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Review

Non-lysosomal degradation pathway for *N*-linked glycans and dolichol-linked oligosaccharides

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ABSTRACT

There is growing evidence that asparagine (*N*)-linked glycans play pivotal roles in protein folding and intra- or intercellular trafficking of *N*-glycosylated proteins. During the *N*-glycosylation of proteins, significant amounts of free oligosaccharides (fOSs) and phosphorylated oligosaccharides (POSs) are generated at the endoplasmic reticulum (ER) membrane by unclarified mechanisms. fOSs are also formed in the cytosol by the enzymatic deglycosylation of misfolded glycoproteins destined for proteasomal degradation. This article summarizes the current knowledge of the molecular and regulatory mechanisms underlying the formation of fOSs and POSs in mammalian cells and *Saccharomyces cerevisiae*.

Contents

1. Introduction

N-Glycosylation is one of the most common co- and posttranslational modifications of eukaryotic proteins occurring in the lumen of the endoplasmic reticulum (ER) [1–5]. *N*-Glycans affect the physicochemical (*e.g.*, solubility or thermal stability) and physiological (*e.g.*, bioactivity or intra-/intercellular trafficking) properties of modified proteins [6]. We, former trainees of Dr. Lennarz as post-doctoral researchers, have investigated the mechanism regulating the "birth and death" of *N*-glycans [7–22]. The biosynthetic pathways leading to *N*-glycosylation in mammalian cells or yeast are well clarified [2], while the molecular details of catabolic pathways involved in glycan breakdown are less well understood, especially for processes occurring outside of the lysosome. In this article, we present an overview of the current knowledge of the "non-lysosomal degradation pathway" for *N*-glycans and dolichol-linked oligosaccharides (DLOs), focusing on mammalian cells and the budding yeast, *Saccharomyces cerevisiae*.

2. fOSs formed in the ER

During the translocation of proteins into the ER, the oligosaccharyltransferase (OST) enzyme complex transfers oligosaccharide (OS) moieties from the DLO substrate to the asparagine residue located within the consensus sequence –Asn-Xaa-Ser/Thr– (where

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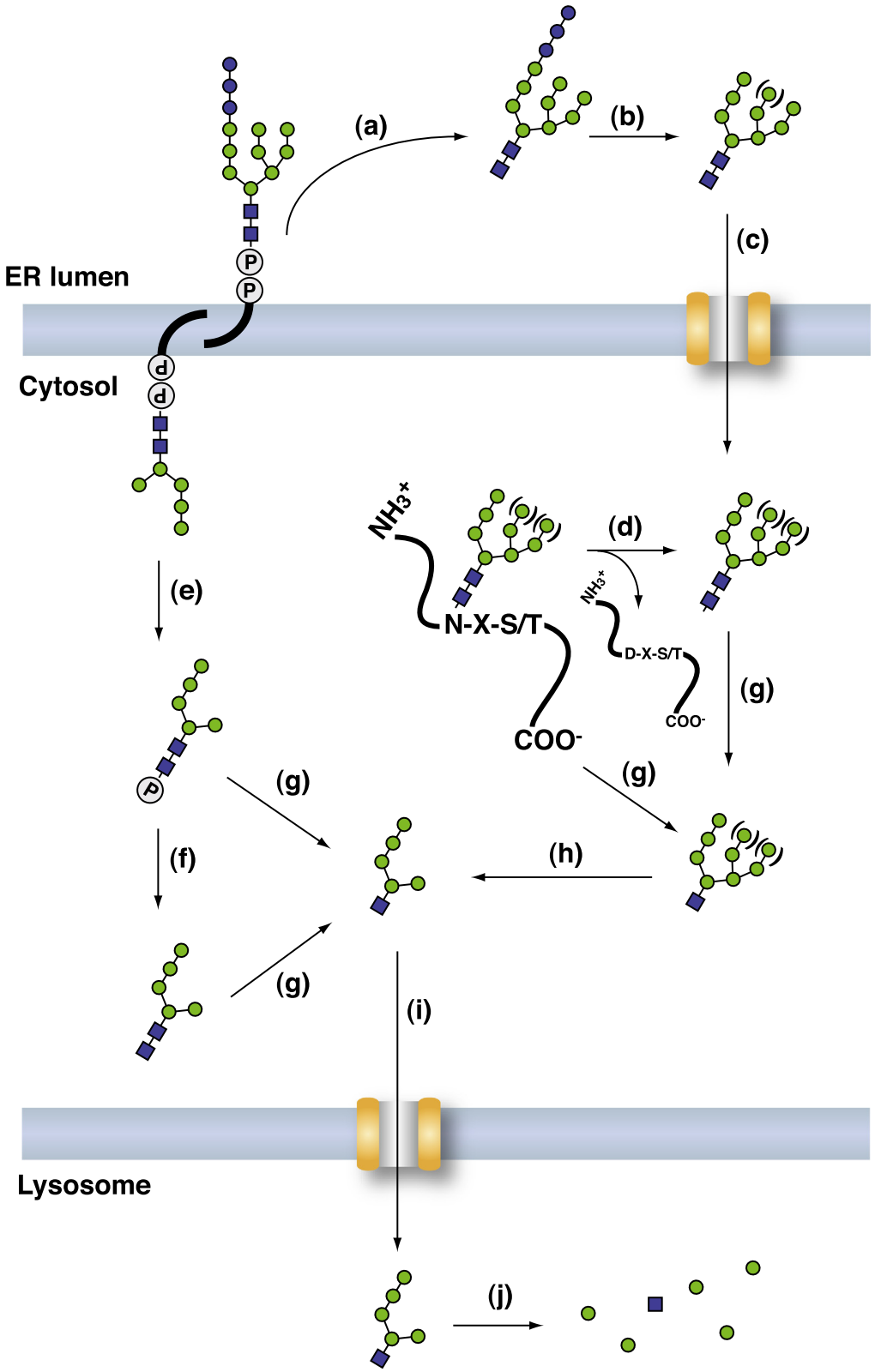

Fig. 1. Current proposed model for the fate of fOSs formed in and outside of the ER in mammalian cells. Gn2-type fOSs are generated in the lumen of the ER by an undefined mechanism (step (a)). After quick deglycosylation by α-glucosidases I/II (and sometimes ER α-mannosidase I) (step (b)), $Man_{8\text{-}9}GlcNAc_2$ is transported into the cytosol by an oligosaccharide transporter in the ER membrane (step (c)). Gn2-type fOSs can also be generated by the action of cytoplasmic PNGase on misfolded glycoproteins (step (d)). Putative pyrophosphatase activity, which has been proposed to be located on the cytosolic side of the ER membrane, releases POSs from the DLOs (step (e)). POSs may be converted to Gn2-type fOSs by the putative POS phosphatase (step (f)). In the cytosol, ENGase acts on Gn2-type fOSs, and possibly on POSs or misfolded glycoproteins, to form Gn1-type glycans (steps (g)). The Gn1-type glycans are susceptible to the action of Man2C1, giving rise to the specific $Man_5GlcNAc$ structures (step (h)). The isomeric structure of Man_5 is identical to that of the last biosynthetic intermediate of pyrophosphoryl dolichol oriented to the cytosolic side of the ER membrane. The $Man_5GlcNAc$ glycan (and possibly more trimmed $Man_{3\text{-}4}GlcNAc$ glycans) may be transported into the lysosomes by an unidentified oligosaccharide transporter (Step (i)). In the lysosome, the incorporated fOSs are hydrolyzed into monomeric sugars by lysosomal glycosidases for reutilization (step (j)). For more details, please see text.

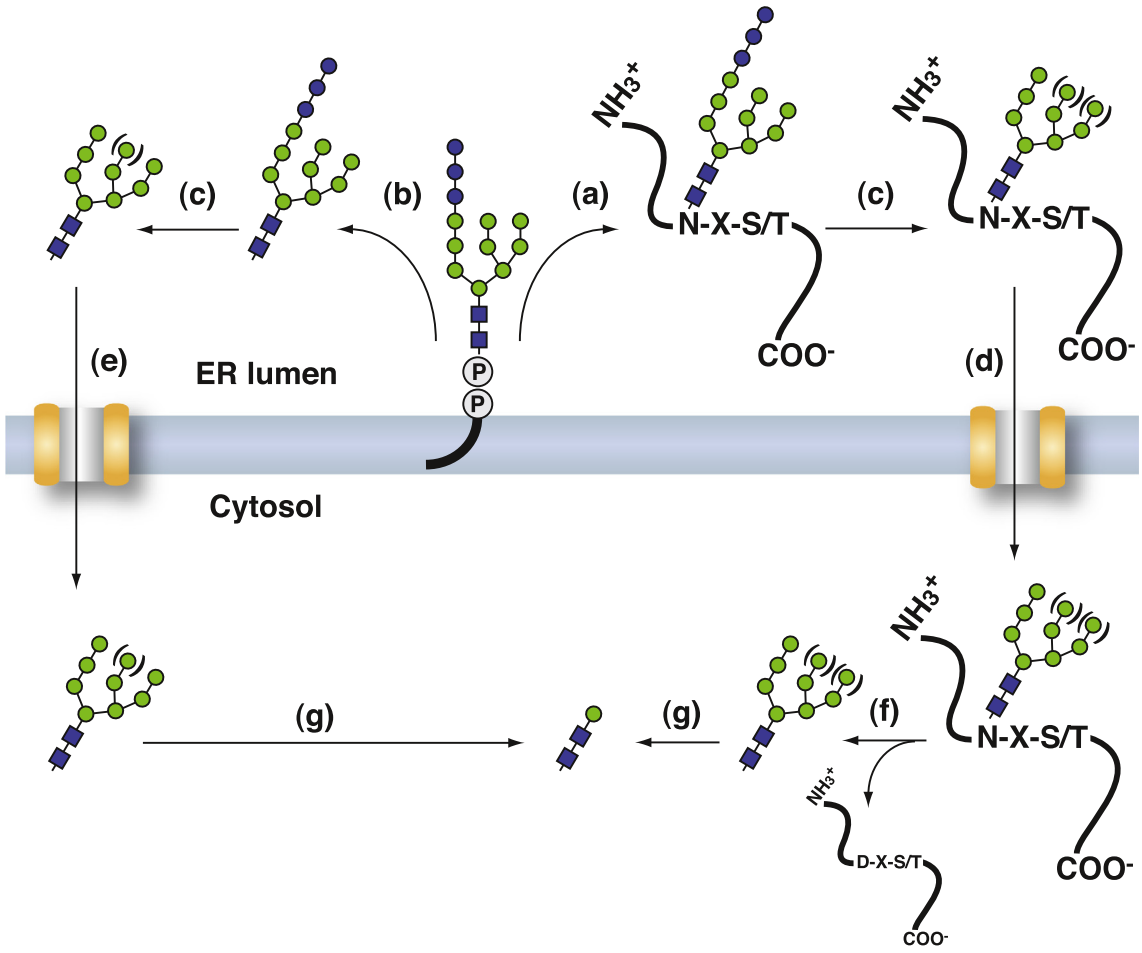

Fig. 2. Current proposed model for the fate of fOSs formed in and outside of the ER in *S. cerevisiae*. In the ER lumen, the fully assembled DLO is transferred onto either proteins (step (a)) or water (hydrolysis; step (b)) by oligosaccharyltransferase. The *N*-glycans or fOSs thus formed are rapidly processed by Gls1 (yeast orthologue of ER glucosidase I) and Gls2 (yeast orthologue of ER glucosidase II), and possibly by Mns1 (yeast orthologue of ER α-mannosidase I) (step (c)). When the glycoproteins are terminally misfolded, Htm1, another ER α-mannosidase, removes a mannose from the C-arm of $Man_{8\text{-}9}GlcNAc_2$ (step (c) on glycoproteins), allowing for the specific sorting of misfolded glycoproteins from folded ones, and for the retrotranslocation of misfolded glycoproteins back to the cytosol through the retrotranslocon (step (d)). The luminal fOSs are also transported to the cytosol by unclarified mechanisms (step (e)). In the cytosol, Png1, the yeast orthologue of PNGase, deglycosylates *N*-glycans from the misfolded glycoproteins, resulting in the release of Gn2-type of fOSs (step (f)). The cytosolic fOSs generated from both DLOs and glycoproteins are catabolized to $ManGlcNAc_2$ by Ams1, the sole cytosol/vacuolar α-mannosidase in yeast (steps (g)). The fate of $ManGlcNAc_2$ remains unknown. For more details, please see text.

Xaa can be any amino acid, except for Pro) to form *N*-linked glycans on the nascent polypeptide chains [1,3–5]. Although the biosynthesis of DLOs, as well as the processing of *N*-linked glycan chains on glycoproteins, is understood in detail, certain aspects of the *N*-glycosylation process require clarification. For instance, it has been established that neutral fOSs, which bear an *N*,*N*′-diacetylchitobiosyl structure at their reducing termini (Gn2-type fOSs), can be released from the microsomal membranes during glycoprotein biosynthesis [23–25] (Fig. 1). It has been suggested that the release should occur in the luminal side of the microsomes [24,25]. While the possible involvement of OST in this fOS-releasing event has been suggested [25,26], no direct experimental evidence was available until our recent demonstration that OST can indeed release peptide:*N*-glycanase (PNGase)-independent fOSs (see below) in the ER lumen of yeast [27] (Fig. 2, step (b)). In this study, genetic analysis using *S. cerevisiae* strongly suggested that the generation of PNGase-independent fOSs could be tightly correlated with the glycan-transferring activity of OST. Furthermore, biochemical purification of the OST complex unequivocally showed that OST can generate fOSs by direct hydrolysis of DLOs. It was also shown in the bacteria *Campylobacter jejuni* that PglB, an orthologue of Stt3 (catalytic subunit of OST), is responsible for the release of fOSs [28]. Moreover, we found that mammalian OST may also mediate hydrolysis of DLOs (Harada, et al., unpublished observation). Collectively, these results indicate that the fOS-releasing activity of OST is evolutionarily conserved. It should be noted, however, that the PNGase-independent fOSs account for only less than 5% of the total fOSs in budding yeast [27,29,30], while the situation seems to be quite distinct in mammalian cells [29], where PNGase-independent fOS release appears to be a dominant process.

In terms of the fOS-releasing activity in mammalian cells, it is interesting to note that mannose 6-phosphate treatment of permeabilized human fibroblasts accelerates the breakdown of $Glc_3Man_9GlcNAc_2$-PP-Dol, resulting in the formation of triglucosylated fOSs [26]. The mechanism by which mannose 6-phosphate regulates fOS-releasing activity, however, remains unknown.

2.1. Free oligosaccharide transport system from the ER to the cytosol

Although it is not yet known whether fOSs have a physiological role in the ER, one would imagine that the accumulation of vast amounts of fOSs in the ER could interfere with the glycan-based quality control system for nascent luminal proteins [31]. Therefore, it is not surprising that cells have the machinery to eliminate fOSs

from the ER lumen. Using permeabilized cells and/or microsomes from mammalian sources, it was demonstrated that fOSs in the ER can be exported into the cytosol [32–35]. It has been suggested that fOS transport is an ATP-dependent process [33–35]. The typical structure of fOSs released from the ER to the cytosol has been shown to be $Man_{8-9}GlcNAc_2$ in the case of mammalian cells [32,33] and $Man_{7-9}GlcNAc_2$ in the case of *S. cerevisiae* [27].

2.2. Putative DLO pyrophosphatase: another pathway for the generation of unconjugated oligosaccharides from DLOs

Another enzyme that could potentially generate unconjugated OSs from DLOs is the putative pyrophosphatase (Fig. 1, step (e)). This enzymatic activity generates phosphorylated OSs (POSs) and has been detected in mammalian cells [23,36–38] and in microsomes from *S. cerevisiae* [39]. *In vivo* evidence for the occurrence of POS in *S. cerevisiae*, however, has yet to be presented. The occurrence of POSs in various congenital disorder of glycosylation type I (CDG-I) patient-derived fibroblasts implied that the degradation of DLO biosynthetic intermediates may facilitate the recycling of dolichyl phosphate, another reaction product of pyrophosphatase, for its reutilization in DLO biosynthesis. We recently showed that under low-glucose conditions, maturation arrest of DLOs occurs and the premature DLOs undergo degradation by pyrophosphatase [40] (Fig. 3). This result suggests that pyrophosphatase-mediated degradation of premature DLOs may function as a quality control system to avoid abnormal *N*-glycosylation under conditions that impair efficient DLO biosynthesis. However, the nature of the pyrophosphatase remains to be clarified. Another key question is the active site of this enzyme, as POS species originate from both cytosolic ($Man_{0-5}GlcNAc_2$-PP-Dol) and luminal ($Man_{6-7}GlcNAc_2$-PP-Dol) DLO sources, and whether the activity resides on the luminal or cytosol side of the ER remains to be determined. However, POSs are almost exclusively recovered in the cytosolic fraction, and are not observed in the ER lumen [37,38,40].

Under low-glucose conditions, the most abundant POS structure in mouse embryonic fibroblasts was found to be $Man_2GlcNAc_2$-P (Fig. 3), a reaction product of ALG2 protein. This observation is notable as ALG2 protein mediates the successive addition of two Man residues, the first α1,3-linked and the second α1,6-linked, to $ManGlcNAc_2$-PP-Dol [41–43]. Since the release of POSs appears to be tightly coupled with the reduction of GDP-Man, it is tempting to speculate that the second step of the ALG2 reaction may be arrested under low GDP-Man concentrations, possibly due to a much higher *K*m value for GDP-Man than the first reaction [40].

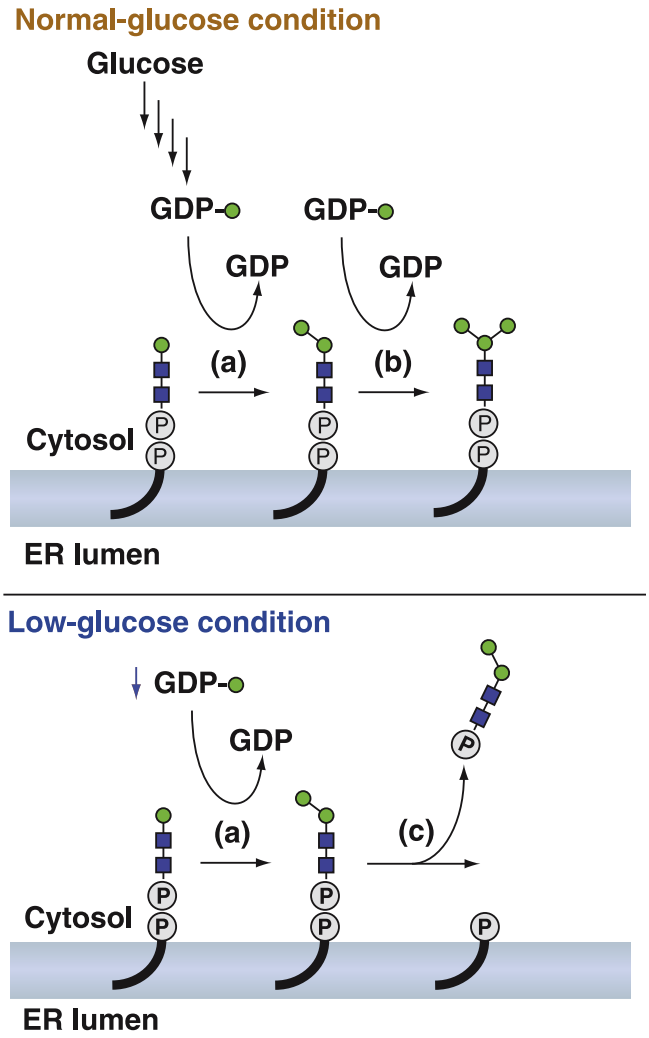

Fig. 3. Reaction of ALG2 protein and possible regulation mechanism of POS release [40]. ALG2 synthesizes $Man_3GlcNAc_2$-PP-Dol by a sequential addition of two mannose residues from GDP-Man to $ManGlcNAc_2$-PP-Dol. The first and second reactions of ALG2 form the α1,3- (step (a)) and α1,6-linked (step (b)) branches on the glycan, respectively. Under low-glucose conditions, the level of GDP-Man is drastically reduced, resulting in the arrest of the second ALG2 reaction. Although the mechanism requires further elucidation, it is tempting to speculate that the second ALG2 reaction might have a higher *K*m value for GDP-Man than the first reaction. The biosynthetic arrest of DLO induces the release of POSs from the premature DLO by the putative DLO pyrophosphatase (step (c)).

2.3. Free oligosaccharide formation in the cytosol: The connection with ER-associated degradation

Recent evidence clearly shows that ER has quality control machineries that can differentiate between misfolded (glyco)proteins and correctly folded proteins, so that only the latter exit from the ER to be delivered to their respective destinations. On the other hand, proteins that fail to fold or form functional complex structures are retained in the ER and interact with various luminal chaperones that assist in their maturation into functional structures. Proteins that consistently fail to acquire the correct folding state, however, are eventually degraded by the ER-associated degradation (ERAD) system [44–46], in which proteasomes play a central role in the degradation of misfolded proteins. It has been widely recognized that the *N*-glycan structures on ERAD substrates play a pivotal role in the recognition of their folding status, which is mediated by various luminal lectins [2,47–52].

When misfolded glycoproteins are retrotranslocated into the cytosol, cytoplasmic PNGase can remove *N*-glycans from them before or during proteasomal degradation [7,15,19,53–55] (Fig. 1), releasing Gn2-type fOSs in the cytosol. Recently, patients bearing mutations in *NGLY1*, a mammalian gene orthologue of the cytoplasmic PNGase [18], have been identified [56,57]. This observation clearly indicates the functional importance of this protein for human life. This is in sharp contrast to observations in yeast, where no notable phenotypes were observed for a *PNG1* (the yeast PNGase orthologue)-deletion mutant [10].

2.4. Processing of fOSs and POSs in the cytosol

When neutral Gn2-type fOSs are released into the cytosol, either from misfolded glycoproteins or DLOs, they can be converted into Gn1-type fOSs, bearing only a single GlcNAc residue at the reducing terminus (Fig. 1). This reaction is catalyzed by endo-β-*N*-acetylglucosaminidase (ENGase) [17,58,59]. Cytoplasmic ENGase is widely distributed in eukaryotes, while some yeasts, such as *S. cerevisiae* or *Schizosaccharomyces pombe*, do not have this

enzyme [17]. The possibility exists that, in some cases, ENGase may release Gn1-glycans directly from glycoproteins or DLOs. In this connection, it is of note that, when ENGase is expressed in budding yeast, increase in the amount of Gn1-type fOSs was observed, while the origin of these fOSs, *i.e.*, glycoproteins, DLOs, or Gn2-type fOSs, remains to be determined [29]. It should also be noted that a number of proteins with a single *N*-linked GlcNAc, which is potentially formed by the action of ENGase, have been identified through proteomic analysis [60–62]. Moreover, at least some *N*-GlcNAc proteins in plants were found to be formed by cytoplasmic ENGase activity [63], strongly indicating that the formation of *N*-GlcNAc proteins by the direct action of ENGase may occur more abundantly than currently envisaged. On the other hand, as *S. cerevisiae* does not possess ENGase, the fOSs formed remain as Gn2-type (Fig. 2) [27,29,30,64–66].

In contrast to the case with neutral fOSs, the catabolic pathway of POSs has been largely uncharacterized. Interestingly, a reconstitution of the DLO pyrophosphatase reaction using radiolabeled DLOs and human liver microsomes indicated the presence of phosphatase(s) acting on POSs (Fig. 1), evidenced by the dramatic accumulation of POSs upon addition of phosphatase inhibitors [38]. Another possible route for POS processing involves cytoplasmic ENGase (Fig. 1), which is predicted to release GlcNAc-P from POSs, leaving Gn1-type fOSs in the cytosol. Irrespective of the mechanism involved, it has been shown that a POS with $Man_5GlcNAc2$ glycan is rapidly cleared in mammalian cells [38].

In mammalian cells, Gn1-type glycans formed in the cytosol can be further catabolized by the cytoplasmic α-mannosidase, Man2C1 (Fig. 1) [67–69]. This enzyme is well conserved in vertebrates, but is not found in other eukaryotes [67]. Suppression of Man2C1 expression or inhibition of Man2C1 activity results in accumulation of Gn1-type high mannose-type oligosaccharides in the cytosol, clearly indicating that Man2C1 is involved in the catabolism of fOSs [29,67,70–74]. It has been shown that cytosolic α-mannosidase activity prefers Gn1 over Gn2 glycans as a substrate [75–77], suggesting that reactions involving ENGase and Man2C1 are well-ordered, *i.e.*, Man2C1 action comes after ENGase. The final product of Man2C1 is primarily $Man_5GlcNAc$, which possesses the same isomeric structure as one of the biosynthetic intermediates of DLOs (Fig. 1).

The $Man_5GlcNAc$ glycan in mammalian cells is thought to be delivered into lysosomes by a putative transporter [78,79] for further degradation into monomeric sugars for reutilization (Fig. 1). The nature of the lysosomal OS transporter remains unknown. It has also been shown that cytosolic fOSs can be catabolized, at least in part, by a starvation-induced autophagic process, implying that autophagy can also serve as an alternative mechanism for catabolizing cytosolic fOSs [80].

In *S. cerevisiae*, it has been shown that cytosol/vacuolar α-mannosidase, Ams1, appears to be the only catabolic enzyme acting on fOSs (Fig. 1) [27,30,64–66]. While this protein is targeted to the vacuole through a non-classical transport machinery, called the cytosol-to-vacuole targeting (Cvt) pathway, catabolism of fOSs was found to be enhanced in a mutant where vacuolar targeting of Ams1 was compromised, strongly indicating that cytosolic Ams1 can efficiently degrade fOSs [27,30] .

3. Concluding remarks

As outlined in this review, we now know that fOSs can be formed in both the ER and the cytosol, from DLOs or glycoproteins. Although the formation mechanisms of fOSs are well conserved between *S. cerevisiae* and mammalian cells, the mechanisms contributing to fOS production differ greatly between the two organisms. Therefore, the source of fOSs in other organisms requires careful examination.

We believe that much remains to be explored regarding the non-lysosomal degradation of *N*-glycans and DLOs. The importance of the cytosol and the ER as sites for "non-lysosomal" degradation of glycans is evident. While it has long been held that lysosomes are the predominant site for catabolism of glycoconjugates, the possible functional importance of cytosolic glycosidases [81,82], as well as a variety of deglycosylation enzymes/reactions [83–85] should attract the interest of not only glycobiologists but also a much wider audience. At the minimum, it is now obvious that PNGase in mammals plays pivotal roles in life (the occurrence of *NGLY1* patients speaks for itself), and it is vital that a concerted effort is made to clarify the biological functions of this enzyme.

Looking back to 16 years ago, when Bill and I (TS) wrote a review describing fOS formation and trafficking [7], we have come to the realization that much progress has been made since that time. However, we are still a long way from completely understanding the overall processes, and the schemes presented in Figs. 1 and 2 will hopefully be rigorously scrutinized and revised by many researchers in the future.

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