 isoprene units in length (Dol-19) [21], as do Chinese Hamster Ovary (CHO) cells [42,89]. Rats contain mostly Dol-18 [21,90], while *Drosophila* contain mainly Dol-16 and Dol-17 [91]. Dol-P is required in a number of reactions in the N-glycosylation pathway and the availability of Dol-P is often one of the rate limiting factors in the synthesis of lipid-linked oligosaccharides [63,92-94].

Though dolichol pyrophosphate is the lipid donor of choice in animals and all eukaryotes, a CHO cell line designated Lec9 was isolated that synthesizes polyprenol, rather than dolichol and utilizes this polyprenol to synthesize its N-glycosylation oligosaccharide donor [21,95].

The polyprenols in Lec9 cells have the same distribution of chains lengths as dolichols in the parental CHO cells [95]. The Lec9 phenotype is thought to be caused by a deficiency in polyprenol reductase, which reduces polyprenol into dolichol (Fig. 4) [95]. This reduction reaction appears to be a rate limiting step in the synthesis of dolichol [96]. Thus, polyprenol reductase appears to be important in the regulation of N-glycosylation in mammalian cells. This reduction step is also important because many glycosyltransferases involved in the formation of lipid-linked mono- or oligo-saccharides prefer polyisoprenoids with particular saturation states (e.g. Dol-P in animals) as a substrates [42,67]. For example, in CHO cells, the glycosyltransferase that catalyzes the first step in the N-glycosylation pathway (the formation of GlcNAc-PP-Dol from UDP-GlcNAc and Dol-P) is significantly impaired in its ability to utilize Poly-P compared to Dol-P [64]. Mannosylphosphoryldolichol synthase, which is responsible for the formation of the lipid activated monosaccharide Dol-P-Man, also has a clear preference for the Dol-P substrate [65].

3.2 Fungi

Fungi, represented most commonly in research by the yeast *Saccharomyces cerevisiae*, also utilize the traditional MVA pathway in their biosynthesis of dolichols [21]. Dolichols in yeast, like mammalian cells, are produced in the ER and exist as a family of different chain length molecules ranging in length from 14-17 isoprene units [52]. The most prevalent dolichols in *S. cerevisiae* contain 15 – 16 isoprene units, while those in *Schizosaccharomyces pombe* contain 17 isoprene units [42].

3.3 Plantae

Plants are especially interesting with regard to isoprenoids synthesis, because these photosynthetic organisms are the only ones that regularly utilize both the MVA and DOXP pathways in their production of polyisoprenoids. The isoprenoid metabolic pathways in photosynthetic organisms are involved in the biosynthesis of chlorophyll [97] as well as various plant hormones [98,99], defensive toxins and antibiotics [100,101], essential antioxidants [102,103], and pigments and aromas found in flowers and fruit [104,105]. Most relevant to this review, however, is the synthesis of dolichol and its derivatives for use in protein N-glycosylation.

Higher plants use both the MVA and DOXP pathways to synthesize isoprenoid compounds, oftentimes in concert. One divergent feature of the two possible routes for IPP and DMAPP synthesis is their different subcellular localizations. The enzymes involved in the MVA pathway are typically located within the cytosol [106,107], while the DOXP pathway is sequestered within the chloroplast [108-110]. Isoprenoids that are fundamental to photosynthesis, including chlorophyll, carotenoids, and plastoquinone are primarily formed within the chloroplast [106,111], while other isoprenoids, such as sterols and ubiquinone are mainly formed in the cytosol [106]. The same is true for most diatomaceous algae (i.e. diatoms) [112]. In contrast, the majority of green algae maintain only DOXP isoprenoid metabolism and, as a result, all algal isoprenoids are derived from chloroplastic IPP and DMAPP [113, 114]. The chloroplasts of plants and green algae are believed to represent remnants of prokaryotic photosynthetic cyanobacteria, which sought endosymbiosis within eukaryotic cells [115]. Interestingly, cyanobacteria utilize only the DOXP pathway to produce isoprenoids [113,116-118], further supporting the idea that chloroplasts, which also contain the DOXP isoprenoid pathway, evolved via endosymbiosis.

Despite the compartmentalization of the MVA and DOXP pathways, molecular exchange of isoprenoid precursors such as IPP between the cytosol and chloroplast occurs routinely in higher plants [119-124]. Researchers have recently elucidated the extent of this interaction (crosstalk) during the synthesis of dolichol [125]. Skorupinska-Tudek and colleagues

investigated the metabolic network responsible for dolichol synthesis in the hairy root of the *Coluria geoides* plant. Their results showed that dolichol synthesis begins with IPP molecules in the chloroplast. Due to a continuous exchange of intermediates between the MVA and DOXP pathways, the IPP in this reservoir is equally likely to have been produced by the local DOXP pathway or imported from the cytosol (Fig. 7) [124,125]. Consequently, the carbon found at the ω -terminus of dolichol is not specific to either pathway. The polyisoprenoid chain is then elongated within the chloroplast using IPP from either pathway to form a Poly-PP with less than 14 isoprene units. Finally, this intermediate is exported to the cytosol wherein its conversion to dolichol is completed using IPP derived solely from the MVA pathway [125]. This results in dolichol's α -terminus being comprised solely of carbon of the MVA pathway (Fig. 7). Ultimately, 40-50% of the dolichol molecule was found to originate from the DOXP pathway, indicating significant contributions from both isoprenoid biosynthetic pathways [125].

Notable diversity has been observed when comparing the families of different length dolichols produced in higher plants and algae. In various model plant systems, including *Arabidopsis thaliana*, *Glycine max* (soybean), and *Coluria geoides*, as well as common plants such as magnolia, spinach, parsley, and perilla, the length of most dolichol molecules falls within the range of 14-18 isoprene units [125-128]. Interestingly, dolichols found in soybean seeds contain one additional isoprene unit compared to those in the roots, shoots, and leaves, which typically have 16 – 17 isoprene units [127]. In the microalga *Prototheca zopfii*, however, dolichols were found to be considerably longer, ranging from 18 – 21 isoprene units [129]. Conversely, in the green alga *Chlamydomonas reinhardtii*, dolichol was observed to have the unexpectedly short chain-length of just 11 isoprene units. This structure is similar to the undecaprenyl pyrophosphate used as the oligosaccharide carrier in prokaryotes; however, the molecule does contain the saturated α -isoprene unit that is characteristic of eukaryotes [130].

Since photosynthetic organisms depend on sunlight as both a source of energy and an important environmental cue to elicit metabolic and developmental changes, it is not surprising that sunlight is used to regulate isoprenoid biosynthesis in these organisms. Along the DOXP pathway, the enzyme IspE is responsible for the phosphorylation of the isoprenoid intermediate CDP-ME. In *Nicotiana benthamiana*, expression of the gene that encodes IspE is induced by light [40]. In *Arabidopsis*, expression of the gene encoding Dxs is also induced by light [131]. In the MVA pathway, however, expression of the rate-limiting enzyme HMGR appears to be suppressed in *Arabidopsis thaliana* by prolonged light stimulus [132,133]. In this case light triggers photoreceptor-mediated signaling cascades which converge to downregulate HMGR expression [133,134]. Generally, exposure to light causes downregulation of genes in the MVA pathway, while upregulating expression of DOXP pathway genes [133]. The result of this is activation of isoprenoid synthesis in the chloroplast, where many of the key photosynthetic isoprenoids are formed.

4. Bacteria

Although it has been known for decades that polyisoprenoids were involved in the formation of bacterial peptidoglycan [135], Bacteria was the last domain of life in which N-glycosylation was discovered, perhaps because it is such a rare event in these organisms [2,4,73]. The DOXP pathway was first discovered in bacteria [9,12], and while many gram-positive cocci use the traditional MVA pathway for isoprenoid synthesis, many gram-negative bacteria utilize only the DOXP pathway [108,136]. Bacteria typically employ either the MVA or the DOXP pathway on an exclusive basis [35]. Interestingly, photosynthetic cyanobacteria utilize only the DOXP pathway to isoprenoids [113,116-118].

In bacteria that utilize the MVA pathway for isoprenoid synthesis, an IPP isomerase is essential for conversion of IPP into DMAPP (Fig. 2). In 2001, Kaneda et al. discovered a novel IPP isomerase in *Streptomyces* which was not homologous to any previously identified IPP isomerase [137]. Orthologs of this enzyme were subsequently found in the genomes of 35 out of 124 eubacterial families [35]. These novel IPP isomerases were designated type II, while the more traditional IPP isomerases found in eukaryotes and some other bacteria, were designated type I [35,137]. Bacteria exist that utilize every combination of type I and type II IPP isomerases in combination with the MVA and DOXP pathways. No isomerase at all is detected in about 70% of bacteria that utilize only the DOXP pathway [35]. One study showed that the IPP isomerase gene is not essential in *E. coli* because they utilize the DOXP pathway and can therefore synthesize both IPP and DMAPP [138]. *E. coli* do typically express an IPP isomerase, however, which is thought to help adjust the ratio of DMAPP to IPP to optimum levels [35,50,138].

Interestingly, the lipid sugar donor involved in N-glycosylation in bacteria is a single polyprenol composed of 11 isoprene units, as opposed to the family of different length isoprenoids that are seen in eukaryotes [21,42,139]. The bacterial *cis*-prenyl transferase, undecaprenyl pyrophosphate synthase (UPPS), is a soluble enzyme that is responsible for adding 8 IPP units to FPP, forming undecaprenyl pyrophosphate (Und-PP) [21,140]. As opposed to the lipid donor in eukaryotes, the bacterial undecaprenyl pyrophosphate is a polyprenol, in that its α -isoprene unit is unsaturated [52,84]. Although the 11 isoprene unit undecaprenyl pyrophosphate is the rule, some exceptions exist. In *Mycobacterium smegmatis* the oligosaccharide is assembled on a Poly-PP either 10 or 7 isoprene units in length [52,93,141], while *Mycobacterium tuberculosis* synthesize only decaprenyl pyrophosphate (10 isoprene units) [93].

Bacterial N-glycosylation was first discovered in *C. jejuni* and most of our current understanding of the process is based on studies of this organism [2,142]. Bacteria, like archaea, lack cellular organelles, so glycosylation occurs across the cell membrane [2,143]. Nucleotide-activated sugars are transferred via glycosyltransferases onto the undecaprenyl pyrophosphate lipid carrier which is located on the cytoplasmic face of the cell membrane [144]. This process is controlled by the *pgl* gene cluster (for a review see [4]) and results in a heptasaccharide with the following structure: GlcGalNAc₅Bac-PP-Und (Bac = bacillosamine, 2,4-diacetamido-2,4,6-trideoxyglucopyranose; GalNAc= N-acetylgalactosamine) (Fig. 6B) [4, 73,142,145]. This lipid-linked oligosaccharide is then flipped across the cell membrane to the periplasm (also by a member of the *pgl* gene family) and the glycan is transferred *en bloc* onto select asparagine residues by an oligosaccharide transferase (encoded by *pglB* in *C.jejuni*) [4, 73,142,146]. There is no evidence that the lipid-linked oligosaccharide is further modified after it is translocated across the membrane [143]. It is still uncertain whether bacterial N-glycosylation occurs co- or post-translationally [4], although it appears to occur independently of the protein translocation machinery since research indicates that the oligosaccharide transferase can transfer N-glycans to completely folded proteins [142,147].

5. Archaea

The production of isoprenoids in Archaea is typically of interest, not due to its role in glycosylation, but due to the fact that isoprenoids make up a major component of archaeal membranes [88,148]. In contrast with bacterial and eukaryotic membrane lipids, which consist of fatty acid side chains linked to glycerol via ester linkages [50,149], archaeal membrane lipids are made up of isoprenoid chains linked to glycerol via ether linkages [88, 150]. Archaea are generally thought to produce isoprenoids through the traditional MVA pathway [35,88]. This idea was supported by the discovery of homologs to several genes in the MVA pathway, such as HMG-CoA synthase and HMG-CoA reductase, in the Archaeal

genome [151-153]. Homologous genes for the last two enzymes in the pathway, however, were not identified [88,149]. It was thought that these enzymes, phosphomevalonate kinase and mevalonate-5-pyrophosphate decarboxylase, could be nonorthologous enzymes, thus making them more difficult to identify and characterize [88]. In 2005, however, Grochowski et al. proposed an alternative route to produce IPP from acetyl-CoA in Archaea. They discovered an enzyme that catalyzed the formation of IPP from isopentenyl phosphate (IP) called IP kinase and proposed a archaeal variation of the MVA pathway (Fig. 2) [88]. Instead of mevalonate phosphate being phosphorylated, then decarboxylated (as in the typical MVA pathway), they propose a pathway in which mevalonate phosphate is first decarboxylated by a phosphomevalonate decarboxylase into IP and then phosphorylated, via the IP kinase they discovered, into IPP [88].

Until recently the method of DMAPP production in Archaea was a mystery, due to the failure of genomic analysis to identify any archaeal homologs of IPP isomerase [35]. The discovery of the type II IPP isomerase in bacteria [137] resolved this issue, however, and orthologs of this type II enzyme have now been found in all 11 archaeal families [35]. Current evidence suggests that archaea utilize a variation on the traditional MVA pathway, in combination with type II IPP isomerases, in their production of isoprenoids [50].

Unlike eukaryotes and bacteria, archaea do not use a single polyisoprenoid lipid donor in their N-glycosylation pathway. Archaea, like eukaryotes, use dolichol in their synthesis of LLOs but evidence of both Dol-P and Dol-PP being used as oligosaccharide carriers has been reported [73,143]. In *Haloferax volcanii*, oligosaccharides are linked to dolichol via a monophosphate bridge [154], while in *Halobacterium salinarum*, both monophosphate and pyrophosphate linked oligosaccharides are found [84,155,156]. The dolichol used by *H. volcanii* is also unique, in that the ω -terminal isoprene, in addition to α -isoprene unit, is saturated [154]. Archaeal dolichols typically appear to be 10-12 isoprene units in length [84,154].

Evidence suggests that archaeal N-glycosylation occurs on the external surface of the cell membrane [73,84,157]. A process similar to the eukaryotic N-glycosylation system is assumed, in which monosaccharides from nucleotide-activated sugar donors are sequentially added to the dolichol mono- or pyro-phosphate on the cytoplasmic face of the cell membrane [5,84,157]. After the oligosaccharide is assembled on the carrier lipid, it is translocated, by an unknown enzyme, across the membrane to the extracellular surface. In a final step, the oligosaccharide is transferred to an asparagine residue of the nascent protein by an oligosaccharide transferase [5,84,143]. At present, it is unknown whether this modification occurs post- or co-translationally [5]. An overview of the current understanding of the genes and enzymes involved in this process can be found in an excellent recent review on archaeal N-glycosylation by Yurish-Doutsch et al. [5].

Unlike eukaryotes, archaea display wide diversity in the linking sugars and sugar composition of their glycoproteins [5,73]. Evidence of glycans containing a variety of saccharides, such as galactofuranose, galactouronic acid, glucose, glucuronic acid, iduronic acid, mannose, *N*-acetylgalactosamine, *N*-acetylglucosamine, and rhamnose, has been reported [84,158-160]. Archaeal N-glycans are typically smaller than their eukaryotic counterparts and thus far, no antennary (branched) structures been detected in archaea [73,84]. There is also evidence that some LLO are either transiently or permanently chemically modified by sulfation or methylation, and that methylation may be involved in translocation of sulfated oligosaccharides across the membrane [84,158,161,162]. The archaeal N-glycosylation pathway is extremely interesting in its use of aspects from both the eukaryotic (MVA pathway, use of dolichol donor) and bacterial (Type II IPP isomerase) N-glycosylation pathways, but also in the aspects that are unique to the archaeal domain (variety in sugar structure, LLO methylation).

6. Conclusion

Although the eukaryotic isoprenoid and LLO biosynthetic pathways seem well understood, there remain many questions that could be addressed by further research in this area. For example, the final steps in the dolichol recycling mechanism, involving the dephosphorylation of dolichol pyrophosphate and the translocation of the non-glycosylated lipid intermediate back across the ER membrane, remain unclear. The evolving story of the Rft-1 “flippase” illustrates that the identity and specificity of the proteins that catalyze the flipping reactions in N-linked glycosylation remain to be elucidated. The details of polyprenol reductase activity also remain unknown. These basic enzymatic questions remain primarily because of the difficulty in producing substrates as well as the difficulty in isolating and studying the enzymes due to the hydrophobic nature of the membrane proteins themselves.

The bacterial pathway has been studied extensively in *C. jejuni*, but given the great diversity of this domain, it will be important to identify and fully understand N-glycosylation processes in other representative bacteria. Archaea have the potential to provide a rich source of information on the different pathways possible for generation of variety in both the lipid and oligosaccharide portions of the LLO. Hopefully our understanding of eukaryotic and bacterial N-glycosylation pathways will aid in the elucidation of the process in Archaea and other species. However, the variety and diversity seen in sugar structure and isoprenoid synthesis across the three domains of life show that nature still offers many surprises in the generation and modification of lipid-linked oligosaccharides for protein N-glycosylation.

Acknowledgement

This work was supported by Grant number R01 GM077530 from the National Institute of Health.

List of Abbreviations

LLO, lipid-linked oligosaccharide
 OST, oligosaccharide transferase
 IPP, isopentenyl pyrophosphate
 DMAPP, dimethylallyl pyrophosphate
 MVA, mevalonate
 DOXP, 1-deoxy-D-xylulose-5-phosphate
 HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA
 HMGS, HMG-CoA synthase
 HMGR, HMG-CoA reductase
 MPPD, MVA-5-pyrophosphate decarboxylase
 Dxs, DOXP synthase
 IspC, DOXP reductase
 MEP, 2-C-methyl-D-erythritol,4-phosphate
 CTP, cytidine triphosphate
 CDP-ME, 4-pyrophosphocytidyl-2-C-methyl-D-erythritol
 IspD, CDP-ME synthase
 IspE, 4-pyrophosphocytidyl-2C-methyl-D-erythritol Kinase
 CDP-MEP, CDP-ME-2-phosphate
 MECPP, 2C-Methyl-D-erythritol-2,4-cyclopyrophosphate
 IspF, MECPP synthase
 CMP, cytidine monophosphate
 HMBPP, (*E*) 1-hydroxy-2-methyl-2--butenyl-4-pyrophosphate
 GPP, geranyl pyrophosphate
 FPP, farnesyl pyrophosphate

GGPP, geranylgeranyl pyrophosphate
 FGPP, farnesylgeranyl pyrophosphate
 Poly-PP, polyprenyl pyrophosphates
 Poly-P, polyprenyl phosphate
 Dol-P, dolichol phosphate
 Dol-PP, dolichol pyrophosphate
 ER, endoplasmic reticulum
 UDP-GlcNAc, uridine diphosphate N-acetylglucosamine
 GDP-Man, guanosine diphosphate mannose
 Glc, glucose
 Oligo-PP, oligoprenyl pyrophosphate
 UPPS, undecaprenyl pyrophosphate synthase
 Und-PP, undecaprenyl pyrophosphate
 bacillosamine, Bac, 2,4-diacetamido-2,4,6-trideoxyglucopyranose
 GalNAc, N-acetylgalactosamine
 IP, isopentenyl phosphate

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Fig 1.

Schematic overview polyisoprenoid alcohol formation and utilization in N-glycosylation. The MVA and DOXP pathways provide the isoprenoid precursors DMAPP and IPP, which are used to synthesize dolichol and polyprenol. Dolichol and polyprenol are modified by sugar additions into a completed LLO, then the oligosaccharide is transferred off of the carrier lipid and onto asparagine residues on the protein.

MVA pathway

Fig 2.

The MVA biosynthetic pathway for IPP and DMAPP. Acetyl-CoA is used to synthesize IPP and DMAPP with MVA as an intermediate. In the modified archaeal pathway, MVA-5-phosphate is first decarboxylated then phosphorylated to form IPP, the opposite of what occurs in the traditional MVA pathway. *Enzyme is theoretical and has yet to be discovered. For a review of the MVA pathway with chemical structures see [108].

DOXP pathway

Fig 3.

The DOXP biosynthetic pathway for IPP and DMAPP. Glyceraldehyde and pyruvate are used to synthesize IPP and DMAPP with DOXP as an intermediate. For a review of the DOXP pathway with chemical structures see [108].

Fig 4.

Polyprenol is reduced via polyprenol reductase to form dolichol. The structure of polyprenol and dolichol differ based on the saturation of the α -isoprene unit. The three isoprene units originating from farnesyl pyrophosphate (FPP) at the ω end of the molecule are in the *trans* configuration, while the remaining isoprene units are in the *cis* configuration.

Fig 5.
The biosynthesis of Poly-PP from IPP and DMAPP through the intermediates GPP and FPP.
 n = number of isoprene units.

Fig 6.
The sugar structures of the final lipid-linked oligosaccharide in eukaryotes (A.) and bacteria (B.)

Fig 7.

Dolichol synthesis in *Coluria geoides* hairy root. Cytosolic IPP from the MVA pathway can enter the plastid. Dolichol synthesis begins in the plastid where a Poly-PP with less than 14 isoprene units is formed using IPP from both the MVA and DOXP pathways. The Poly-PP is then transported to the cytosol, where synthesis continues using only IPP from the MVA pathway.