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Chapter Title	The Alg1, Alg2, and Alg11 Mannosyltransferases of the Endoplasmic Reticulum	
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Abstract	Asparagine (*N*)-linked glycans are common posttranslational modifications found on many glycoproteins in bacteria to man. These glycans are not produced by addition of individual sugars directly on an Asn residue side chain of a protein, but rather as lipid-linked precursors that only after their assembly are transferred to nascent proteins. Once attached to protein, these glycans are modified in a variety of different ways. The structures of mature protein *N*-linked glycans vary enormously among eukaryotic species and even among cells of the same species. This is in stark contrast to the highly conserved structure of the preassembled lipid-linked precursor $Glc_3Man_9GlcNAc_2$ oligosaccharide (LLO), which, with few exceptions, is shared by all eukaryotes (Fig. 14.1). The conserved structure of the LLO is a reflection of the evolutionary conservation of the 12 different glycosyltransferases that catalyze its production. LLO synthesis begins on the cytoplasmic face of the endoplasmic reticulum and is completed in the lumen (Fig. 14.2). Two *N*-acetyl-glucosamines (GlcNAc) and five mannoses (man) are covalently attached to dolichol pyrophosphate (PP-Dol) on the cytosolic face of the ER. After flipping across the membrane, seven sugars (four man and three glucoses (glc)) are attached in the lumen and then transferred to nascent proteins by oligosaccharyltransferase (Fig. 14.2).	

14 The Alg1, Alg2, and Alg11 Mannosyltransferases of the Endoplasmic Reticulum

Neta Dean

Contents

Introduction

Asparagine (*N*)-linked glycans are common posttranslational modifications found on many glycoproteins in bacteria to man. These glycans are not produced by addition of individual sugars directly on an Asn residue side chain of a protein, but rather as lipid-linked precursors that only after their assembly are transferred to nascent proteins. Once attached to protein, these glycans are modified in a variety of different ways. The structures of mature protein *N*-linked glycans vary enormously among eukaryotic species and even among cells of the same species. This is in stark contrast to the highly conserved structure of the preassembled lipid-linked precursor $Glc_3Man_9GlcNAc_2$ oligosaccharide (LLO), which, with few exceptions, is shared by all eukaryotes (Fig. 14.1). The conserved structure of the LLO is a reflection of the evolutionary conservation of the 12 different

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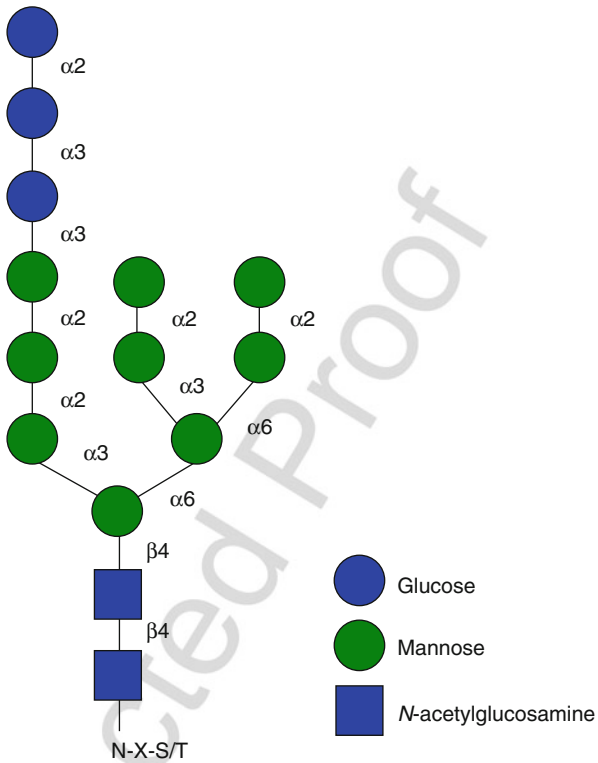

Fig. 14.1 Structure of the core *N*-linked oligosaccharide. This figure depicts the glycosidic linkage and sugars of the $Glc_3Man_9GlcNAc_2$ tetradecasaccharide core covalently attached to the side chain of an asparagine in the N-X-S/T consensus sequence for N-linked glycosylation

glycosyltransferases that catalyze its production. LLO synthesis begins on the cytoplasmic face of the endoplasmic reticulum and is completed in the lumen (Fig. 14.2). Two *N*-acetyl-glucosamines (GlcNAc) and five mannoses (man) are covalently attached to dolichol pyrophosphate (PP-Dol) on the cytosolic face of the ER. After flipping across the membrane, seven sugars (four man and three glucoses (glc)) are attached in the lumen and then transferred to nascent proteins by oligosaccharyltransferase (Fig. 14.2).

The Alg1, Alg2, and Alg11 mannosyltransferases, which together catalyze the first five man additions on the ER cytoplasmic face, are the focus of this chapter. In the past decade since the last "Handbook of Glycosyltransferases and Related Genes" was released in 2002, there has been significant progress in our understanding of how these mannosyltransferases function. Notably, as is true for the vast majority of cellular proteins, we now know that in vivo these mannosyltransferases function as part of a larger mannosyltransferase protein complex rather than as individual polypeptides (Gao et al. 2004). Furthermore, the Alg2 and Alg11 enzymes each catalyze

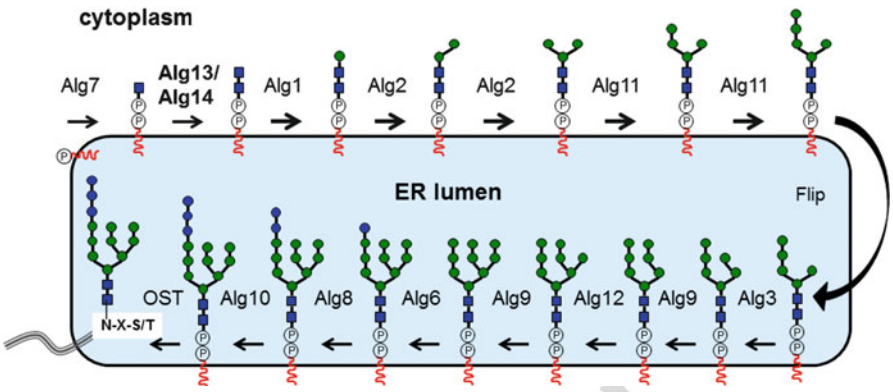

Fig. 14.2 Synthesis of $GlcNAc_2Man_9Glc_3$-PP-Dol at the endoplasmic reticulum. Shown is a schematic depiction of the sequential assembly of the 14 sugars on dolichol pyrophosphate by the ER mannosyltransferases. Seven sugars are added on the cytoplasmic face and then another seven in the lumen. The first GlcNAc is added by Alg7, in which GlcNAc-1-P from UDP-GlcNAc is added to dolichol phosphate (P-Dol) to generate GlcNAc-PP-Dol. GlcNAc from UDP-GlcNAc is then added by the Alg13/Alg14 hetero-oligomeric GlcNAc transferase to generate $GlcNAc_2$-PP-Dol. The next five mannoses are added sequentially using GDP-man by the Alg1, Alg2, and Alg11 mannosyltransferases, which are the focus of this chapter. Alg1 adds the first β1,4-linked man, and as described in the text, Alg2 and Alg11 each catalyze two sequential reactions. Alg2 adds the second and third α1,6- and α1,3-linked mannoses, while Alg11 adds the fourth and fifth α1,2-linked mannoses, generating Man_5Glc_2-PP-Dol. This intermediate flips into the lumen, where it is further extended by four mannose and three glucose residues from dolichol-linked sugars cytoplasmic face of the ER. Oligosaccharyltransferase transfers this "core" oligosaccharide from dolichol to nascent polypeptides

two reactions of LLO biosynthesis, rather than a single transfer reaction (O'Reilly et al. 2006; Kampf et al. 2009; Absmanner et al. 2010). Thus, we now know the identity of all the glycosyltransferases for each and every step of yeast LLO biosynthesis. This knowledge has had direct applicability to human glycobiology and disease.

Databanks

The Alg1, Alg2, and Alg11 mannosyltransferases are highly conserved among all eukaryotes. For simplicity, Tables listed below gives information about the yeast and human orthologues, including enzyme names as recommended by the *Nomenclature Committee of the International Union of Biochemistry and Molecular Biology* (NC-IUBMB), useful gene and protein accession numbers, and alternative names that are found in the literature. Au1

Alg1 (GDP-Man: GlcNAc2-PP-Dolichol mannosyltransferase)

NC-IUBMB enzyme classification: *EC 2.4.1.142*

The Alg1 mannosyltransferases of the endoplasmic reticulum

Species	Gene	Protein
Saccharomyces cerevisiae	AAA66322	P16661 (UniProt)
		CAA85067.1
Homo sapiens	AB019038.1	Q9BT22 (UniProt)
		NP_061982.3
Alternative name(s):		
Chitobiosyldiphosphodolichol beta-mannosyltransferase Asparagine-linked glycosylation protein 1 homolog Beta-1,4-mannosyltransferase GDP-mannose-Dolichol diphosphochitobiose mannosyltransferase Mannosyltransferase-1		
HMAT1/HMT1(human homologue)		

Alg2 (alpha-1,3/1,6-mannosyltransferase).

NC-IUBMB enzyme classification: EC number 2.4.1.257

The Alg2 mannosyltransferases of the endoplasmic reticulum

Species	Gene	Protein
Saccharomyces cerevisiae	X87947	P43636 (UniProt)
		CAA96768
Homo sapiens	85365	Q9H553 (UniProt)
		NP_149078
Alternative names[a]:		
GDP-Man: Man1GlcNAc2-PP-Dol alpha-1,3-mannosyltransferase GDP-Man: Man2GlcNAc2-PP-Dol alpha-1,6-mannosyltransferase GDP-Man: Man1GlcNAc2-PP-Dol alpha-1,3/alpha-1,6-mannosyltransferase		
Alpha-1,3-mannosyltransferase		

[a]Note that there is an unrelated human gene also known as ALG2, for apoptosis-linked gene 2

Alg11 (GDP-Man: Man(3)GlcNAc(2)-PP-Dol alpha-1,2-mannosyltransferase)

NC-IUBMB enzyme classification: EC number 2.4.1.131

The Alg11 mannosyltransferases of the endoplasmic reticulum

Species	Gene	Protein
Saccharomyces cerevisiae	U62941	P53954 (UniProt)
		CAA95916.1
Homo sapiens	440138	Q2TAA5 (UniProt)
		NP_001004127.2
Alternative names[a]:		
Alpha1, 2 mannosyltransferase GDP-Man: $Man_3GlcNAc_2$-PP-Dol alpha-1, 2-mannosyltransferase Asparagine-linked glycosylation protein 11		
Glycolipid 2-alpha mannosyltransferase		

Name and History

Much of what we know about the early steps of N-linked glycosylation in the ER comes from extensive genetic and biochemical analyses of this process in the budding yeast, *S. cerevisiae*. Although there are some differences in the structure and processing of the lipid-linked oligosaccharide among different species, overall, the entire process is highly conserved. The insights from yeast studies have been directly applicable to other systems, especially humans where mutations in this process manifest as a family of diseases, collectively known as congenital defects in glycosylation (CDG). The gene name asparagine-***l***inked ***g***lycosylation (*ALG*) was first coined by Huffaker and Robbins to describe a set of yeast mutants with temperature-sensitive defects in glycosylation (Huffaker and Robbins 1982). To isolate these mutants, they used a ^{3}H-mannose suicide selection scheme, whereby mutagenized cultures incubated with ^{3}H-mannose were frozen at −80 °C for about a month. The rationale behind this scheme was that wild-type yeast whose walls are very rich in mannosylated glycoproteins incorporates large amounts of H^{3}-man into their walls and would therefore be unable to withstand long-term tritium-induced radiation damage. In contrast, glycosylation mutants that incorporate less mannose into their glycoproteins were predicted to survive. These predictions were borne out and led to identification of a number of different *alg* mutants, including *alg1* and *alg2* (Couto et al. 1984; Jackson et al. 1993). Additional *alg* mutants (through to *alg14*) with specific defects in LLO biosynthesis have been isolated using different strategies since then and were named in the order of their discovery, rather than the order of the reaction catalyzed (see Fig. 14.2). The *alg11* mutant was identified based on its resistance to sodium vanadate, a drug that enriches for glycosylation mutants (Cipollo et al. 2001). Tables in the Databanks provide links to databases that provide further information about the *ALG1*, *ALG2*, and *ALG11* mannosyltransferase genes, proteins, and alternative names in the literature for each enzyme.

Enzyme Activity

The Alg1, Alg2, and Alg11 mannosyltransferases are ER membrane proteins with catalytic domains that face the cytosol and that use GDP-man as the sugar donor for mannosylation. Alg1 is a β1,4 mannosyltransferase that adds the first mannose onto $GlcNAc_2$-PP-Dol using GDP-mannose to produce $Man_1GlcNAc_2$-PP-Dol (Fig. 14.2).

Alg2 carries out the next two mannosylations, adding both α1,3- and α1,6-linked mannose to $Man_1GlcNAc_2$-PP-Dol (O'Reilly et al. 2006; Kampf et al. 2009; Absmanner et al. 2010) (Fig. 14.2). This leads to the first branched trimannosyl chitobiose intermediate $Man_3GlcNAc_2$-PP-Dol. This bifunctionality distinguishes Alg2 as a glycosyltransferase with both α1,3- and α1,6-mannosyltransferase activity. Biochemical analysis of extracts derived from both yeast and human *alg2* mutants suggests that the preferred order of addition proceeds by the addition of an α1,3-linked mannose followed by addition of the α1,6-linked mannose

(Thiel et al. 2003). However, microsomal extracts from wild-type human cells are able to elongate Man(α1,6)Man-GlcNAc$_2$-PP-dolichol acceptor to Man$_5$-GlcNAc$_2$-PP-dolichol (Thiel et al. 2003), implying there is not an absolute requirement for the α1,3- mannose linkage prior to the α1,6-mannosylation. Further experiments are required to understand what regulates the acceptor substrate specificity of this unusual enzyme.

Alg11 carries out the next two mannosylations, sequentially adding the fourth and fifth α1,2-linked mannoses on the α1,3 arm of the branched intermediate (Cipollo et al. 2001; O'Reilly et al. 2006; Absmanner et al. 2010). This produces the Man$_5$GlcNAc$_2$-PP-Dol intermediate, the precursor for the putative "flippase" that catalyzes the transbilayer translocation of Man$_5$GlcNAc$_2$-PP-dol from the cytoplasm to the ER lumen, where it undergoes further elongation and then transfer to the nascent protein (Fig. 14.2).

Alg1, Alg2, and Alg11 have been purified as functional transferases in vitro, from endogenous microsomal membrane fractions (primarily yeast or mammalian cells) or as recombinant proteins expressed in *E. coli* (Couto et al. 1984; O'Reilly et al. 2006). Incubation of detergent extracts from microsomal membranes with [^{14}C]GlcNAc$_2$-PP-Dol and unlabeled GDP-Man leads to the production of Man$_5$GlcNAc$_2$-PP-Dol. Synthetic water-soluble phosphorylated analogues of dolichol or GlcNAc2-PP-Dol (e.g., citronellol-P/Dol$_{10}$ or GlcNAc-PP- citronellol) can also be extended to Man$_5$GlcNAc$_2$-PP-Cit by membrane proteins isolated from the CHO Lec15 mutant that lacks the Man-P-Dol synthase (Rush and Waechter 2005). Lipid-linked oligosaccharide products of these enzymatic reactions are commonly analyzed by descending chromatography, HPLC, or matrix-assisted laser desorption/ionization tandem time of flight (MALDI-TOF/TOF). Protein-linked *N*-glycans are commonly analyzed via molecular weight shift assays of reporter glycoproteins, such as carboxypeptidase Y (CPY) in yeast, or serum transferrin in humans. The hypoglycosylation of these reporters results in a characteristic electrophoretic pattern due to decreased glycan site occupancy (see below). As an alternative to analyzing individual reporters, *N*-linked glycans attached to bulk proteins are analyzed by preparing whole cell lysates from normal or LLO mutant cells and incubating the lysates with glycosidases, such as peptide *N*-glycanase (PNGase) that specifically cleaves between the innermost GlcNAc of the *N*-linked glycan and asparagine. The released glycans can be purified and analyzed as described above or by fluorophore-assisted carbohydrate electrophoresis (FACE).

Normal Function/Biological Aspects

Synthesis of the LLO is sequential, so a block anywhere along the pathway results in an accumulation of truncated oligosaccharide. In yeast, mutations that interfere with the early LLO biosynthetic steps on the cytoplasmic face of the ER lead to very severe growth phenotypes. In contrast, mutations that interfere with the later luminal steps have less severe phenotypes, despite the fact that these mutations

lead to the production of glycoproteins that are decorated with fewer glycans. The reduced occupancy of glycosylation sites in LLO mutants occurs because oligosaccharyltransferase preferentially recognizes and transfers only fully assembled LLO.

The sequential, stepwise order of sugar additions leading to core oligosaccharide assembly is a consequence of several factors. First, these reactions are catalyzed by enzymes whose catalytic domains have evolved different ER membrane topologies. Enzymes catalyzing the cytosolic reactions have their catalytic domains facing the cytosol, while those catalyzing the luminal compartment have their catalytic domains facing the lumen. These topologies are coincident with the biosynthetic location of sugar donors; enzymes catalyzing the reactions on the cytosolic face of the ER use nucleotide sugar donors, while enzymes catalyzing the luminal reactions use dolichol-linked sugar donors. In addition to these topological constraints, the order of reactions is dictated by the substrate specificity of each enzyme, where the product of the preceding reaction is the substrate for the next.

Finally, an additional factor that may regulate the activity of these related mannosyltransferases in vivo is their physical interaction with one another in larger multi-protein complexes. Biochemical analyses have identified two distinct ER mannosyltransferase complexes. Both of them contain two or more copies of Alg1 and are distinguished from each other by the presence of Alg2 or Alg11 (Gao et al. 2004). Support for the idea that formation of these complexes is important for in vivo function comes from genetic data; over expression of catalytically inactive *alg1* alleles producing Alg1 proteins that maintain the ability to both homodimerize and interact with Alg2 and Alg11 displays dominant-negative phenotypes. It is unknown how the interaction of Alg2 and Alg11 with Alg1 modifies their activity. One explanation is that the interaction of these related mannosyltransferases that catalyze sequential reactions limits the requirement for substrate diffusion. Alg7 (aka DPAGT1), Alg13, and Alg14, which catalyze addition of the first and second GlcNAcs of the LLO, also form a hetero-oligomeric GlcNAc transferase. This finding lends support to the idea that physical interactions between related glycosyltransferases may be a common mechanism to optimize or modulate GTase activity in vivo (Noffz et al. 2009). Testing this idea remains a major challenge.

Human Disease

Early on, clinical assays for glycosylation defects, based on isoelectric focusing of serum transferrin, distinguished two distinct classes of defects: a reduction in glycan site occupancy, termed CDG type I, and a reduction in glycan chain length, termed CDG type II. Earlier studies of LLO yeast mutants whose shared common phenotype was reduction in *N*-glycan transfer by oligosaccharyltransferase thus predicted that CDG type I defects would result from mutations that interfere with LLO synthesis. This has proven to be the case, and every CDG-I mutation mapped thus far affects enzymes involved directly or indirectly with the LLO pathway. With regard to Alg1, Alg2, and Alg11, these deficiencies are named CDG-Ik,

CDG-Ii, and CDG-Ip, respectively (Thiel et al. 2003; Kranz et al. 2004; Rind et al. 2010). In each case, these mutations have been mapped, characterized as enzyme deficiencies in lysates from patient's fibroblasts, and the cloned human wild-type and mutant alleles assayed for gain or loss of function through complementation of the corresponding yeast *alg* mutant. These are relatively rare mutations so the phenotypic consequences cannot be generalized; phenotypes range from slight mental or psychomotor impairment to multiple organ dysfunction and infantile lethality. Most mutations in *hALG1*, *hALG2*, and h*ALG11* identified thus far are missense or partial-loss-of-function mutations. Since *ALG1*, *ALG2*, and *ALG11* are essential genes in other eukaryotes, it seems likely that complete loss of function of any of these mannosyltransferases in humans would lead to embryonic lethality.

Future Perspective

Despite our detailed biochemical knowledge of the oligosaccharide biosynthetic pathway, there remain many outstanding questions. There is good evidence that changes in the flux through this pathway leads to increase or decreased levels of glycosylation, and a major goal is to understand how this pathway is regulated and feedbacks into other metabolic pathways. The importance of glycosylation for correct protein folding, stability, and activity has long been appreciated. With increasing technical efficiency of biopharmaceutical protein production has come the increasing demand to engineer host systems that correctly glycosylate these proteins (Chiba and Jigami 2007). Yeast cells efficiently glycosylate heterologous proteins, but modify them in a nonhuman way, with excessive amounts of mannose. Host expression systems, such as *S. cerevisiae*, *Pichia pastoris*, *and Yarrowia lipolytica*, have been genetically engineered in a variety of different ways to produce more "humanized" glycans (for instance, see De Pourcq et al. 2012). Recently the bioengineering of bacterial strains that co-express yeast *ALG13*, *ALG14*, *ALG1*, and *ALG2*, along with the *Campylobacter jejuni* PglB oligosaccharyltransferase, has demonstrated successful production of lipid-linked $Man_3GlcNAc_2$ and its transfer to eukaryotic proteins in vivo (Valderrama-Rincon et al. 2012). This raises the hope that customized glycosylation of recombinant glycoproteins in bacteria will facilitate the production of useful biopharmaceutical products.

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