Biochimica et Biophysica Acta 1833 (2013) 2430–2437

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Review

N-linked protein glycosylation in the ER☆

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ARTICLE INFO

Article history:
Received 10 January 2013
Received in revised form 25 March 2013
Accepted 1 April 2013
Available online 10 April 2013

Keywords:
Glycosylation
Oligosaccharyltransferase

ABSTRACT

N-linked protein glycosylation in the endoplasmic reticulum (ER) is a conserved two phase process in eukaryotic cells. It involves the assembly of an oligosaccharide on a lipid carrier, dolichylpyrophosphate and the transfer of the oligosaccharide to selected asparagine residues of polypeptides that have entered the lumen of the ER. The assembly of the oligosaccharide (LLO) takes place at the ER membrane and requires the activity of several specific glycosyltransferases. The biosynthesis of the LLO initiates at the cytoplasmic side of the ER membrane and terminates in the lumen where oligosaccharyltransferase (OST) selects N-X-S/T sequons of polypeptide and generates the N-glycosidic linkage between the side chain amide of asparagine and the oligosaccharide. The N-glycosylation pathway in the ER modifies a multitude of proteins at one or more asparagine residues with a unique carbohydrate structure that is used as a signalling molecule in their folding pathway. In a later stage of glycoprotein processing, the same systemic modification is used in the Golgi compartment, but in this process, remodelling of the N-linked glycans in a protein-, cell-type and species specific manner generates the high structural diversity of N-linked glycans observed in eukaryotic organisms. This article summarizes the current knowledge of the N-glycosylation pathway in the ER that results in the covalent attachment of an oligosaccharide to asparagine residues of polypeptide chains and focuses on the model organism *Saccharomyces cerevisiae*. This article is part of a Special Issue entitled: Functional and structural diversity of endoplasmic reticulum.

1. Introduction

N-linked protein glycosylation of proteins occurs in all three domains of life. The pathway at the periplasmic membrane of prokaryotes and the process at the ER membrane are considered to be homologous [1]. The high conservation of the process across all domains of life makes it possible to identify the underlying principles of the pathway and to develop general concepts of N-linked protein glycosylation. The assembly of an oligosaccharide on an isoprenoid lipid (bactoprenol in the bacterial systems and dolichol in archaea and eukaryotes) from nucleotide activated monosaccharides on the cytoplasmic side of the membrane by a set of specific glycosyltransferases and the subsequent translocation of the lipid-linked oligosaccharide is a unifying scheme in all systems. In eukaryotes, the oligosaccharide is further extended by lumen-oriented glycosyltransferases, but these enzymes use dolichylphosphate-bound monosaccharides as substrates. The acceptors of the oligosaccharide are selected asparagine residues of polypeptide chains that have entered the periplasm or the lumen of the ER, respectively. Oligosaccharyltransferase, the central enzyme of the N-glycosylation pathway, catalyses the formation of an N-glycosidic linkage of the oligosaccharide to the side-chain amide of asparagine residues that are specified by the consensus sequence N-X-S/T.

Attached to the protein, the hydrophilic carbohydrates alter the biophysical properties of the polypeptide and thereby affect their folding either directly [2–4] or indirectly [5]. For the later function, the defined structure of the carbohydrate is essential because in eukaryotes, processing products serve as universal signals that display the folding status of the underlying protein [6]. This subsequent use of a branched carbohydrate structure as a defined signalling molecule after the transfer to the polypeptide requires on the one hand a high substrate specificity of the oligosaccharyltransferase and a fine-tuning of the temporal order of glycosylation and polypeptide folding.

2. Biosynthesis of the lipid linked oligosaccharide (LLO)

2.1. Dolichol, the lipid carrier in ER glycosylation

The isprenoid lipid dolichol serves as a carrier of the oligosaccharide and it localizes the biosynthetic pathway to the membrane of the ER. Dolichol is synthesized by cis-prenyltransferase, starting from farnesylpyrophosphate and the sequential addition of C_5 isoprenoid units [7,8]. Alterations in dolichol biosynthesis severely affect the N-glycosylation process [9] and can result in a recently described class of congenital disorders of glycosylation (CDG) [10]. However, alterations in dolichol biosynthesis can also affect the metabolism of

☆ This article is part of a Special Issue entitled: Functional and structural diversity of endoplasmic reticulum.

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http://dx.doi.org/10.1016/j.bbamcr.2013.04.001

dolichol-derived molecules such as dolichol esters but little is known about the biological function of these isoprenoids [11,12].

Interestingly, the chain length of dolichol is species specific and it is determined by cis-prenyltransferase [9]. It is important to note that isporenoid lipids are used as membrane anchors in many biosynthetic pathways that involve the translocation of monosaccharides or oligosaccharides across the membrane [13,14]. Bacterial O-antigen [15] and murein biosynthesis [16] or different cell wall biogenesis pathways in mycobacterial cell wall biogenesis [17] are based on this principle. It is tempting to speculate that the biophysical property of the isoprenoid lipid plays an essential role in the translocation process of complex molecules across the membrane [18].

2.2. The building blocks for oligosaccharide biosynthesis

The three carbohydrate building blocks of the LLO substrate (GlcNAc, Man and Glc) emerge from the primary metabolism and enter the pathway as nucleotide activated sugars. UDP-GlcNAc and GDP-Man serve as substrates for the transferases that act on the cytoplasmic side of the ER membrane, whereas Dol-P-Man and Dol-P-Glc, created in the cytoplasm from Dol-P, GDP-Man and UDP-Glc, respectively, are translocated across the membrane and are used as substrate for the lumen-oriented mannosyl- and glucosyltransferases. Dol-P-Man is synthesized by the Dol-P-Man synthase, encoded by the *DPM1* locus [19], whereas the *ALG5* locus encodes Dol-P-Glc synthase [20]. The translocation of Dol-P-Man and Dol-P-Glc requires a directed transport from the cytoplasm to the lumen of the ER and biochemical studies indicate that this translocation is protein-mediated but does not require ATP [21–24]. The corresponding flippases are not known and no mutant strains with matching phenotypes have been identified. Dol-P-Man is an essential component in yeast and a deficiency of Dol-P-Man synthase yields a lethal phenotype. This is not due to the lack of Dol-P-Man for the synthesis of the LLO because deficiencies of the lumen oriented mannosyltransferases have no detrimental effect. The lethal phenotype stems from the fact that Dol-P-Man is used as a substrate for the essential O-mannosylation of proteins [25] and the biosynthesis of GPI-anchored proteins [26]. The situation for Dol-P-Glc is different because it is only used in the N-glycosylation pathway. A deficiency of Dol-P-Glc synthase in yeast does result in a hypoglycosylation of proteins but not in a severe growth phenotype. It is therefore surprising that in the quite extensive searches for mutations affecting the N-glycosylation pathway no mutants deficient in the Dol-P-Glc flippase have been detected (the expected phenotype is similar to a deficiency in the Dol-P-Glc synthase). A possible explanation is that both Dol-P-Man and Dol-P-Glc are translocated by a single flippase, resulting in an essential nature of this enzyme, or there are two flippases with redundant functions. This would make it difficult to identify the corresponding locus in conventional genetic screens. Alternatively, Dol-P-Man- and Dol-P-Glc synthase translocate their respective products.

2.3. The topology of lipid-linked oligosaccharide assembly

The bipartite nature of the LLO assembly pathway is one of the main characteristics of the N-linked protein glycosylation process and it has several important features (Fig. 1):

1) Two types of glycosyltransferases act on the LLO: glycosyltransferases that utilise nucleotide-activated sugars (in the cytoplasm) and glycosyltransferases that require dolichylphosphate-bound sugars as a substrate.
2) The process requires the translocation of lipid-linked biosynthetic intermediates across the membrane, resulting in a topologically different location of the initiating (cytoplasm) and terminating (lumen) steps of the pathway.

As a consequence of this bipartite pathway, the biosynthesis of a single oligosaccharide transferred to an acceptor protein by oligosaccharyltransferase results in the translocation of 1 dolichylpyrophosphate and 7 dolichylphosphate molecules from the cytoplasmic leaflet of the ER membrane to the lumen of the ER. It has been postulated that the lipid carrier is recycled, resulting in a dolichol cycle that transports the molecule back to the cytoplasm [9]. Phosphatases might be involved in this process and indeed, a deficiency of a dolichylpyrophosphate phosphatase (Cwh8p) results in a severe N-glycosylation deficiency [27]. The recycling of the dolichol carrier offers the possibility to regulate the flux thru the LLO biosynthetic pathway. Under the assumption that the utilisation of the LLO substrate by OST is the rate-limiting step of the N-glycosylation process, it is the level of dolichylphosphate in the ER that determines the LLO substrate level present in the cell. Such a regulatory mechanism can compensate for the lack of a feedback regulation of the committed step of LLO biosynthesis (catalysed by Alg7p) that is due to the topological separation of the initiating and the terminating steps of the pathway. Such a model, however, asks for a regulation of the activity of the Dol-P utilising enzymes [28].

It is important to note that it is not the absence of nucleotide-activated sugars in the lumen of the ER that makes the use of Dol-P-Man or Dol-P-Glc a prerequisite for lumen-oriented glycosyltransferases. It is well established that UDP-Glc is found in the lumen of the ER and Golgi-localized glycosyltransferases that act on secretory glycans all use nucleotide-activated sugars as substrates [29]. Specific transporters ensure the availability of these components in the lumen of the respective compartments. It has rather been speculated that the use of dolichylphosphate-linked monosaccharides by ER-localized glycosylatransferases reflects the prokaryotic origin of the process of N-linked protein glycosylation biosynthetic pathway [1].

2.4. The assembly of the lipid-linked oligosaccharide

LLO biosynthesis is performed by a series of glycosyltransferases that are encoded by the *ALG* (asparagine linked glycosylation) genes (Fig. 1). The process is initiated by *ALG7* N-acetylglucosamine-phosphate transferase that adds GlcNAc-P to Dol-P, forming the anhydride dolichylpyrophosphate-GlcNAc (Dol-PP-GlcNAc). Alg7p is the target of the well-known N-glycosylation inhibitor tunicamycin [30]. A protein complex, encoded by the *ALG13* and *ALG14* loci transfers the second GlcNAc residue [31–33]. Interestingly, the UDP-GlcNAc utilising enzymes form a complex composed of Alg7p, Alg13p and Alg14p, whereby Alg14p is the organizing subunit [34,35].

Three enzymes are responsible for the addition of 5 mannose residues using GDP-Man as a substrate. As is the case for the UDP-GlcNAc utilising enzymes, they form a protein complex [36]. The first Man is added to Dol-PP-$GlcNAc_2$ by the *ALG1* encoded β-1,4 mannosyltransferase [37], whereas the subsequent addition of the two branching mannose residues is catalysed by a single enzyme, Alg2p. The ordered addition of α-1,3 and subsequently the α-1,6 linked mannose is mechanistically not understood [38,39]. Elongation of the $Man_3GlcNAc_2$ pentasaccharide is performed by the *ALG11* enzyme [40], resulting in the $Man_5GlcNAc_2$ oligosaccharide that represents the final product of cytoplasmic LLO biosynthesis.

The subsequent steps in LLO biosynthesis occur at the luminal side of the ER, requiring the translocation (flipping) of the glycolipid across the membrane [21,23,41,42]. As mentioned above, such translocation events are characteristic for other biosynthetic pathways such as O-antigen biosynthesis in Gram-negative bacteria [15], or murein biosynthesis [43], arabinomannan biosynthesis in Mycobacteria [17] and this reaction has attracted the attention of both biochemists and geneticists alike. Genetic studies identified loci that encode potential flippases based on the phenotype of the corresponding loss-of-function mutations. In case of the $Man_5GlcNAc_2$ flippase, *rft1* mutant strains prevent N-linked protein glycosylation and accumulate the lipid-linked $Man_5GlcNAc_2$ oligosaccharide, the phenotypes expected for a loss of flippase function [44]. However, in vitro translocation assays reported Rft1p-

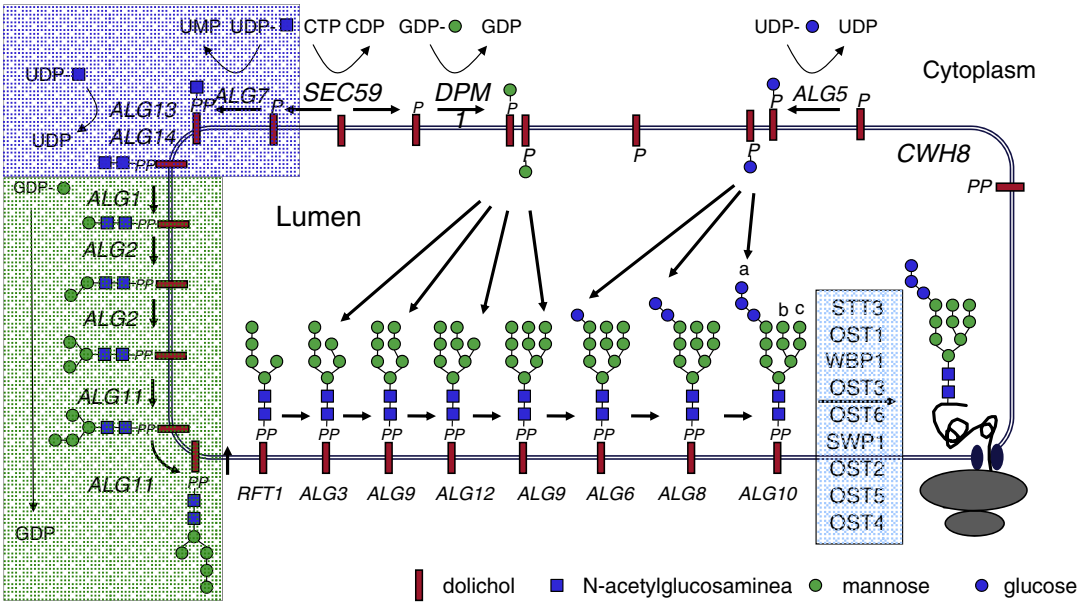

Fig. 1. The pathway of lipid-linked oligosaccharide biosynthesis and the transfer of the oligosaccharide to protein at the membrane of the endoplasmic reticulum. The topology of lipid-linked oligosaccharide biosynthesis catalysed by glycosyltransferases that are encoded by the different *ALG* loci is shown. Glycosyltransferases active at the cytoplasmic side of the ER membrane that utilise UDP-GlcNAc and GDP-Man, respectively, form two enzyme complexes indicated by a blue and a green square. After translocation of the lipid-linked $Man_5GlcNAc_2$ oligosaccharide, further extension by lumen-oriented glycosyltransferases yields the $Glc_3Man_9GlcNAc_2$ oligosaccharide (a,b and c refer to the three antennae of the oligosaccharide) that is transferred to selected asparagine residues of translocated polypeptides by the oligosaccharyltransferase complex.

independent LLO translocation [24,45–47]. These contradicting results question the function of Rft1p as a flippase but also ask for a different function of the *RFT1* protein in the N-glycosylation process.

Four mannose and three glucose residues are added to the LLO in the lumen of the ER by Dol-P-Man- and Dol-P-Glc-dependent glycosyltransferases. These highly conserved enzymes are characterised by the presence of multiple transmembrane segments and they belong to the GT-C clade of glycosyltransferases [48]. They are inverting enzymes that catalyse the formation of an α-glycosidic linkage. Lumenal biosynthesis is initiated by the α-1,3 mannosyltransferase encoded by the *ALG3* locus [49], followed by the addition of an α-1,2 linked mannose by the *ALG9* mannosyltransferase [50], resulting in the b-antenna of the oligosaccharide (see Fig. 1 for the nomenclature of the antenna). The c-antenna is initiated by the *ALG12* α-1,6 mannosyltransferase ([51] and terminated by the addition of the α-1,2 linked mannose (catalysed by Alg9p) [50]. It is not before the $Man_9GlcNAc_2$ oligosaccharide is finished that Alg6p initiates the glucosylation of the a-antenna of the oligosaccharide [52]. Alg8p subsequently adds the second α-1,3 linked Glc residue to the LLO [53]. The final step in LLO synthesis, catalysed by Alg10p, attaches a third Glc residue in α-1,2 linkage [54].

It is important to note that the branched nature of the oligosaccharide allows for multiple biosynthetic paths after the translocation of the oligosaccharide. However, one biosynthetic route is primarily taken, but alternative assembly pathways can be detected in mutant cells deficient in defined mannosyltransferases [55,56]. Due to the high substrate specificity of the glycosyltransferases involved a single linear pathway that terminates with the addition of the "capping" α-1,2-linked glucose on the a-antenna of the oligosaccharide takes place. It is this capping glucose that represents the key-determinant for substrate recognition by oligosaccharyltransferase. The ordered assembly of the lipid-linked oligosaccharide, in conjunction with the substrate specificity of oligosaccharyltransferase favours the transfer of completely assembled oligosaccharides to protein (Fig. 2). Deficiencies in late steps of LLO assembly result in the accumulation of biosynthetic intermediates and a hypoglycosylation of proteins. It is this hypoglycosylation and not the transfer of incomplete oligosaccharides that is the primary cause for the severe deficiencies observed in patients with Congenital Disorder of Glycosylation [57]. Nevertheless, in view of a functional role of oligosaccharide structure in the processing of N-glycoproteins in the ER, it is of central importance that completely assembled oligosaccharides are transferred to protein [5]. In view of the signalling function of the protein-bound glycan it is noteworthy that the removal of the α-1,2-linked mannose of the b-antenna of protein-bound N-linked glycans by ER mannosidase I generates glycan structures that differ from biosynthetic lipid-linked oligosaccharide intermediates [6].

3. Transfer of the oligosaccharide to protein: oligosaccharyltransferase

3.1. Composition of oligosaccharyltransferase

Oligosaccharyltransferase (OST) is the central enzyme in the pathway of N-linked protein glycosylation. It catalyses the transfer of the oligosaccharide from the lipid carrier dolichylpyrophosphate to the amide group of selected asparagine residues of polypeptide chains. In animal, plants and fungi, the membrane-bound enzyme is a heterooligomeric complex and in yeast, it consists of eight subunits, all membrane proteins. *STT3*, *OST1*, *OST2*, *WBP1* and *SWP1* encode the essential subunit, whereas depletion of either the *OST3*, *OST4*, *OST5* or *OST6* encoded subunit results in a general hypoglycosylation of N-glycoproteins [58]. The complex exists in two isoforms containing either Ost6p or Ost3p and the two isoforms differ in their polypeptide substrate specificity [59–62]. Mammalian OST has a very similar composition and contains proteins homologous to the yeast OST components: DAD1 (Ost2p), N33/Tusc3 and MagT1 (Ost3p and Ost6p), OST48 (Wbp1p), ribophorin I (Ost1p), ribophorin II (Swp1p), STT3A and STT3B (Stt3p) [63]. Mammalian isoforms of the OST complex contain either STT3A or STT3B and N33/TUSC3 or MagT1, respectively [64]. Recently, two additional components specific for the mammalian OST have been identified: KCP2 and DC2 associate with mammalian OST complexes [65–67].

The characterisation of N-glycosylation pathways in prokaryotes and in unicellular eukaryotes has led to the identification of homologous N-glycosylation pathways [1] that rely on less complex OST enzymes: *Cryptosporidium parvum* encodes most of the OST subunits found in

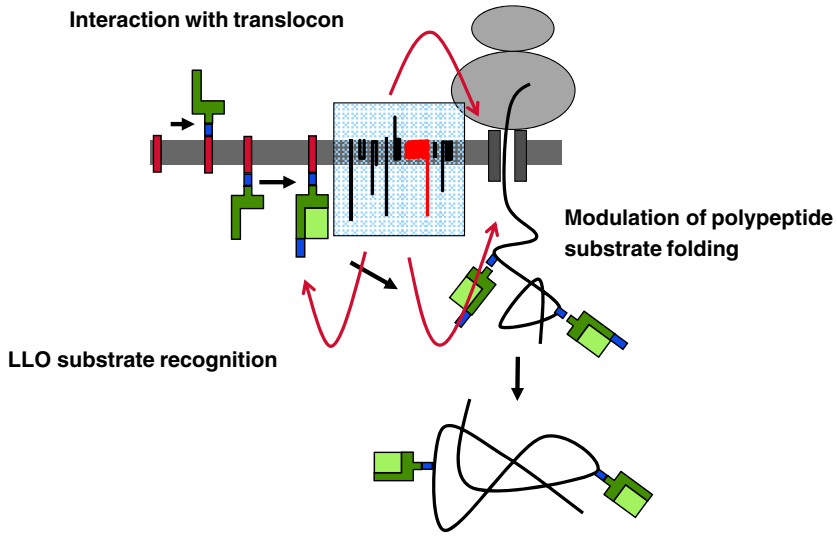

Fig. 2. Specific functions of eukaryotic oligosaccharyltransferase. The enzyme preferentially transfers completely assembled oligosaccharides and not biosynthetic intermediates. It can interact with the translocation machinery and it can modulate the folding of polypeptide substrates. These functions are mediated by eukaryote-specific components of the oligosaccharyltransferase (shown in black) that form a complex with the catalytically active STT3 subunit (red).

yeast, except for *OST5* and *SWP1*, more simple OST complexes are present in *Trichomonas vaginalis*, *Entamoeba histolytica*, *Plasmodium falciparum* genomes [58]. Here, homologues of Ost1p, Ost2p, Stt3p and Wbp1p were identified. *Giardia lamblia* and kinetoplastids encode only STT3 homologues [58]. Some kinetoplastids encode several paralogues of STT3 that have been demonstrated to function as single subunit OSTs [68–70] and prokaryotic OST enzymes are homologous to the eukaryotic STT3 proteins. This phylogenetic analysis clearly identifies STT3 as the catalytically active subunit of the multimeric OST complex.

The reaction catalysed by OST is unique and it is the basis for the "generality" of the N-linked modification of proteins. On the one hand, OST has high substrate specificity for the lipid-linked oligosaccharide but it modifies a large number of proteins. Glycosylation sites are characterised by the consensus sequence NxS/T, where x can be any amino acid except proline [71]. In the following, the specificity of OST towards its substrates is discussed.

3.2. Substrate specificity of oligosaccharyltransferase

The LLO substrate of OST is a large and complex molecule that is anchored in the membrane of the ER. In vitro studies revealed Dol-PP-$GlcNAc_2$ is the minimal oligosaccharide structure that is accepted by OST [72] but in vivo studies show a clear preference for the fully assembled oligosaccharide and it is the terminal α-1,2 Glc residue of the a-branch that is a key determinant for substrate recognition of OST while the Man residues of the b- and c-branch are of limited importance [51] (Fig. 2). However, the molecular details of LLO substrate recognition by the multimeric OST remain unknown but a regulatory mechanism that increases substrate specificity of OST has been proposed [73]. It is important to note that in *Trypanosoma brucei*, oligosaccharyltransfrase is a single subunit enzyme, homologous to the STT3 protein family. Three paralogues are encoded in the genome and the corresponding OST differ with respect to their LLO and peptide specificity [68,74]. TbSTT3A transfers the $Man_5GlcNAc_2$ oligosaccharide to selected acceptor sequences of a VSG protein, whereas the TbSTT3B protein utilises the $Man_9GlcNAc_2$ LLO as a substrate to other glycosylate acceptors. These data show that a single subunit OST is able to distinguish oligosaccharide substrates that differ in their structure, asking for a lectin domain of the enzyme located at a distance from the catalytic center.

Apart from the oligosaccharide moiety of the LLO also the lipid carrier may interact with the OST. In yeast, OST seems to possess certain flexibility towards the length of the LLO dolichol moiety. Biosynthesis of truncated dolichol with 11–12 isoprene units by a cis-prenyltransferase from *G. lamblia* in *Saccharomyces cerevisiae* did not result in a hypoglycosylation phenotype, indicating that the truncated dolichol is sufficient for the yeast OST to glycosylate proteins [75]. However, yeast OST cannot utilise very short lipid carriers with only three to four isoprene units in vitro [76].

It is noteworthy that in eukaryotes the oligosaccharide transferred to protein is remarkably conserved but shorter "high mannose" oligosaccharides are transferred in different protists. It has been proposed that they represent intermediates in the evolution of the N-glycosylation pathway [1]. Alternatively, these pathways might represent reduced forms that all originate from a $Glc_3Man_9GlcNAc_2$ pathway [77]. Irrespective of the origin of the diversity, the oligosaccharide structure transferred in the N-glycosylation pathway is remarkably conserved in eukaryotic species.

In contrast to the LLO substrate, the polypeptide acceptor of OST seems to be very diverse: a large number of proteins are N-glycosylated in the secretory pathway. However, N-glycosylation sites are highly conserved in the primary sequence of these acceptor proteins: the well known NxS/T sequon [78] where x can be any amino acid except proline defines a potential N-glycosylation site of proteins. The same sequon is also present as a key determinant of N-glycosylation sites in the homologous prokaryotic N-glycosylation systems [79–81]. Based on the crystal structure of the bacterial OST PglB from *Campylobacter lari* in complex with an acceptor peptide, the substrate specificity of the N-glycosylation system can now be explained at a molecular level. The acceptor peptide forms a 180° turn and the hydroxyl amino acid at the +2 position is recognized by a binding pocket of the enzyme formed by a highly conserved WWDYG motif found in the STT3 protein family [82]. Assuming that polypeptide substrate recognition of eukaryotic OST is similar to the bacterial enzyme (the conserved nature of the peptide binding site supports this hypothesis), the crystal structure of PglB explains the preferences of NxT over NxS for OST reactivity in vitro [83] as well as the use of rare non-canonical N-glycosylation sites found in N-glycoproteins [84–90]. NxC glycosylation sequons represent about 1% of all efficiently used sites in the mouse glycoproteome [90] and both in vivo and in vitro studies reveal that the NxC sequon can be viewed as an infrequently used canonical glycosylation site [91–93] whereas the detection of NG glycosylation sequons using PNGase digestion of glycoproteins might be due to the deamidation of asparagine–glycine sequences during sample preparation [94]. It is important to note that not all potential N-glycosylation sequons are glycosylated in vivo. It is estimated that

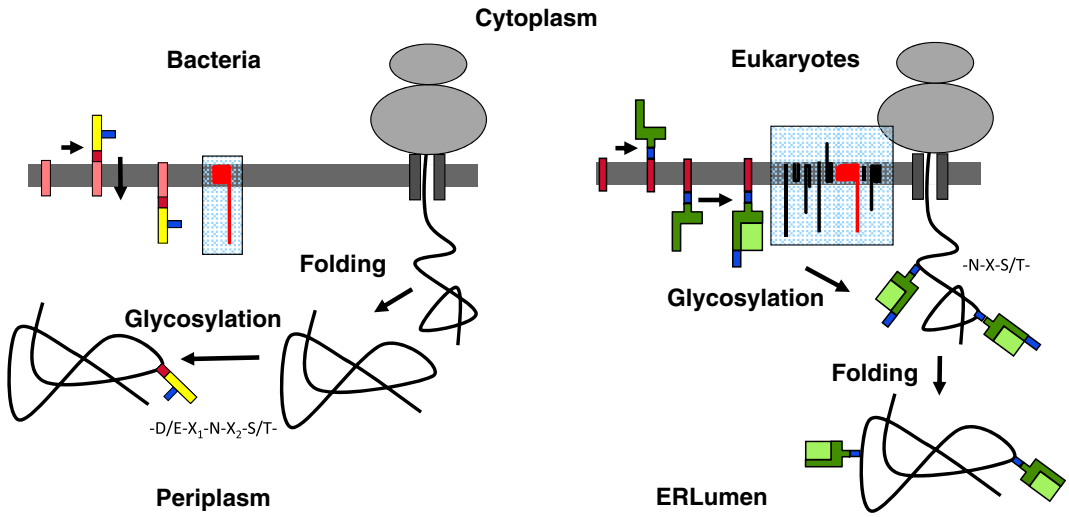

Fig. 3. Schematic comparison of the biosynthesis of N-glycoproteins in pro- and eukaryotes. In prokaryotes specific oligosaccharide is transferred by a single-subunit oligosaccharyltransferase to folded proteins in the periplasm. In eukaryotes, the multisubunit oligosaccharyltransferase can interact with the translocation machinery and can modulate the folding of polypeptide substrates. This can result in the modification of proteins prior to their folding.

about 2/3 of the potential sites are used as substrate by oligosaccharyltransferase [90,95] and it has been postulated that sequences surrounding the glycosylation site affect glycosylation [96]. In addition, distance from a transmembrane domain or to the next N-glycosylation site might influence glycosylation [97].

In the course of eukaryotic evolution, N-linked protein glycosylation has become the most widely used protein modification, more than 2300 N-glycoproteins with over 5000 glycosylation sites have been identified in the mouse glycoproteome [90]. It is the unique substrate specificity of the oligosaccharyltransferase that made such a wide distribution possible. The short, linear NxS/T acceptor sequence can be inserted at many different sites of the proteins. Indeed, the analysis of the N-glycoproteome of different species revealed a high diversity of glycosylation sites [98], demonstrating the flexible use of this modification on an otherwise conserved proteome. In addition, N-glycosylation is much more frequent in multicellular than in unicellular species [98], supporting the hypothesis that diversifying glycosylation plays a major role in the evolution of multicellularity [99]. These data also suggest that there is a selective pressure in the course of eukaryotic evolution to increase the degree of N-glycosylation in secretory proteins. Due to the short acceptor sequon only few mutations are required to alter the N-glycosylation of a given protein, thereby changing the biophysical property of the protein dramatically. Efficient N-glycosylation of a given site is under positive selection pressure: the preference of the NxT over the NxS sequon found in all species so far examined [98] correlates with the substrate specificity of OST (NxT is preferred over NxS). However, the presence of the N-X-S/T sequon in the sequence of a polypeptide is not sufficient to define a glycosylation site. The molecular analysis of the bacterial OST reveals that the acceptor sequence has to be located in a flexible domain of the polypeptide [82], restricting the possible placement of N-glycosylation sites to flexible domains of proteins. Indeed, a majority of N-glycosylation sites is found in potential loop and turn domains of proteins, but a significant portion of N-glycosylation sites is present in defined secondary structures of proteins and β-sheet structures are even enriched in N-glycosylation site [98].

Therefore, protein folding can be viewed as a competing event in the glycosylation process of sequons that end up in a structured domain of the terminally folded protein. Accordingly, the positive selection for an increased number of glycosylated sites in a protein results in the arrangement of glycosylation before folding in the protein maturation pathway. An altered timing of the N-glycosylation in the biogenesis of secretory proteins can be achieved by additional functionalities of OST, performed by additional, non-catalytic subunits of the enzyme. Indeed, an increase in the complexity of OST, together with an increase in the number of glycosylated sites in a proteome is observed in the course of evolution. Within the framework of this hypothesis, non-catalytic subunits of complex OST provide additional substrate range by modulating the folding of OST client proteins.

One direct way to time glycosylation before folding is the localisation of OST at the translocation machinery (Fig. 2). Nascent polypeptide chains enter the lumen of the ER in an extended and flexible conformation [100,101], the optimal substrates for OST. Placing OST at the translocation site also increases the local OST substrate concentration and favours the glycosylation process. The close proximity of OST and the translocon has been confirmed experimentally: components of the OST complex have been cross-linked to translocating ribosomes [102], antibodies directed against the cytoplasmic domain of the OST component ribophorin I prevent protein translocation [103] and OST can be found in a complex with the translocon and the TRAP complex [66]. However, co- and post-translocational glycosylation are not excluding each other and both processes can take place in the ER. It has been suggested that the composition of the OST complex differs for the two modification events: The STT3A OST isoform glycosylates substrate polypeptide chains co-translationally, while STT3B isoforms can glycosylate sites that were skipped by STT3A complexes in mammalian cells [104]. A similar specificity of the two distinct OST complexes has been proposed for yeast [105].

An alternative way to modulate the folding of client proteins is the direct interaction of OST with folding intermediates (Fig. 2). The role of the STT3 subunit as the catalytically active protein is well established [64,69,70,79,106–108] and small subunits like Ost4p and Ost5p might be involved in the assembly of the OST complex [58]. In contrast, lumen-exposed domains of larger subunits can directly interact with polypeptide substrates and prevent their folding before glycosylation has taken place. Depletion of the mammalian ribophorin I and KCP2 subunit affected the glycosylation of only a subset of proteins [109–111] compatible with their role as substrate-specific chaperone. An enzymatically active role of one of the OST subunits has been demonstrated for the yeast Ost6p: the luminal domain of this protein forms has a thioredoxin fold and can function as an oxidoreductase in vitro [112]. Based on the observation that oxidative protein folding might interfere with glycosylation [113] and the analysis of glycosylation sites affected by an Ost6p deficiency it has been proposed that the OST6 (and the homologue OST3) subunit forms a transient mixed-disulphide with client proteins. This covalent interaction modulates protein folding and may allow (or prevent) the modification of N-glycosylation sites that are in direct proximity of cysteine residues [112].

Within the framework of this hypothesis, N-linked protein glycosylation has evolved from the prokaryotic "folding–glycosylation" to the eukaryotic "glycosylation–folding" process (Fig. 3). As a consequence, many different sites in a large number of secretory proteins can be glycosylated. In addition, this shift in the temporal order of maturation events is a prerequisite for using N-linked glycans as "universal signals" to modulate and control N-glycoprotein folding [6]: N-linked glycans can direct the folding machinery to defined regions of the polypeptide and the differentially processed N-linked glycan is used as a covalently attached signal to control and to direct the processing path of the polypeptide in the ER [5]. These "general" functions of the N-linked glycan are confined to the ER compartment and are a conserved feature of eukaryotic cells. Subsequent processing of the same N-linked glycan in a protein-, cell- and species-specific manner in the Golgi can result in a wide variety of N-linked glycan structure that have many different functions on (primarily) surface-exposed proteins. This additional role of N-linked glycans adds another unique feature to this protein modification pathway and makes it possible to generate the functionally and structurally very diverse N-glycoproteome in eukaryotic organisms. The unique chemical properties of carbohydrates and the substrate specificity of oligosaccharyltransferase are the molecular determinants that make N-linked protein glycosylation the most prominent protein modification of eukaryotic cells.

Acknowledgements

I am thankful to the past and present members of my research group for their cooperation and the many fruitful discussions. Work in my research group was supported by the Swiss National Science Foundation and ETH Zurich.

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