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Influence of aglycone structures on *N*-glycan processing reactions in the endoplasmic reticulum

Kiichiro Totani [a, *], Kenta Yamaya [a], Makoto Hirano [a], Yukishige Ito [b]

[a] Department of Materials and Life Science, Seikei University, 3-3-1 Kichijoji-kitamachi, Musashino, Tokyo, 180-8633, Japan
[b] Synthetic Cellular Chemistry Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama, 351-0198, Japan

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ABSTRACT

Glycoprotein N-linked oligosaccharides in the endoplasmic reticulum function as tags to regulate glycoprotein folding, sorting, secretion and degradation. Since the *N*-glycan structure of a glycoprotein should reflect the folding state, *N*-glycan processing may be affected by the aglycone state. In this study, we examined the influence of aglycone structures on *N*-glycan processing using synthetic substrates. We prepared $(Glc_1)Man_9GlcNAc_2$ linked to hydrophobic BODIPY-dye with a systematic series of different linker lengths. With these compounds, glucose transfer, glucose trimming and mannose trimming reactions of an endoplasmic reticulum fraction were examined. The results showed that substrates with shorter linkers between the *N*-glycan and hydrophobic patch had higher activities for both the glucose transfer and the mannose trimming reactions. In contrast, the glucose trimming reaction showed lower activity when substrates had shorter linkers. Thus, the reactivity for N-linked oligosaccharide processing of glycoproteins in the endoplasmic reticulum might be tunable by the aglycone structure, e.g., protein portion of glycoproteins.

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1. Introduction

In general, glycosyl hydrolases and glycosyl transferases have more or less strict glycan specificity [1,2]. Thus, specificity analysis against various glycan substrates is often conducted to determine the target substrate of glycan processing enzymes. However, whether *N*-glycan processing reactions are affected by the aglycone structure has not been studied systematically, except in limited cases [3,4].

Most glycan processing enzymes act on N-linked oligosaccharides bound to aglycones such as proteins or lipids. For example, newly synthesized high-mannose type ($Glc_3Man_9GlcNAc_2$) glycoprotein in the endoplasmic reticulum (ER) is sequentially processed by various glycosyl hydrolases and glycosyl transferases to give diverse glycoforms through glucose trimming, glucose transfer and mannose trimming reactions [5]. Each glycoform plays important roles in regulating glycoprotein quality control [6] as a marker for diverge glycoproteins into separate pathways, such as folding, secretion and degradation (Fig. 1). $Glc_1Man_9GlcNAc_2$ (G1M9) oligosaccharide functions as a signal for folding acceleration, and is recognized by the membrane-bounded lectin-like molecular chaperone calnexin (CNX) and its soluble homologue calreticulin (CRT). Since $Man_9GlcNAc_2$ (M9) is a substrate for the ER folding sensor enzyme UDP-glucose:glycoprotein glucosyltransferase (UGGT), this N-linked oligosaccharide is recognized as a signal for scrutinizing the folding status. Moreover, mannose trimmed $Man_{8\text{-}5}GlcNAc_2$ (M8-5) are believed to be discharge signals involved in secretion and degradation. Therefore, glycoprotein N-linked oligosaccharides in the ER can be seen to reflect the protein's folding status. Namely, ER *N*-glycan processing reactions may be affected by their aglycone structures.

In our previous specificity analysis of UGGT, we showed that this enzyme has higher activity for an M9-derivative with a hydrophobic patch positioned closer to the reducing end [3]. This demonstrates that ER *N*-glycan processing reactions can be regulated by the aglycone structure. While this previous study showed that purified UGGT has aglycone specificity, it is unclear whether the aglycone structure influences the *N*-glycan processing reactions in the ER that are mediated by various *N*-glycan processing enzymes and lectins.

In this report, we examined the preference of ER glucose trimming, glucose transfer and mannose trimming reactions, which play major roles in glycoprotein quality control (Fig. 1), towards

* Corresponding author.
E-mail address: ktotani@st.seikei.ac.jp (K. Totani).

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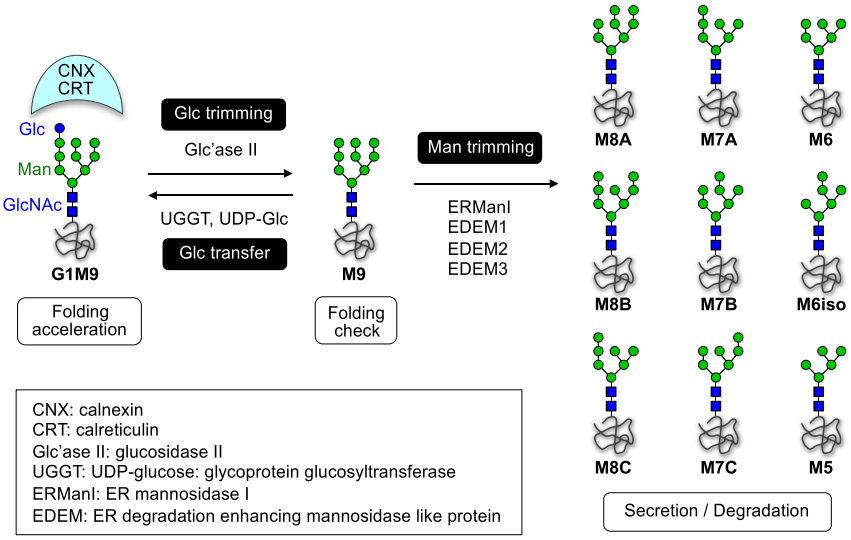

Fig. 1. High-mannose-type glycan processing in ER glycoprotein quality control.

synthetic N-linked oligosaccharide substrates that differed systematically in length and were connected to a hydrophobic patch. The glucose transfer and the mannose trimming reactions had higher activity towards substrates when the hydrophobic substituent resides closer to the reducing end, whereas the glucose trimming reaction preferentially acted on substrates with longer linkers. Since these results do not conflict with the roles of the generated glycoforms on glycoprotein quality control, reactivity tuning of *N*-glycan processing by the aglycone structure might contribute significantly to maintaining cellular homeostasis.

2. Results

2.1. Preparation of ER-enriched fraction and N-glycans for glycan processing analysis

To examine the ER *N*-glycan processing reactions *in vitro*, we initially prepared the enriched ER fraction as an enzymatic source that included whole glycosyl transferase, glycosyl hydrolases and lectins related to *N*-glycan processing. We have conducted reconstruction analysis of *N*-glycan processing using synthetic N-linked oligosaccharide substrates and an ER fraction centrifugally fractionated from rat/mouse livers [7,8]. In a similar manner, the ER fraction was prepared from C57BL/6 mouse livers. Each centrifugal fraction was analyzed by western blotting using anti-ER and *cis*-Golgi marker protein antibodies to provide an enriched ER fraction (Fig. 2).

N-Linked oligosaccharide substrates were designed so that a systematic change of the aglycone structures can be made. We reported previously total synthesis of a series of ER high-mannose oligosaccharides [9] and the related *N*-glycan probes [3,10,11]. Among the synthetic probes, we decided to choose M9-G-EG(n)-BODIPY [3] as mimics of molten globule-like glycoproteins, which were used in aglycone specificity analysis of UGGT (Fig. 3). These compounds consist of the M9-glycan and BODIPY-dye (a hydrophobic substituent [12–14]), which are connected by oligoethylene glycol as a hydrophilic linker. Various linker lengths facilitated systematic change of the position of the hydrophobic substituent. The actual distances between the reducing end of the M9-glycan and BODIPY-dye, which are connected by different ethylene glycol linker lengths, was confirmed by molecular dynamics calculations [3]. Although M9-G-EG(n)-BODIPY has a

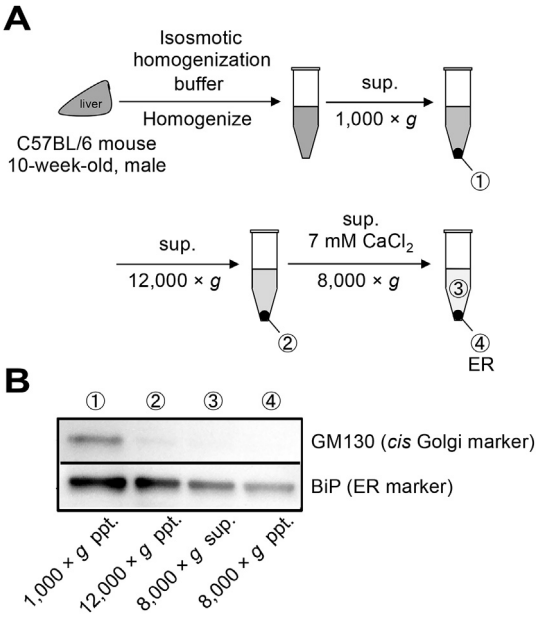

Fig. 2. Extraction of the rough ER fraction used in this study. (A) Schematic diagram of centrifugal fractionation of the ER fraction. Extraction buffer contained: 10 mM HEPES (pH 7.8), 250 mM sucrose, 1 mM EGTA, 25 mM KCl. (B) Western blotting of the centrifugal fraction using anti-BiP (ER-marker) and anti-GM130 (*cis*-Golgi-marker) antibodies.

compact conformation with a bent EG-linker, we found that enzyme activity changed was dependent on the linker length and we therefore concluded that enzymes recognize the changing of molecular shape and the topology of hydrophobicity around the reducing end of M9-oligosaccharide. Hence, we used previously synthesized M9-G-EG(n)-BODIPY (n = 0, 4, 8, 12) for aglycone specificity analysis of glucose transfer and mannose trimming reactions in the ER fraction. Moreover, we prepared the related G1M9-G-EG(n)-BODIPY by elongation of the ethylene glycol linker for G1M9-G[11] followed by BODIPY-labeling with a reported method [3]. With these G1M9-derivatives, we examined the glucose trimming reaction in the ER fraction.

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