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REVIEW

Free *N*-linked oligosaccharide chains: Formation and degradation

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Abstract There is growing evidence that *N*-linked glycans play pivotal roles in protein folding and intra- and/or intercellular trafficking of *N*-glycosylated proteins. It has been shown that during the *N*-glycosylation of proteins, significant amounts of free oligosaccharides (free OSs) are generated in the lumen of the endoplasmic reticulum (ER) by a mechanism which remains to be clarified. Free OSs are also formed in the cytosol by enzymatic deglycosylation of misfolded glycoproteins, which are subjected to destruction by a cellular system called "ER-associated degradation (ERAD)." While the precise functions of free OSs remain obscure, biochemical studies have revealed that a novel cellular process enables them to be catabolized in a specialized manner, that involves pumping free OSs in the lumen of the ER into the cytosol where further processing occurs. This process is followed by entry into the lysosomes. In this review we summarize current knowledge about the formation, processing and degradation of free OSs in eukaryotes and also discuss the potential biological significance of this pathway. Other evidence for the occurrence of free OSs in various cellular processes is also presented.

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Abbreviations

Dol	dolichol
EDEM	ER degradation enhancing α-mannosidase-like protein
ENGase	endo-β-*N*-acetylglucosaminidase
EST	Expression sequence tag
Gn1	oligosaccharide with single GlcNAc at its reducing terminus
Gn2	oligosaccharide with N,N'-diacetylchitobiose at its reducing terminus
ER	endoplasmic reticulum
ERAD	ER-associated degradation
OS	oligosaccharide
OST	oligosaccharyltransferase
PNGase	peptide:*N*-glycanase
UNG	unconjugated *N*-glycan
MDBK	Mardin-Darby bovine kidney
CHO	Chinese Hamster Ovary
UGGT	UDP-glucose:glycoprotein glucosyltransferase

1. Introduction

It is known that the ER is the site for assembly of polypeptide chains destined either for secretion or routing into various subcellular compartments. *N*-Glycosylation is one of the most common co- and post-translational modifications of eukaryotic proteins that occurs in the lumen of the ER. Recent studies have shown that the *N*-linked glycan chains on glycoproteins play important roles in facilitating to correct folding

of proteins as well as in degrading proteins which fail to fold properly [1–4].

For more than two decades, the occurrence of "free" *N*-linked OSs has been known [5–7]. The free OSs with a high mannose-type structure can be classified into two species: OS-phosphate (OS-P) and neutral OS [5]. Although the origin of OS-P, because of its structural similarity, was assumed to be from dolichylpyrophosphoryl OSs (Dol-PP-OSs), the sources as well as the enzyme(s) responsible for the formation of free OS (without phosphate) are still a matter of debate. However, the discovery of cytoplasmic peptide:*N*-glycanase (PNGase) which cleaves *N*-linked glycans from *N*-glycoproteins [8], in addition to the newly-found process called "ERAD" [9–11] provides the occurrence of glycoprotein-originated OSs in the cytosol. Thus it is clear that the formation of OS, which is now believed to occur both in the cytosol and in the lumen of the ER, is revealed to be much more complicated than it has been envisioned.

2. Free OSs; in the ER

During the translocation of proteins in the ER, an enzyme complex called OST (oligosaccharyltransferase) transfers oligosaccharide moieties from the dolichol-linked donor substrates to asparagine residues located within the consensus sequence -Asn-Xaa-Ser/Thr- (Xaa: any amino acids except Pro) to form *N*-linked glycans on nascent polypeptide chains (Fig. 1). Although the biosynthesis of lipid-linked OSs as well as the processing of *N*-linked glycan chains on glycoproteins is well understood, certain aspects of phenomena occurring during *N*-glycosylation reaction remain unclarified. One of unanswered questions is how free OSs are generated in the lumen of the ER [5–7].

It has been proposed that, in the absence of sufficient acceptor sequences, the OST exhibits hydrolytic activity and transfers OSs ($Glc_3Man_9GlcNAc_2$ in most organisms) on Dol-PP to water, presumably to control the amount of donor substrates under the condition that less acceptor molecules are around [12,13]. However, recent studies using mammalian cells as well as yeast cells have challenged this scheme by demonstrating that the inhibition of protein synthesis also causes complete inhibition of free OS formation [14,15]. OSs formed by OST should bear *N*,*N*′-diacetylchitobiose (GlcNAcβ1-4GlcNAc) at their reducing termini, collectively called Gn2 species. On the other hand, most of free OSs found in the cytosol contain only a single GlcNAc at their reducing termini, i.e. Gn1. It is now believed that the OSs formed in the ER are Gn2 species [15,16]. Whether there are any other enzyme activities responsible for the release of free OSs in the lumenal side of the ER is still controversial and has not been unequivocally demonstrated in any systems.

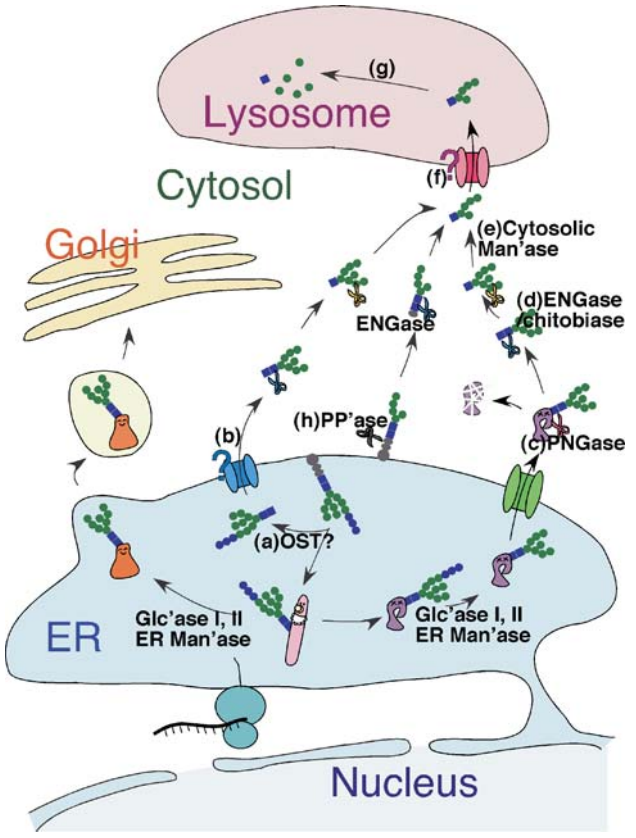

Fig. 1 Model for the fate of free oligosaccharides (free OSs) generated in and out of the ER in mammalian cells. (a) Free OS generated in the lumen of the ER possibly by the hydrolytic activity of oligosaccharyltransferase (OST) bears *N*,*N*′-diacetylchitobiose (Gn2) at its reducing terminus. (b) After quick deglucosylation by α-glucosidase I and II (and sometimes ER α-mannosidase I), $Man_{8\sim9}GlcNAc_2$ is transported into the cytosol by a putative transporter. (c) In the cytosol, PNGase removes *N*-linked glycan from misfolded glycoproteins and thereby release Gn2 glycans bearing *N*,*N*′-diacetylchitobiose. (d) Once in the cytosol, ENGase (or in some cases a chitobiase) forms Gn1 ($Man_{8\sim9}GlcNAc$). (e) Gn1 is now susceptible to the action of a cytosolic α-mannosidase, giving rise to $Man_5GlcNAc$ structure. The isomeric structure Man_5 is identical to that of last dolichol intermediate oriented to the cytosolic face. (f) The $Man_5GlcNAc$ is transported into the lysosomes by a specific transporter. (g) In lysosome the $Man_5GlcNAc$ is hydrolyzed into monomers (Man and GlcNAc) by lysosomal α- and β-mannosidases. (h) Putative pyrophosphatase, activity of which is reported in the cytosolic face of the ER membrane, can also release free OS from the dolichol-linked OS (presumably with the structure of $Man_5GlcNAc_2$) in the cytosol. Probably this oligosaccharide-phosphate will be processed by ENGase to give rise to $Man_5GlcNAc$, which is readily transported into lysosome. Note that this scheme cannot be applied to other organisms: for example, *S. cerevisiae* does not possess ENGase so there must be a distinct path. Blue square: GlcNAc, green circle: mannose. For more details, see text

It has also been proposed that the lumenal OS can be released by lumenal PNGase activity [15,17,18]. Although lumenal PNGase activity was observed in rat liver [19] the molecular nature of this enzyme has not yet been revealed. In *S. cerevisiae PNG*1 encodes cytoplasmic PNGase (Png1p; [20]) and the *png1*-null mutant is devoid of PNGase activity

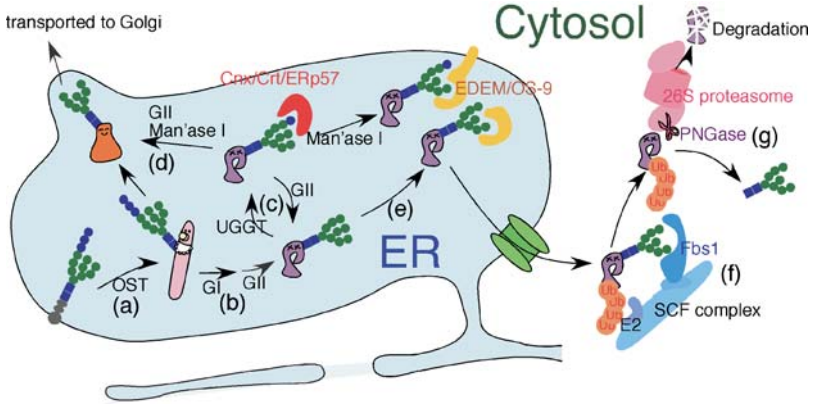

Fig. 2 Glycoprotein ERAD (GERAD [2–4,26]) (a) Tetradecasaccharides, $Glc_3Man_9GlcNAc_2$ synthesized on the dolichol pyrophosphate, are transferred *en bloc* onto the nascent proteins by oligosaccharyltransferase (OST) in the ER lumen. (b) Right after the transfer, glucoses at the non-reducing ends are removed by glucosidase I/II (GI/GII). (c) The calnexin (Cnx) cycle is mediated by the interplay between UGGT and glucosidase II, which are both inhibited by the action of ER-mannosidase (Man'ase) I. If the unfolded proteins are glucosylated by UGGT, Cnx or calreticulin (Crt) (shown in red) binds to the glucose residue at the terminal of *N*-linked glycans and the process is often followed by the correct disulfide formation aided by ERp57. (d) The correctly folded proteins escape from the recognition by UGGT and therefore from the calnexin cycle. (e) On the other hand, glycoproteins bearing Man_8 (and in some cases shorter ones in which more Man are trimmed) also exit the cycle and are recognized somehow by EDEM [149–151] and/or OS-9 (Yos9p in *Saccharomyces cerevisiae*) [152–155] (shown in yellow), which allows these proteins to be targeted for degradation. (f) Once in the cytosol after the retrotranslocation, the SCF (Skp1-cullin-Roc1-Fbox protein) complex bearing sugar-binding Fbs family protein (e.g. Fbs1/Fbx2, shown in light blue) [156–158] binds to the *N*-linked glycans on glycoproteins, and the proteins are polyubiquitinated by the action of this complex. (g) The polyubiquitinated proteins are deglycosylated by PNGase prior to the action of the 26S proteasome (shown in pink)

even when various glycopeptide substrates are tested [20,21], suggesting that the cytoplasmic Png1p is the only PNGase at least in this yeast species. Moreover, recent evidence shows that the Png1p orthologue in mammalian cells is associated with the cytoplasmic face of the ER membrane [22–24], indicating that the ER-associated enzyme activity can be attributed to the ER-associated form of the cytoplasmic PNGase.

Very recently, an interesting observation was made by Gao *et al.* with respect to the source of free OS release [25]: During the analysis of glycosylation in fibroblast derived from a patient of congenital disorder of glycosylation (CDG)-Ia, an increase of glucosylated free OS release was observed upon treatment of the cells with mannose 6-phosphate [25]. Though the detailed mechanism is not known, Gao *et al*. have also observed that addition of an OST acceptor reduced the amount of OSs released, suggesting that the OSs observed is derived from Dol-PP-OS. While the exact cellular compartment where the glucosylated OSs are formed remains to be determined, considering the lack of ER transport activity toward glucosylated OSs on the ER membrane (see below), the formation of those OSs should take place in the lumen of the ER.

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

Although it is not known whether free OSs play a physiological role in the ER, one can easily envisage that accumulation of free OSs in the ER may interfere with the *N*-glycan-dependent quality control system involving recognition of the folding state of proteins in the ER lumen, export of misfolded proteins into the cytosol, and degradation of them by the 26S proteasome [2–4,26] (Fig. 2). Therefore it is not surprising that cells have a machinery to eliminate free OSs from the ER lumen. Using permeabilized mammalian cells, it has been demonstrated that free OSs formed in the ER are, after rapid deglucosylation, exported from the ER into the cytosol [16]. A gene encoding a transporter responsible for the export of free OS on the ER membrane has not been identified, although biochemical studies show that the export is an ATP- and Ca^{2+}-dependent process [16,27]. The OS export is also effectively blocked by the addition of low concentration of mannose or its derivatives but not by other sugars, indicating that this transport machinery recognizes the mannosyl residues at the non-reducing ends, but not the GlcNAc residues at the reducing termini [27].

2.2. Glucosidase-inhibition causes the perturbation of free OS transport from the ER lumen to the cytosol

The main structure of free OS released from the ER to the cytosol has been shown to be $Man_{8\sim9}GlcNAc_2$ [16,28] (Fig. 1). Inhibition of ER glucosidases causes the inhibition of free OS transport [28,29], suggesting the importance of glucose trimming for transporter activity. This observation is in sharp contrast to the case of export of small *N*-glycopeptides, which

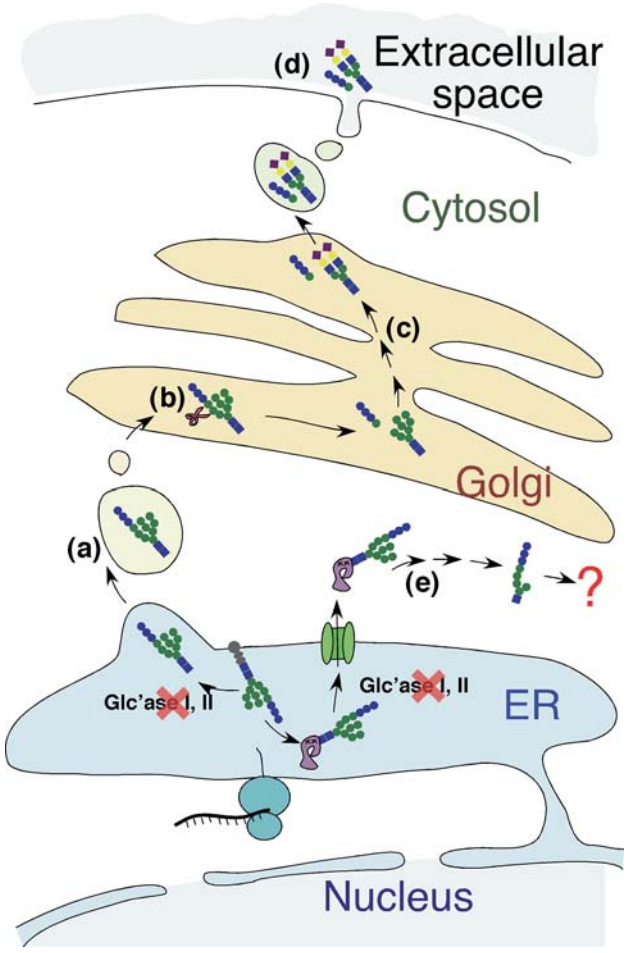

Fig. 3 Overview of the generation/processing of free OS by glucosidase deficient cells. If glucosidase activity is perturbed by the mutation in glucosidase-I (Lec23 cells) or by the addition of glucosidase inhibitor castanospermine, triglucosylated free OSs generated in the ER will be: (a) transported to the Golgi apparatus. (b) digested by the Golgi endo-α-Man'ase to remove triglucose residues. (c) delivered through the Golgi apparatus to acquire further processing of glycans. (d) secreted into the media. (e) Some reports supported the occurrence of glucosidated OSs in the cytosol, most likely generated by the action of cytoplasmic PNGase, but the metabolic pathways for them are not completely elucidated yet. Blue square: GlcNAc, green circle: mannose, yellow circle: galactose, purple square: sialic acid. This figure was made mainly based on the work by Durrant and Moore [29]

is dependent on the Sec61-complex [30], but independent of the presence of glucoses on free OSs [31,32].

If the glucosylated OSs cannot escape from the ER lumen, what is their fate? In fact, accumulations of glucosylated OSs can be observed in the cytosolic fraction upon incubation with glucosidase inhibitor [13,28,33,34], but those are believed to be derived from ERAD system in the cytosol and released in the cytosol by PNGase (Fig. 3; see below). A recent study has clearly shown that if the glucosylated OSs are generated in the lumen of the ER, the transport of glucosylated OSs into the cytosol is perturbed, leading to the secretion of "processed," complex-type OSs and Glc_3Man fragments into the extracellular space [29] (Fig. 3, process d). In this case, the glucose residues are most likely removed by the action of endo-α-mannosidase activity [35,36], which resides in the Golgi and provides the glucosidase-independent processing of *N*-glycan and thereby allows formation of complex-type glycan chains (Fig. 3, process c). Based on their results, we speculate that in case of glucosylated OSs formed in the ER, they are secreted through secretory vesicles, though the detailed mechanism by which these OSs are transported remains to be clarified.

3. Free OSs formed in the cytosol

3.1. Free oligosaccharides formed in the cytosol: its connection with the ERAD

Recent evidence clearly shows that the ER has a "quality control" machinery that distinguishes misfolded (glyco)proteins from correctly folded ones, so that only the latter move from the ER to the Golgi. In this system, proteins that fail to fold or to form subunit structures are retained in the ER by interacting with various lumenal chaperones in order to allow them to mature into correct structure before they exit the ER (Fig. 2). When proteins consistently fail to acquire the correct folding state, they are eventually degraded by the ERAD system [9–11]. It is now clear that this degradation process involves retro-translocation of the defective (glyco)proteins from the ER to the cytosol, followed by their degradation in the cytosol by the action of the 26S proteasome. It is also becoming evident that glycan chains on glycoproteins play critical roles for monitoring the folding state of glycoproteins in the ER; this process involves various intracellular lectins (Fig. 2).

It is feasible to assume that for glycoproteins to be degraded efficiently by the proteasome, bulky modifications of polypeptide side chains such as *N*-glycan chains might be expected to be removed prior to proteolysis by the proteasome [37–39]. The removal of glycans is achieved by the action of ubiquitous, cytoplasmic peptide:*N*-glycanase (PNGase), releasing free OS in the cytosol (Fig. 1). PNGase cleaves the amide bond in the side chain of glycosylated-asparagine residues, and under physiological pH the ammonia is released from OS, generating free OS bearing *N*,*N*′-diacetylchitobiose structure at the reducing terminus. The cytosolic PNGase occurs widely from yeast to mammalian cells [8,37–43], and the gene encoding this enzyme has been identified [20,44].

Another enzyme that can generate free OSs in the cytosol is a pyrophosphatase that would release OS-P moieties from Dol-PP-OSs (Fig. 1). Such a cytosol-oriented enzyme activity has been detected in mammalian cells [45]. A similar enzyme activity has been detected in budding yeast [46], though the gene encoding dolichylpyrophosphate-oligosaccharide

pyrophosphatase remains to be identified. Another possibility is the action of endo-β-*N*-acetylglucosaminidase (ENGase; abbreviation originally proposed by Karamanos [47]), which will cleave the glycosidic bond in the *N*,*N*′-diacetylchitobiose unit. The ENGase action gives rise to Gn1-type free OSs. It is not known if the cytoplasmic ENGase can directly generate free OSs either from Dol-PP-OSs or glycoproteins *in vivo*. Most of the deglycosylated intermediates formed are, so far as examined, deglycosylated by PNGase during ERAD pathway (by confirming the introduction of negative charge(s) into the core peptide) [37 and references therein]. Thus, cytoplasmic *N*-glycoproteins are not likely a physiological substrate for ENGase.

3.2. Cytosolic processing of free oss - conversion of Gn2 to Gn1 species

Irrespective of their source, cytosolic OSs would be expected to be further catabolized, possibly to maximize the reutilization of the component sugars. Since the discovery of free OS in the cytosolic fraction, extensive studies on structural characterization of the cytosolic free OSs have been carried out [7,12,16,28,45,48,49]. In the cytosol, two enzymes mainly contribute to the catabolism of free OS: endo-β-*N*-acetylglucosaminidase and α-mannosidase. For endo-β-*N*-acetylglucosaminidase, two distinct enzyme activities have been reported; one is ENGase [50,51], and the other is a cytosolic, neutral chitobiase [52]. Cytoplasmic ENGase has been known to be distributed widely in animal cells [50,53–57], while it is absent in yeasts such as *S. cerevisiae* or *S. pombe* [42]. Indeed, the gene encoding cytoplasmic ENGase activity does occur widely from *C. elegans* to human [50,51], but is absent in these yeasts.

While a chitobiase catalyses the same reaction with ENGase, it acts only if the reducing terminus of free OS is *N*,*N*′-diacetylchitobiose (Gn2 form), releasing a single GlcNAc from its reducing terminus to generate Gn1 free OSs. Therefore, the chitobiase can be called as a "reducing-end exoglycosidase" in this sense. It has been reported that in Mardin-Darby bovine kidney (MDBK) cells, unlike in Chinese Hamster Ovary (CHO) cells, the cytoplasmic (neutral) ENGase activity was absent, while a neutral chitobiase activity is observed [52]. Therefore the cytosolic, neutral chitobiase has been claimed to be the only responsible enzyme to convert Gn2 to Gn1 free OS in the cytosol of MDBK cell [52]. It should be noted here that a gene orthologue of the cytoplasmic ENGase can be found in bovine EST sequence (Gene accession No. XP_874593). The predicted bovine ENGase appears to be highly homologous to other ENGase proteins from mammalian cells based on the amino acid sequence deduced from EST sequence. It would be interesting to see whether this gene product has lost its enzymatic activity due to mutations or it is somehow silenced in MDBK cells. Since the lysosomal acidic chitobiase [58], which is involved in lysosomal catabolism of *N*-linked glycans, is absent in bovine [59], the detected chitobiase should be a novel one; however at present the molecular nature of the cytosolic chitobiase remains to be unveiled.

3.3. Trimming of mannose by cytosolic α-mannosidase

It has been demonstrated that the "soluble, neutral" mannosidase occurs in a variety of animal cells [60–72]. From its enzymatic properties as well as biochemical studies of free OS in the cytosol, it has been assumed that cytosolic α-mannosidase trims the free OS to form mainly $Man_5GlcNAc$ (Figs. 1 and 4). The enzyme has distinct properties from other ER or Golgi-resident mannosidases such as activation by Co^{2+} and an inhibition profile for various mannosidase inhibitors. The soluble mannosidase has been cloned from rat liver [73], but it has been postulated that the soluble form is derived from the membrane-associated or lumenal form by the post-homogenization process during purification. The "cytosolic" α-mannosidase has been found to be enzymatically, and immunologically related to "ER" form of mannosidase (also termed ER mannosidase II [74]), which is believed to be involved in the processing pathway of *N*-linked glycans [61,75]. Although it has been proposed that the cytoplasmic mannosidase is most likely the precursor of the ER enzyme [74,75] the mechanism by which the cytoplasmic enzyme entered the ER lumen still remains to be determined.

It has been shown that cytosolic α-mannosidase prefers Gn1 to Gn2 as substrates [63,67]. The Man_5 structure yielded by the action of cytosolic α-mannosidase (α1-2,3,6

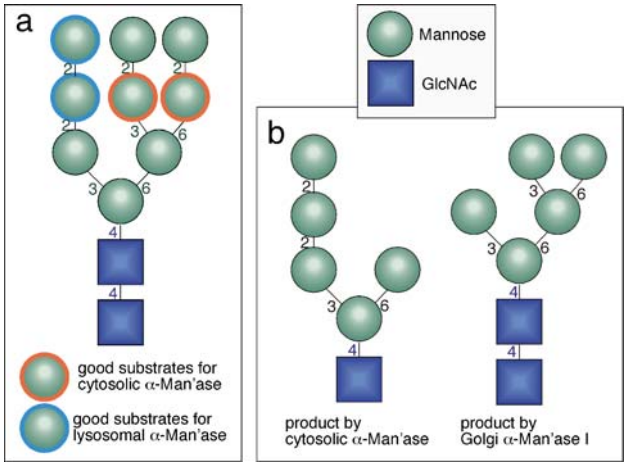

Fig. 4 Specificities of mannosidases. (a) comparison of the specificities of cytosolic or lysosomal mannosidases. The mannose residues circled by the red lines indicate the ones which are preferentially cleaved by cytosolic mannosidase, and the ones by the blue lines show the residues for lysosomal mannosidase [62]. (b) comparison of $Man_5GlcNAc$ free OS structure after the cleavage by cytosolic α-mannosidase (left) or $Man_5GlcNAc_2$ on glycoprotein after the action of Golgi α-mannosidase I (right)

mannosidase) results in different isomeric structure from that formed by Golgi α-mannosidase I (α1-2 mannosidase) (Fig. 4). It has been postulated that cytosolic mannosidase cleaves mannose residues that are not cleaved efficiently by lysosomal enzymes, suggesting the complementary roles of these two enzymes in the catabolic pathway of *N*-linked glycans [62].

3.4. OS transporter on lysosomal membrane

The end product of ENGase (or chitobiase) and α-mannosidase in the cytosol, $Man_5GlcNAc$, is then transported into the vesicular compartment, presumably lysosomes, where further degradation by lysosomal α-mannosidase and β-mannosidase can occur [76,77] (Fig. 1). The transporter on the lysosomal membrane seems to be specific for the $Man_5GlcNAc$ structure (or possibly smaller species as observed in the cytosol [45,49,52]), is ATP-driven, and is inhibited by GlcNAc but not by mannose, indicating that the reducing terminal structure of free OSs, in sharp contrast to the ER transporter, is crucial for free OS transport to lysosomes [76,77]. The molecular nature of this transporter is yet to be elucidated.

Although the glucosylated free OSs generated in the ER may not reach the cytosol [29], the occurrence of these species in the cytosol has repeatedly reported, especially in the presence of glucosidase inhibitor [28,34,45,49]. These OSs are presumed to be derived from cytosolic deglycosylation by PNGase from glycoproteins or, perhaps from small glycopeptides transported from the ER [31,32]. The cytosolic α-mannosidase is shown to act on glucosylated free OS as well as on nonglucosylated ones [68]. What will be the final fate of glucosylated free OSs in the cytosol remains unknown (Fig. 3).

4. Free OSs observed in living organisms other than mammals

4.1. Free OSs in *S. cerevisiae*

Though the most of the works described above have been carried out using animal cells, there is emerging evidence for occurrence of free OSs in the cytosol from other origins. In budding yeast (*S. cerevisiae*), the origin and fate of the free OSs have been examined [14]. It has been revealed that in yeast, 70–80% of free OS was generated by cytoplasmic deglycosylation by Png1p (PNGase in *S. cerevisiae*), while where and how the remaining free OSs are generated are largely unknown. After the release of free OS in the cytosol, vacuolar α-mannosidase, Ams1p, seems the only enzyme involved in their catabolism. Ams1p is a vacuolar enzyme but does not have a signal sequence and therefore reaches the vacuole, independently of the conventional secretory pathway [78]. Recently, it has been found that this enzyme is targeted from the cytosol to the vacuole directly through the mechanism called Cvt (cytoplasm to vacuole targeting) pathway [79]. Whether the free OS can be catabolized by Ams1p in the cytosol, or it also has to be targeted to the vacuole for degradation is not yet determined.

4.2. Free OSs in plants

In plants, it has long been proposed that unconjugated *N*-glycans (UNGs) [80,81] have biological activities on growth, differentiation, and senescence. The UNGs are classified into two subclasses according to their structure (Fig. 5) [82–93]; one is the oligomannose Gn1-type glycan which is composed of only Man and GlcNAc, and the other is the plant complex-type Gn2 glycans, which contains other sugars such as Xyl, Gal, Ara and Fuc α1-3 linked to the proximal GlcNAc residue (Fig. 5). The dose-dependent influence of UNGs on both growth and senescence of plant cells has been observed [83,86,94]. It is also known that the amount of UNGs changes during fruit ripening or cell culture [94,95], suggesting the correlation of OS metabolism and observed biological phenomena. There are two deglycosylating enzymes so far known in plants; plant-specific acid PNGase [84,95–107] and cytoplsmic ENGase [95,105,108–114]. It appears that the acidic PNGase is responsible for the release of complex type glycans, while the ENGase is involved in generation of high mannose-type, Gn1 species. These enzyme activities also appear to be regulated in a developmental stage-dependent manner [95,105,107].

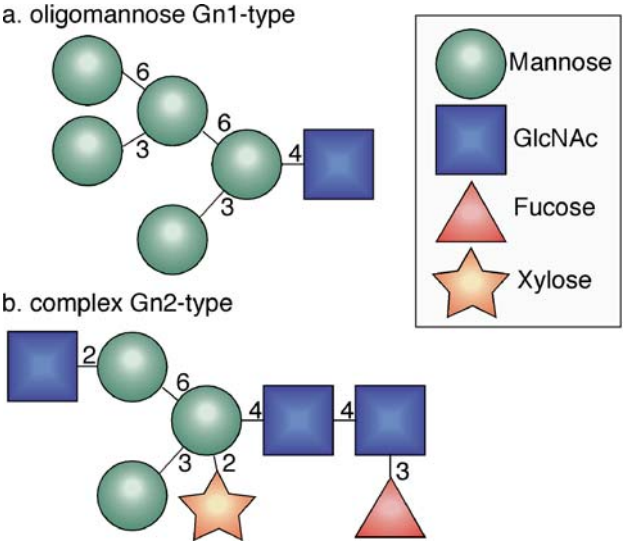

Fig. 5 Example structures of (a) high mannose type and (b) complex type UNG, plant-type free OS. Those OSs were found in both tomato and white campion [82,85]

Despite of the initial finding of both high mannose-type and complex-type free OSs from the culture medium of *Silene alba* cells [82], recent studies by Kimura *et al.* have revealed that high mannose type UNG is accumulated predominantly in the cytosol [93]. The high mannose type free OS (UNG) is therefore predicted to be derived from the glycoprotein and/or Dol-PP-OS by the similar pathway with that of animal cells (Fig. 1). In this context, recently the occurrence of a Co^{2+}-sensitive, soluble mannosidase has also been demonstrated in ginkgo seeds [115]. Given the structural differences of high-mannose type, Gn1 OSs between animal cells and plants (see Figs. 4, 5), it would be interesting to analyze the details of the substrate specificity for the plant α-mannosidase. It should also be noted that while the orthologues of cytoplasmic PNGases have been found in plants through database survey [20,116], and indeed a PNGase-deglycosylated form has been observed during ERAD in plant cells [117], the direct proof of the cytoplasmic PNGase activity in Png1p-orthologue has not yet been provided. It is noteworthy that the orthologue of cytoplasmic PNGase in *Arabidopsis thaliana*, AtPng1p, exhibits transglutaminase activity [118]. Indeed the Png1p and transglutaminases share the homology around catalytic triad [37,119]. If the protein also possesses PNGase activity or it serves as a dual functional enzyme remains to be seen.

4.3. Free OSs in fish

Free OSs with complex-type glycan structure are found in oocytes and early embryos of several fish species, as it is first described by Yasuo and Sadako Inoue's group in 1989 [120–126]. These free OSs can be divided into two classes according to their progenitor proteins (Table 1). One possesses complex-type bi-, tri- and tetraantennary structure, and has been found in large quantities in unfertilised eggs (5×10^{-8} mol (*Plecoglossus altivelis*) and 25×10^{-8} mol (*Tribolodon hakonensis*) per g fresh eggs) [127]. It is speculated that these free OSs are derived from glycophosphoprotein, one of the major forms of phosvitin derived from vitellogenin. The other free OSs are accumulated in the embryos [122,124] and are derived from the cortical alveoli-localized glycoprotein called hyosophorin [128]. Hyosophorin was also discovered and characterized by the Inoues, and the properties and possible physiological functions are well summarized in their review [127].

Because of the structural feature of these free OSs in fish (*i.e.* complex type free OSs), those free OSs may be generated by distinct pathway from the ER or ERAD-related pathway as described above. Most of those free OSs found in *Paralichthys olivaceus* are Gn2 species [122], leading them to search for and successfully identify PNGase activity in the early embryos of medaka fish [129]. It should be noted that it was the first report on the occurrence of PNGase activity in animal origin and that the discovery led them to the new finding of cytoplasmic PNGase activities in mammalian cells and organs [8,130]. More recently two distinct PNGase activities have been reported by them in the same fish, and are called acid and neutral PNGase, respectively [43]. Based on the enzymatic properties, one can assume that neutral PNGase is an orthologue of cytoplasmic PNGase [43]. On the other hand, the acid PNGase [43,129], so far as examined, is fish-specific, and similar acidic PNGase activity is not found in mammalian cells or in hen oviduct [8,41]. In *T. hakonensis* oocytes most of the free OSs exist as Gn1 species, indicating the action of ENGase-like enzyme toward these complex-type free OSs [121]. Whether the cytosolic ENGase can carry out this function or there is another ENGase-type activity in fish is not known.

With respect to the physiological significance of the deglycosylation reaction in fish, the Inoues hypothesized that deglycosylation of vitellogenin-derived protein (most likely by the acid PNGase) could facilitate the recycling of the vitellogenin receptor (after endocytosis of the protein from the serum to cells) or alternatively its further proteolytic processing [43,123,127]. In the case of hyosophorin, little is known with regard to the functional importance, but it has been hypothesized that either deglycosylated protein (peptide) or free OSs generated by PNGase may be a bioactive molecule essential for embryogenesis [41,127]. The similar free OSs has been also found in chicken egg [131], although the detailed biological significance of these molecules in the chicken egg or how these molecules are generated remains to be clarified.

5. Concluding remarks

Cells possess a variety of enzymes and transporters to handle free OS generated by several distinct mechanisms. It should be noted here that the scheme depicted in Fig. 1 is

Table 1 Free OSs found in oocytes and embryos of several fish species

Fish (species)	Cells	Progenitor protein	Reference
Freshwater Trout (*Precoglossus altivelis*)	Unfertilized Eggs	Glycophosphoprotein	[120]
Dace (*Tribolodon hakonensis*)			[121]
Medaka (*Oryzias latipes*)			[125]
Flounder (*Paralichthys olivaceus*)	Fertilized Eggs	Hyosophorin	[122]
Medaka (*Oryzias latipes*)			[124]

largely based on the extensive biochemical studies such as characterization of free OS structures as well as responsible enzymes/transporters as covered in this review. Indeed, although the concept of "cytosolic catabolism of OSs" has not been generally accepted yet, many have proposed the potential importance of cytosolic glycosidases as well as deglycosylation reactions in non-lysosomal compartments, based on these biochemical observation [47,123,127,132–140]. However, the lack of information (*i.e.* genes encoding these molecules involved in this process) has prevented us from further characterization. For instance, free OSs collected in the cytosol are processed by cytosolic ENGase (or a chitobiase) and α-mannosidase before being incorporated into the lysosomes for degradation into monomeric sugars. Why this rather complicated pathway (ER-to-cytosol-to-lysosome) is chosen for free OS-catabolism is a profound mystery. One can imagine, as described above, that this trafficking route enables them to be removed quickly from the ER, thereby preventing them from interfering with the efficient glycan-dependent quality control machinery for newly synthesized glycoproteins in the ER. However, it is still possible that, as proposed in plants, in particular free OS itself may play a role in certain cellular processes.

More characterization of the process involved raises further questions, making the current scenario more intriguing (and complicated as well). For instance, it is now known that some misfolded glycoproteins once exit the ER and proteins with more 'processed' (Golgi-form) glycan can be also exported into the cytosol [141–145]. In fact even some toxin proteins, entering from the cell surface by endocytosis, travel all the way to the ER by retrograde transport and end up with cytosol via retrotranslocation from the lumen of the ER to the cytosol, as if it were a substrate of ERAD [146,147]. We therefore know that not only newly synthesized proteins bearing typical high mannose-type (ER-type) glycan, in certain situations proteins once localized outside of the ER can be redirected to the ERAD pathway. In animal cells, some of those proteins can bear complex type glycans consisting of sialic acid or Gal in addition to Man and GlcNAc. Can proteins bearing complex-type glycans be degraded by a similar ERAD pathway? At least free complex type OSs have been observed in the cytosol of rat liver [49]. We do believe that, now in this "post-genome" era, most of the biosynthetic pathways for *N*-glycan chains have been unveiled, the efforts have to be made to envision an overall pathway for catabolism of *N*-glycan-OS in various biological systems, in addition to the conventional lysosomal process [148]. Whether this non-conventional catabolic pathway is just degrading remnants of OSs or creating bioactive molecules is unknown. Identification of proteins involved in formation, modification and transport of free OS should provide us more insight into the mechanism and function of this pathway, which has been largely overlooked.

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