REVIEW ARTICLE

Bifunctionality of glycan-recognizing proteins in N-glycoprotein biosynthesis

Taiki Kuribara and Kiichiro Totani*

Authors' affiliations:

Department of Materials and Life Science, Seikei University 3-3-1 Kichijoji-kitamachi, Musashino, Tokyo 180-8633, Japan

***Correspondence:** Kiichiro Totani, Department of Materials and Life Science, Seikei University, 3-3-1 Kichijoji-kitamachi, Musashino, Tokyo 180-8633, Japan
Tel.: +81-422-37-3745; Fax: +81-422-37-3871; E-mail: ktotani@st.seikei.ac.jp

Abstract

In an early step of N-glycoprotein biosynthesis, high-mannose type glycans are attached to nascent polypeptides in the endoplasmic reticulum. Subsequently, the glycopolypeptides adopt the correct folding conformations, after which glycans are converted to complex type glycans in the Golgi apparatus. During these processes, over 30% of nascent glycoproteins exist in either misfolded or unfolded forms, therefore proper folding of these proteins is essential for efficient N-glycoprotein biosynthesis. Furthermore, elimination of misfolded glycoproteins from the endoplasmic reticulum via cytoplasmic degradation is essential for maintenance of cellular homeostasis. Through these sorting processes, various glycan-recognizing proteins, such as glycosyltransferases, glycosidases, and lectins, contribute to the conversion of nascent glycoproteins into a variety of glycoforms. Some glycans are believed to be signals for the folding, secretion, and degradation of glycoproteins that are partially folded, correctly folded, and misfolded, respectively. Since targets of the glycan-recognizing proteins are diverse within the body, some of the lectin and glycan processing enzymes may be bifunctional, characterised by dual recognition of both glycan and aglycone moieties. In fact, uridine 5'-diphosphate glucose: glycoprotein glucosyltransferase, which localises in the endoplasmic reticulum, has been known to have both, glucose transfer activity and the ability to sense improperly folded proteins. Similar dual recognition properties have been reported, particularly in glycan-recognizing proteins, with regards to glycoprotein folding, sorting, and degradation processes. However, there have not been any review articles highlighting the bifunctionality of glycan-recognizing proteins thus far.

In this review, we summarise several examples of the bifunctionality of glycan-recognizing proteins involved in N-glycoprotein biosynthesis.

Keywords: N-glycoprotein biosynthesis, glycan-recognizing protein, aglycone recognition, bifunctionality

1. Introduction

In N-glycoprotein biosynthesis, various glycan-recognizing proteins contribute to glycan processing, as well as protein-folding and degradation. The N-glycoprotein biosynthesis processes are categorised as follows: 1) High- mannose-type glycan-processing, glycoprotein folding, and sorting in endoplasmic reticulum (ER), 2) Structural conversion of folded glycoproteins, such as the conversion of high-mannose-type glycans to complex-type glycans, in the Golgi apparatus, and 3) Glycan removal and degradation of misfolded glycoproteins in cytosol (Figure 1).[1-7] Since the targets of glycan- recognizing proteins are extremely diverse within the body, glycan recognition and the related glycan conversion likely depends on both the glycan structure and the folding state of the protein. In fact, activity of uridine 5'-diphosphate (UDP)-glucose: glycoprotein glucosyltransferase (UGGT) has been known to differ between native, misfolded, and unfolded monoglucosylated glycoproteins.[8] A similar phenomenon has been reported for several other glycan processing enzymes and lectins. In this review, we will provide an outline on the bifunctionality (the simultaneous dual recognition of glycan and protein moiety) of glycan-recognizing proteins in N-glycoprotein biosynthesis. A summary of the bifunctionality of typical glycan recognizing proteins highlighted in this review is listed in Table 1.

Table 1. Typical examples for the bifunctionality in N-glycoprotein biosynthesis

Protein	Glycan related function	Other function
Malectin	Recognition of G2M9	Recognition of misfolded protein [ref 12]
CNX/CRT	Recognition of monoglucosylated N-glycan	Molecular chaperone activity [ref 16-19] Discrimination of hydrophobicity [ref 23, 24]
UGGT	Glucose transfer activity	Discrimination of hydrophobicity [ref 32-34]
ERManI	Mannose-trimming activity	Recognition of misfolded protein [ref 50]
EDEM1	Mannose-trimming activity	Covalent-like bond formation with client protein [ref 49] Discrimination of hydrophobicity [ref 51]
EDEM2	Mannose-trimming activity	Discrimination of hydrophobicity [ref 51]
OS-9	Recognition of N-glycan lacking C-branch outermost α1,2-mannose residue	Recognition of misfolded protein [ref 53]
Cytosolic PNGase	Deglycosylation activity	Discrimination of peptide sequence [ref 63]

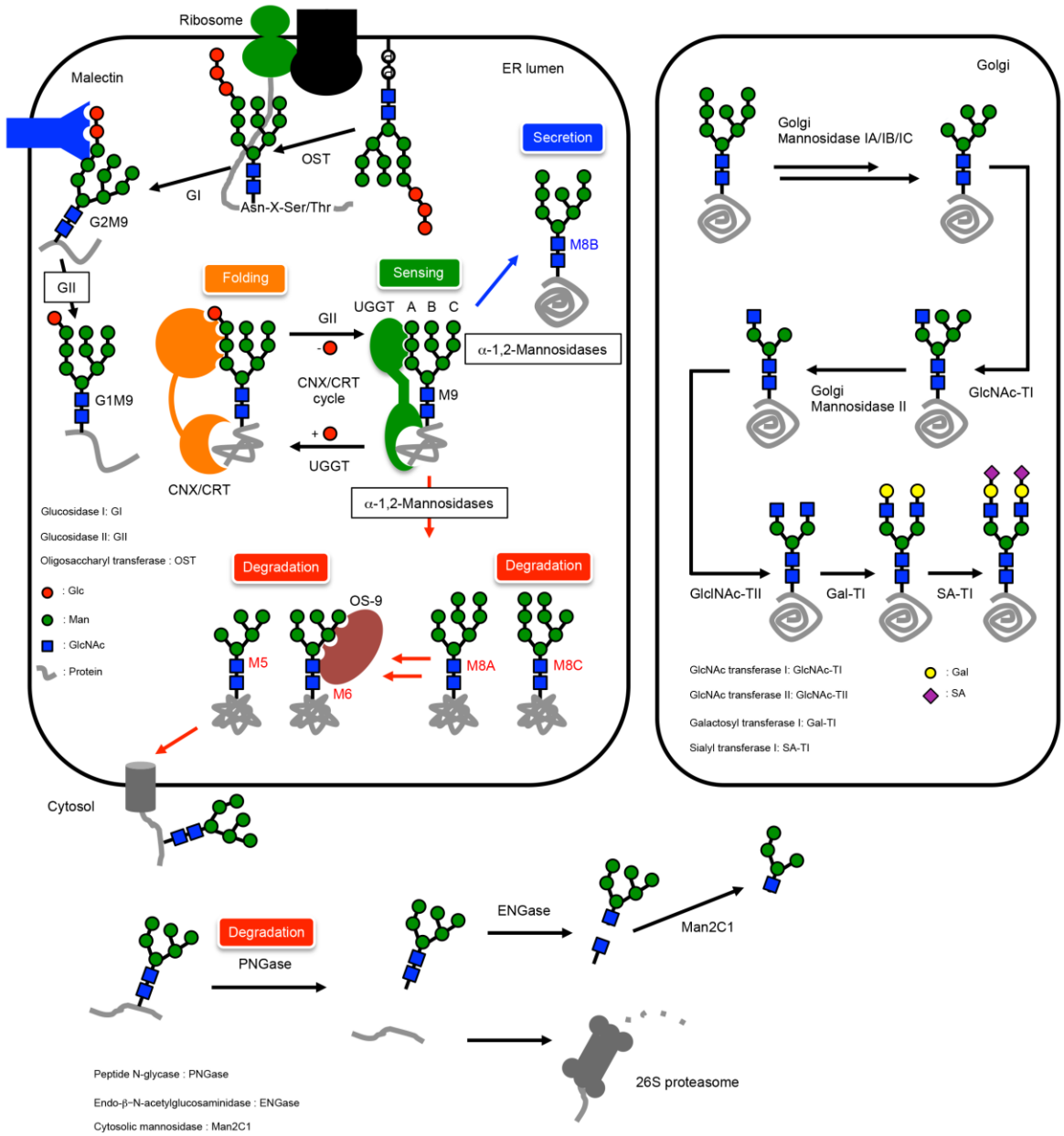

Figure 1. N-glycoprotein biosynthesis

2. Glycoprotein folding and sorting in the ER

Within the ER, glycan structures on glycoproteins play an essential role for the secretion and degradation of glycoproteins.[1-6] In terms of determining signal glycans, the folding state of the glycoprotein could affect glycan-related enzymatic or lectin activities. In this section, we focus on the bifunctionality of ER enzymes and lectins during signalling pathways that involve glycoproteins.

2.1. Malectin

In 2008, Schallus et al. reported a novel lectin named malectin that is involved in the

early steps of N-glycoprotein biosynthesis in ER.[9] Malectin recognises $Glc_2Man_9GlcNAc_2$ (G2M9), which is produced at the initial step of ER glycoprotein processing. The target epitope of malectin was revealed to be the Glcα1-3Glc moiety based on frontal affinity chromatography[10,11] and crystallographic studies.[9,12] Thus, the lectin selectively binds diglucosylated high mannose N-glycan. Several studies have proposed the bifunctionality of malectin. In brief, the lectin associates with the misfolded α1-antitrypsin null Hong Kong variant rather than the wild-type α1-antitrypsin,[12] indicating that malectin selectively associates with a model substrate for ER-associated degradation (ERAD). Furthermore, overexpression of malectin enhances ERAD.[12] Although it has been shown that malectin forms a complex with ribophorin I to recognise misfolded proteins,[13] immunoprecipitation studies highlight that malectin may directly bind to misfolded ERAD substrates, suggesting the bifunctionality of the lectin.[12] Although the precise mechanism of malectin bifunctionality requires further investigation, this activity likely serves to prevent aggregation in early N-glycoprotein biosynthesis via detection of nascent unfolded glycoproteins.

2.2. Calnexin and Calreticulin

Removal of the outermost glucose-residue of the G2M9-protein produces $Glc_1Man_9GlcNAc_2$ (G1M9)-protein, which is recognised by calnexin (CNX) / calreticulin (CRT) to accelerate protein folding. CNX and its soluble homologue CRT are lectin-like molecular chaperones that bind to monoglucosylated glycoproteins in the ER.[14,15,16] CNX binds to the folding intermediate of class I histocompatibility molecules,[16] T cell receptors, membrane immunoglobulins,[17] influenza hemagglutinin,[18] and transferrin.[19] As the protein nears its correct folded conformation, the glycoproteins dissociate from CNX. CRT possesses a similar dissociation mode for monoglucosyated glycoproteins.[20,21] Taken together, CNX/CRT bind to partially folded glycoproteins as part of a glycoprotein quality control system. Inversely, Helenius et al. reported that only monoglucosylated N-glycan is required for CRT *in vitro*.[22] Thus, whether CNX/CRT have bifunctionality towards glycoproteins remains controversial. We focused on the bifunctionality of CRT, and carried out an interaction analysis using a series of structurally-defined G1M9-derivatives with different aglycone moieties on proximal regions at N-glycosylation sites (-OH, -Gly, and -Gly-Glu-^{t}Bu).[23] Binding assays between CRT and these substrates revealed diverse specificity. CRT bound strongly to G1M9-Gly-Glu-^{t}Bu, but showed moderate binding to G1M9-Gly. Moreover, weak binding relative to these derivatives was observed when using G1M9-OH as a ligand. These results indicate that CRT simultaneously recognises the G1M9-moiety and aglycone structures at regions close to N-glycosylation sites depending on their hydrophobicity. In other words, CRT displays bifunctionality when recognising G1M9-derivatives. In order to better understand this bifunctionality, we examined the interaction of CRT with a series of G1M9-derivatives exposed to hydrophobic

BODIPY-dye connected with hydrophilic ethylene glycol linkers of varying lengths (n = 0, 4, 8, and 12).[24] These experiments revealed that CRT preferentially binds to G1M9-BODIPY with a shorter linker (binding orders; $EG_0 > EG_4 > EG_8 > EG_{12}$), highlighting that CRT binding depends on the hydrophobicity of the regions close to the N-glycosylation site.

2.3. UGGT

After G1M9-protein undergoes a folding process mediated by CNX/CRT, the glucose residue is removed by glucosidase II to produce $Man_9GlcNAc_2$ (M9)-proteins.[25] The resulting M9-protein is recognised by the folding sensor enzyme UGGT.[8,26] UGGT specifically glucosylates the M9-glycoprotein, resulting in a molten globule-like folding intermediate state. Conversely, this enzyme displayed little recognition for properly folded and severely misfolded random-coil-like M9-protein.[8] A unique specificity has been observed for not only natural glycoproteins, but also synthetic glycoprobes, such as denatured glycoproteins,[27,28] neoglycopeptides,[29] synthetic homogeneous glycoproteins,[30] and structurally-defined synthetic substrates.[31] To obtain a precise understanding of target recognition via UGGT, Trombetta et al. reported that chemically denatured ribonuclease B (RNaseB) was strongly glucosylated by the enzyme, while native RNaseB was only slightly reglucosylated.[32] Furthermore, no UGGT activity was reported for the fully unfolded RNaseB. These *in vitro* studies clearly showed the bifunctionality of UGGT. Kajihara et al. reported *in vitro* simultaneous monitoring of the reglucosylation and folding process using chemoenzymatic synthesised crambin as a model substrate-glycoprotein.[33] Their findings indicate that UGGT can recognise each of the folding-intermediates during the folding process. Sousa and Parodi reported that UGGT recognises hydrophobic nonapeptides more than hydrophilic ones.[26] On the other hand, Totani et al. previously synthesised non-peptidic substrates of UGGT, i.e. thirteen structurally-defined M9-glycans conjugated with various hydrophobic aglycones.[34] The reactivity of UGGT towards these synthetic substrates and the recognition motif of the enzyme were clearly identified, thus contributing to our understanding of the molecular basis behind the bifunctionality of UGGT. Interestingly, reactivity orders of M9-Gly-Glu-^{t}Bu, M9-Gly, and M9-OH to UGGT were similar to that of G1M9-derivatives to CRT.[23,34] These results indicate that UGGT and CRT recognise these glycoprobes in a similar bifunctional manner. Moreover, binding of synthetic M9-derivative changed the circular dichroism (CD) spectrum of UGGT only when M9-glycan was conjugated with hydrophobic aglycone (5-TAMRA).[35] This change in the CD spectrum strongly suggests that UGGT simultaneously recognises M9-glycan and hydrophobic aglycone. Hachisu et al. reported UGGT has flexible recognition mode for pseudo-misfolded glycoproteins.[36] The authors compared the glucosyltransfer activity of UGGT towards M9-methotrexate complexes with hydrophobic pyrene-labelled dihydrofolate reductase (DHFR), which modifies cysteine groups at different positions of DHFR by

pyrene groups. According to these results, UGGT displays flexibility for sensing hydrophobic surface patches. In fact, Satoh et al. recently reported that UGGT possesses a small catalytic domain and a flexible recognition domain for hydrophobic patches.[37] This attribute likely allows UGGT to sense a variety of glycoprotein folding intermediates. Therefore, the flexible recognition of surface hydrophobicity is important for the bifunctionality of UGGT. Focusing on the proximal regions of N-glycosylation sites, we synthesised a series of chitobiose-pentapeptides as inhibitors of UGGT.[38] Six pentapeptides with systematically controlled hydrophobic leucine and hydrophilic serine sequences were synthesised. With these six pentapetides, UGGT inhibition assays were carried out. UGGT specifically recognised the serine residue directly linked to the C-terminus of N-glycosyl-asparagine. Taken together, these findings increase our understanding of the molecular basis behind the bifunctionality of UGGT.

2.4 α-1,2-Mannosidases in the ER

Through a glycoprotein sorting step in the ER, mannose residues of M9-glycoproteins are sequentially trimmed by α-1,2-mannosidases. Tri-branched M9-glycan contains cleavable α-1,2-mannoside linkages in the outermost sugar-residue of each branch. Several reports suggest that four mannosidases are responsible for trimming α-1,2-mannosides in the ER: ER mannosidase I (ERManI),[39,40] ER-degradation- enhancing α-1,2-mannosidase-like protein 1 (EDEM1),[41] EDEM2,[42] and EDEM3.[43] Based on the lectin/enzyme specificities related to glycoprotein sorting, $Man_{8B}GlcNAc_2$ (M8B) and $Man_{8A/C}GlcNAc_2$ (M8A and M8C) that are produced by α-1,2-mannosidases in the ER are believed to be secretion and degradation signals, respectively.[44] However, it remains unclear which of the α-1,2-mannosidases, particularly among the EDEM family, produce glycoprotein secretion (M8B) and degradation (M8A and M8C) signals. This is likely because EDEM activity has not yet been detected *in vitro*. Therefore, the activity of these mannosidases has been inferred using transgenic cell lines (i.e. overexpression or knockout of EDEMs).[43,45,46] According to these studies, all EDEMs possess mannosidase activity within intact cells. In brief, it is known that EDEM1 trims the A and C branch, EDEM2 trims the B branch, whereas the branch specificity of EDEM3 has not yet been identified. Despite various efforts towards analysing the specificity of α-1,2-mannosidases, it is still unclear whether these glycosignals are produced randomly or regioselectively. Recently, we succeeded in selectively manipulating discrete α-1,2-mannosidase activities using reciprocally selective inhibitors against glycosignal production.[47] The results suggested that secretion and degradation glycosignals are regioselectively produced by discrete α-1,2-mannosidase(s), and that two independent pathways for glycosignal production exist in the ER. Very recently, Hosokawa and co-workers reported that *in vitro* EDEM3 activity requires covalent interaction with ER-resident protein 46.[48] Furthermore, Hebert et al. reported that the mannosidase-like domain of EDEM1 possesses mannosidase activity by using a ER

mannosidase I/Golgi mannosidase IA/B/C quadruple knockout cell.[49] Therefore, the *in vitro* mannosidase activity of the EDEM family has been partially understood, although *in vitro* branch specificity is still under investigation. With regards to aglycone structure recognition by α-1,2-mannosidases, several studies indicate that ER-ManI, EDEM1, EDEM2, and EDEM3 are able to recognise aglycone structures. ER-ManI preferentially trims denatured M9-bovine thyroglobulin and soybean agglutinin.[50] The enzyme can recognise the polypeptide region of substrate M9-proteins, thus suggesting the bifunctional nature of ERManI. EDEM1 and EDEM2 also have been reported to bind to hydrophobic surfaces of model misfolded proteins.[51] Thus, EDEM1 and EDEM2 seem to have bifunctionality towards misfolded glycoproteins. In addition, putative ERAD target proteins have been reported to bind to the hydrophobic surface patch in EDEM1's mannosidase-like domain.[49] In any case, EDEM1 and EDEM2 display bifunctionality via their glycan-binding ability and ability to discriminate between different hydrophobicities. In addition, we examined mannose trimming of M9-glycan linked to BODIPY-dye with a systematic series of linker lengths in the ER of mouse liver samples.[24] A higher mannose trimming velocity was observed for the substrates with shorter linkers. Since multiple α-1,2-mannosidases were tested in these experiments, it remains unclear which mannosidase(s) is responsible for the mannose-trimmings. However, at least a single α-1,2-mannosidase might discriminate hydrophobic surfaces on the proximal region at N-glycosylation sites. Interestingly, in terms of redox conditions in the ER, EDEM1 showed additional bifunctionality, such as covalent-like disulfide bond formation between cysteine residues of EDEM1 and the target protein.[49]

2.5. OS-9

The action of ER α-1,2-mannosidases provide several glycoforms of high mannose glycans. Particularly, the glycoforms lacking an outermost mannose residue at the C-branch are believed to serve as degradation signals.[44] Proteins with these glycosignals are captured by osteosarcoma 9 (OS-9) located at a dislocon channel, which aids in delivering misfolded glycoproteins from the ER to the cytosol.[52,53] Based on its role in facilitating degradation of misfolded glycoproteins, OS-9 might possess discrimination ability based on hydrophobicity. In fact, OS-9 preferentially binds to a folding-defective, glycoprotein-null, Hong Kong variant of α1-antitrypsin.[53] It has also been reported that OS-9 forms a complex with glucose-regulated protein 94, which functions as a molecular chaperone in the ER.[54] Although several findings suggest the bifunctionality of OS-9, further investigation will be necessary for precise understanding of its molecular basis.

3. Glycoprotein degradation in cytosol

Retrotranslocated misfolded glycoproteins are deglycosylated in the cytosol, followed by proteasomal degradation.[55] A deglycosylation reaction likely contributes to efficient degradation of misfolded proteins.[56] In light of the fact that deglycosylation is ne-

cessary for misfolded glycoprotein degradation, we focused on the bifunctionality of cytosolic deglycosylation enzyme.

3.1. PNGase

Peptide: N-glycanase (PNGase) is a type of amidase that cleaves amide bonds between N-glycan and asparagine.[57] Although various types of PNGase have been identified in nature,[58-61] biological significance has only been linked to cytosolic PNGase, which plays an essential role in misfolded glycoprotein degradation.[57] Glycan specificity of cytosolic PNGase has been reported,[62] although the related peptide specificity has not been precisely understood. We therefore analysed the peptide specificity of PNGase towards chitobiose-pentapeptides using systematically controlled hydrophobic leucine and hydrophilic serine sequences as substrates.[63] Yeast cytosolic PNGase showed not only broad capacity for these substrates, but also preferential deglycosylation for chitobiose-pentapetides with peptide sequences that were more hydrophobic. Namely, PNGase would discriminate between both glycan-moieties and peptide sequences. Interestingly, subsequent kinetic analysis revealed that cytosolic PNGase may act not only as a K_m dependent enzyme for some substrates, but also as a V_{max} dependent enzyme for other substrates. The high catalytic range of yeast cytosolic PNGase may be one of the reasons for the broad capacity toward a variety of misfolded glycoproteins. This seems to be a kind of another bifunctionality. A similar glycoprotein quality control machinery has also been reported in yeast.[64] Therefore, similar bifunctionality could be conserved in mammalian cytosolic PNGase.

4. Future perspectives

In this review, we focused on the bifunctionality of N-glycoprotein biosynthesis. Several lectins and enzymes possess the ability to recognise not only glycans, but also aglycones (hydrophobicity and/or peptide sequence). In the early steps of N-glycoprotein biosynthesis in the ER, newly synthesised G2M9-polypeptides are recognised by malectin. Malectin likely possesses bifunctionality towards G2M9-polypeptides to prevent aggregation of nascent misfolded G2M9-polypeptides. After removal of the outermost glucoside linkage of G2M9-protein, the resulting G1M9-protein is recognised by CNX and its soluble homologue CRT. In this step, these chaperones simultaneously recognise glycan- and aglycone-moieties. The bifunctionality contributes to accelerated protein folding and delivery to glucosidase II for further glucose cleaving. The resulting M9-protein is then recognised by the enzyme UGGT, which possesses glucose transfer activity and folding sensor ability. The bifunctionality allows for the monitoring of folding states and the reglucosylation of misfolded M9-protein to provide G1M9-protein to maximise folding with CNX/CRT. After sensing the folding status of M9-protein, α-1,2-mannosidases in the ER trim the mannose-residues to produce glycosignals for secretion and degradation. During these steps, the bifunctionality of α-1,2-mannosidases might be useful for discriminating properly folded and mis-

folded glycoproteins. During downstream N-glycoprotein biosynthesis in the ER, misfolded glycoproteins must be properly transferred to degradation pathways. The proposed bifunctionality of OS-9 might assist in the delivery of misfolded glycoproteins to dislocon channels that retrotranslocate misfolded glycoproteins from the ER to the cytosol. Subsequently, retrotranslocated misfolded glycoproteins in the cytosol are successfully degraded upon deglycosylation. The adjustable catalytic properties of PNGase might be useful for accepting a wide variety of misfolded glycoproteins. Taken into account the aforementioned examples of bifunctionality during N-glycosylation biosynthesis, the ability of glycan-recognizing proteins might be essential glycoprotein folding, sensing, sorting, and degradation. Moreover, various folding diseases i.e. Alzheimer's diseases, osteoporosis, and type 2 diabetes are believed to stem from disruption of glycoprotein quality control systems.[65] Therefore, insight into glycoprotein bifunctionality may reveal new opportunities for understanding protein folding diseases.

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