Reconstructed glycan profile for evaluation of operating status of the endoplasmic reticulum glycoprotein quality control

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Glycoprotein oligosaccharides function as tags for protein quality control in the endoplasmic reticulum (ER). Since most of proteins are glycosylated and function only after they are properly folded, glycoprotein glycan profiles in the ER might be useful to analyze various cellular status including diseases. Here, we examined whether ER glycan-processing profiles in diabetic rats and osteoporotic mice as models might have different cellular status from those of normal controls. Direct analysis of glycoprotein-processing profiles in the ER is often hampered by glycoforms that are retro-translocated to the ER from other cellular compartments. Moreover, when we focus on the mixture of glycoproteins as the processing substrates, the glycoprocessing efficiencies are influenced by the aglycon states including their polypeptide folding. To overcome this problem, we reconstructed glycan profiles using ER extracts as an enzymatic source and synthetic glycoprotein mimetic having homogeneous aglycon as a substrate, resulted in disease-specific glycan profiles. To understand such differences, we also analyzed the activity, and expression level, of each glycan-related enzyme. These glycan profiles are expected to be useful indexes for operational status of the ER glycoprotein quality control, and may also give information to classify some diseases.

Keywords: diabetes / osteoporosis / protein quality control / synthetic glycoprobe

Introduction

Polypeptides function as mature proteins only after they are properly folded in the endoplasmic reticulum (ER). Protein folding in the ER takes place in several steps, including folding acceleration, folding check as well as transport of folded and terminally misfolded proteins to the Golgi apparatus (for processing and secretion) and cytosol (for degradation), respectively, in order to ensure protein quality control (Määttänä et al. 2010). When newly generated polypeptides are translocated from the ribosome to the ER, a majority of asparagine residues of the consensus tripeptide sequence (Asn-Xaa-Thr, where Xaa is any amino acid with the exception of proline) are modified with a high-D-mannose (Man)-type tetra-decasaccharide that consists of three glucose (Glc, α-glucose), nine Man, and two N-acetyl-D-glucosamine (GlcNAc) residues (Weerapan and Imperiali 2006). Combined action of various glycan-processing enzymes in the ER produces diverse glycoforms from these glycoprotein oligosaccharides. The glycoforms that emerges during processing of the Glc1 Man9GlcNAc2 (G1M9) protein to the Man8GlcNAc2 (M8) protein have been determined to correlate intimately with glycoprotein quality control (Aebi et al. 2010; Benyair et al. 2011) (Figure 1). For instance, G1M9 protein is recognized by membrane-bounded lectin-like molecular chaperone calnexin (CNX) and its soluble homolog calreticulin (CRT), allowing this glycoform to function as a folding acceleration signal (Paquet et al. 2005). Subsequent action of glucosidase II (GII) generates M9 protein, which is recognized by protein-folding sensor enzyme uridine diphosphate (UDP) Glc:glycoprotein glucosyltransferase (UGGT) for checking the folding status (D’Alessio et al. 2010). This enzyme specifically glucosylates misfolded M9 protein in a molten globule state to regenerate the G1M9 ligand for CNX/CRT. This step is considered as a sorting signal, discriminating properly folded, misfolded or an unfolded protein. Glycoproteins obtain proper folding by repeating the CNX/CRT cycle, which consists of deglucosylation and reglucosylation. Mannose-trimming enzymes mannosidase I (MI; Avezov et al. 2008), ER degradation enhancing α-mannosidase-like protein (EDEM)1 (Hosokawa et al. 2010b) and EDEM3 (Hirao et al. 2006) generate Man8GlcNAc2 (M8) with several structural isomers. Evidence pointing to a mannosidase activity of the EDEMs has recently been found in vitro in lower eukaryotes, in the case of an EDEM-like protein in Saccharomyces cerevisiae, where Htm1 acts in a complex with...
the oxidoreductase such as protein disulfide isomerase (Gauss et al. 2011). M8B has been suggested to function as a secretion signal through association with cargo receptor vesicular integral-membrane protein of 36 kDa-like protein (VIPL) and ER–Golgi intermediate compartment protein of 53 kDa (ERGIC53) (Yamamoto 2009). On the other hand, M8A, M8C and further trimmed glycans (M7, M6 and M5) are recognized by osteosacroma amplified 9 (OS9) and XTP3-transactivated gene B protein (XTP3B) that binds with retrotranslocation channel to enhance the unfolded protein degradation (Hosokawa et al. 2010a). The conversion step from M9 to M8 can be considered as a turning point of glycoprotein’s lifetime. Thus, analysis of ER glycoprotein glycan processing helps to estimate the status of glycoprotein quality control.

Since protein folding connects with its function, operational changes in the protein quality control should affect its cellular phenotype, including diseases. For instance, accumulation of misfolded proteins has been known to develop various diseases such as Alzheimer’s disease, Parkinson’s disease, diabetes and osteoporosis (Yoshida 2007; Kim et al. 2008; Murakami et al. 2009; Makareeva et al. 2011). Although there have been many reports concerning composition changes of Golgi-type (complex-type) glycans in cancer (Kondo et al. 2006; An et al. 2009; Tharmalingam et al. 2010), efforts to correlate between diseases and ER-type (high-mannose-type) glycans has been far less successful. Since glycoprotein quality control is constantly operating, careful examination of related glycan-processing profiles might detect signs of abnormal cellular status. However, the slight operational changes may be overlooked by several reasons as follows: (i) Direct analysis of ER glycoprotein oligosaccharides is hampered by glycoproteins that are retro-translocated from other cellular compartments (Hammond and Helenius 1994; Yamamoto et al. 2001; Hirayama et al. 2010), (ii) Some glycan-processing efficiencies in the ER are diverse for substrates having heterogeneous aglycon with various folding status as natural glycoproteins (Taylor et al. 2003; Totani et al. 2009). If a structurally defined synthetic glycan derivative is accepted as a substrate for ER glycan-processing enzymes, we can overcome these problems. In this study, we proposed a glycan-based technology to detect the operating status of the ER glycoprotein quality control, using test tube experiments in which synthetic oligosaccharide substrates are reacted with ER fractions extracted from organs. Based on this approach, we carried out evaluation of the glycoprotein quality control in model animals of type 2 diabetes and osteoporosis.

**Results and discussion**

**Direct glycan analysis of diabetic model rat**

Diabetes mellitus can be roughly classified into type 1 (insulin dependent) and type 2 (non-insulin dependent); type 2 is further divided into obese and non-obese types (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997). For this study, we employed Goto-Kakizaki (GK) rat (Toyota et al. 1981), an animal model of non-obese type 2 diabetes. The silent outbreak of this disease is easily overlooked by general medical examination. This is why we focused on this disease as a target for evaluation in this study. Since abnormal protein folding has been known to occur in an obese type 2 diabetes (Ozcan et al. 2004), such a difference on the glycoprotein quality control may also occur under non-obese-type diabetes in principle. Established procedure for the extraction of the ER fraction was a technical reason to choose the liver as a target organ. The most straightforward approach for obtaining glycoprotein glycan-processing profiles in the ER is glycoprotein glycan analysis of the ER fraction. We therefore carried out centrifugal extraction of an ER fraction.
from GK (diabetic model) and Wistar (WT) (healthy control) rat livers using an ER enrichment kit (IMGENEX Co.) (Composition and purity, see Supplementary data, Figure S1). We then purified glycoproteins from these fractions, followed by PNGase F-mediated glycan liberation (Maley et al. 1989) and pyridylamination (Hase and Ikenaka 1990) to provide fluorescent-labeled glycans. High-performance liquid chromatography (HPLC) analysis of the resulting glycan mixtures indicated no significant differences in their profiles between the diabetic model (GK) and the healthy model (WT) (Figure 2). If all of these glycoproteins were involved in the target glycan processing, then this result would have changed our working hypothesis. However, the entire spectrum of glycoproteins that are included in the ER fraction will contain undesired glycoproteins that had been translocated from the Golgi apparatus (Hirayama et al. 2010). In fact, there is some evidence in mammalian cells showing ER–Golgi recycling of misfolded ER associated degradation substrate proteins (Hammond and Helenius 1994; Yamamoto et al. 2001). Participation of Golgi α1,2-mannosidase in the mannos trimming by cycling of the enzyme and substrates between the ER and the Golgi has also been reported (Hosokawa et al. 2007). Thus, the reason as to why no differences were observed between the two glycan profiles shown in Figure 2 may derive from contaminants which masked the differences in the operational status of glycoprotein quality control. Moreover, glycan-processing enzymes in the ER may have diverse efficiency for each glycoprotein because of its own folding status. Especially, reaction efficiency of UGGT has been known to be highly dependent on the folding status of substrate glycoprotein (Taylor et al. 2003; Totani et al. 2009). If the expression balance of newly generated proteins or the folding status of each proteins is different between the disease model and the normal control, a slight operational change of glycoprotein quality control may be overlooked by direct glycan analysis.

**Development of reconstruction approach to obtain glycan profile**

To overcome the problems on the masking of disease-specific glycan profile, we developed a reconstruction approach to obtain ER glycan-processing profiles in test tubes. Thus, a structurally defined set of synthetic high-mannose-type glycoprobos was incubated with an extracted ER fraction that contains a series of enzymes associated with glycoprotein processing. Subsequent HPLC analysis of the reaction mixture allowed the reconstruction of the glycan profiles. Since the glycan profiles obtained using this reconstruction approach can exhibit only glycan-processing steps associated with glycoprotein quality control, the above-described uncertainty originates from contamination of glycoproteins from other cell compartments that can be avoided. Another advantage of using structurally defined synthetic glycoprobe is to be fixed its reaction efficiency for each glycan-processing enzymes. This methodology will also reconstruct a dynamic glycan profile, which shows changes in the profile over time, by controlling the incubation time. Therefore, this approach can provide information that will supplement the snapshot approach that is shown in Figure 2. To carry out the analysis, a set of synthetic substrates must be designed so that it can be widely recognized by ER lectins and by glycan-processing enzymes in order to simulate processing steps similar to those that occur in vivo. In contrast to most of the glycan-converting enzymes that bind glycan moieties, the folding sensor enzyme UGGT displays unique multi-site substrate recognition (Trombetta and Parodi 2005). This enzyme specifically recognizes the M9-glycoprotein, that is in a molten globule state, and glucosylates, the non-reducing end of the glycan to regenerate G1M9. On the other hand, correctly folded or extensively misfolded glycoproteins are poorly recognized as a substrate by UGGT even if they have M9 glycans. Although it has been believed for a long time that UGGT recognizes only glycoproteins in a molten globule state, we reported the first non-peptidic synthetic substrate for this enzyme in 2005 (Totani et al. 2005). Moreover, we have also reported that synthetic M9-boron-dipyromethene (BODIPY) (Figure 3A), which has a fluorescent group whose distance from the reducing end of the glycan can be finely tuned by alteration in the linker size, is a highly reactive second-generation substrate, suitable for sensitive assay of UGGT (Totani et al. 2009). In this substrate, BODIPY functions both as a fluorescent tag and as a mimic of hydrophobic patch exposed in misfolded protein. Since an analytical database of various pyridylaminated glycans has been reported (Tomiya et al. 1988; Takahashi et al. 1995), pyridylamination is the most widely used oligosaccharide fluorescence-labeling method (Hase and Ikenaka 1990). We therefore synthesized G1M9-pyridylamino (PA) (Figure 3A) from the previously reported G1M9 (Matsuo et al. 2006), and investigated the substrate efficiency of BODIPY-labeled glycan and PA-labeled glycan for glycan profile reconstruction (Figure 3B).

![Fig. 2. Glycoprotein oligosaccharide analysis of ER fractions from WT and GK rat liver (10 weeks old) using HPLC. Analysis conditions: TSK-gel Amide-80 5 μm column (4.6 mm Φ × 25 cm), mobile phase CH3CN/3% acetic acid (AcOH)-Et3N (pH 7.3), linear gradient from 80:20 to 40:60 over 50 min, flow rate 1.0 mL min −1 at 40°C.](image-url)
Our study focused on the CNX/CRT cycle (D’Alessio et al. 2010), the central mechanism of glycoprotein quality control, which involves interconversion of G1M9 and M9 glycoforms. Glycan transformation steps in this cycle were reconstituted with both of the labeled glycans, using enzymatic activities of GII and UGGT that are contained from the ER fraction. First, Glc trimming of G1M9-PA and G1M9-BODIPY by the ER fraction from WT rat liver in the presence of the mannosidase inhibitor deoxymannojirimycin was compared after 30 min incubation, and the resulting HPLC profiles showed that both reactions proceeded with the same efficiency. Secondly, the efficiency of the Glc transfer was examined by addition of UDP-Glc and the glucosidase inhibitor deoxynojirimycin to the reaction mixtures. The reaction profiles after 20 h revealed that the UGGT product G1M9 can only be regenerated from the BODIPY-labeled glycan. Because UGGT has been suggested to use the chitobiose structure at the reducing end for its substrate recognition (Totani et al. 2005), the ring-opened structure of the innermost GlcNAc associated with PA attachment might prevent glucosylation. This result indicates that a specifically designed glycan, rather than a simple fluorescent-labeling glycan, is necessary for glycan profile reconstruction. We therefore further investigated glycan profile reconstruction utilizing our original BODIPY-labeled glycans.

Reconstructed glycan analysis of diabetic model rat
We focused on glycan processing that is associated with glycoprotein quality control as shown in Figure 1. Reconstructed glycan profiles were obtained from WT (control) and GK (diabetes) rat livers using HPLC after each liver extract was incubated with G1M9-BODIPY for 8 h (Figure 4A). The resulting glycan profiles that only reflect ER glycan processing were different between WT and GK, indicating that no significant changes between WT and GK on the direct glycan profiles shown in Figure 2 would include several uncertainties for reflecting the operational state of glycoprotein quality control, just as we suspected. Interestingly, the HPLC chromatograms by the reconstruction approach of the GK rat indicated significantly increased production rates of M8, M7 and M6 compared with those obtained from the WT rat. Although G1M9 and M9 are considered to function as retention signals in the ER.

Fig. 3. Reactivity of fluorescent-labeled glycans to CNX/CRT cycle-related glycan-processing enzymes. (A) Structures of fluorescent-labeled synthetic glycans. (B) HPLC profiles of de-glucosylation and re-glucosylation of G1M9-PA and G1M9-BODIPY. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 1 μM substrate, 30 μg ER fraction (WT, 10 weeks old), 5 mM deoxymannojirimycin, 5 mM deoxynojirimycin (after 30 min), 5 mM UDP-Glc (after 30 min), 10 mM CaCl2, 0.6% Tween20, 10 mM HEPES (pH 7.4), 37°C. Analysis conditions: TSK-gel Amide-80 5 μm column (4.6 mm Ø × 25 cm), mobile phase CH3CN/H2O, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL min⁻¹ at 40°C.
because of their association with the CNX/CRT cycle, participation of M8, M7, M6 and M5 in secretion or degradation pathways makes it possible to consider them as discharge signals from the ER. Redrawing of the glycan ratios shown in Figure 4A as graphs that present retention and discharge signals from the ER. Thus, it was found that glycan profile reconstruction could detect a slight operational change in glycoprotein quality control, which does not appear as the difference in glycan composition obtained by direct glycan analysis.

We next evaluated the activity of each glycan-processing enzyme that controls glycan profile alterations. The activity of both GII, which mediates processing of G1M9 to M9 (Figure 4C), and of UGGT, which mediates regeneration of G1M9 from M9 (Figure 4D), was found to decrease in the diabetic model rat (GK) compared with the healthy rat (WT). In contrast, the GK rat showed a higher value for combined mannose trimming activity (M9 to M8, M7, M6 and M5) than the WT rat (Figure 4E). These results seem to corroborate with the fact that the ratio of M8 to M5 was increased in type 2 diabetes as shown in Figure 4A. Moreover, incubation of a 1:1 mixture of G1M9-BODIPY and M9-BODIPY with UDP-Glc and the ER fraction showed that the rate of M9 generation by the ER fraction of GK rat is much higher than that of the WT rat (Figure 4F). These results seem to corroborate with the measured difference in activity of this enzyme between the WT and the GK rat (Figure 4G). We also analyzed the expression levels of these glycan-processing enzymes using western blot analysis (Figure 4G). The expression level of GII in the GK rat liver was found to be lower than that in the WT rat liver. This result is consistent with the measured difference in activity of this enzyme between the WT and the GK rat (Figure 4C). On the other hand, the expression level of UGGT was similar in both WT and GK rats, even though the activity of this enzyme decreased in the GK rat (Figure 4D). This result is likely to be caused by insufficient folding of UGGT. Thus, our activity-based approach can reveal the practical metabolic pathway. MI

Fig. 4. Analysis of non-obese type 2 diabetes using reconstructed glycan profiles. (A) HPLC chromatograms of reconstructed ER glycan processing starting from G1M9-BODIPY for WT (control) and GK (diabetes). Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 1 μM G1M9-BODIPY, 30 μg ER fraction (male, 10 weeks old), 5 mM UDP-Glc, 10 mM CaCl2, 0.6% Tween20 and 10 mM HEPES (pH 7.4) and were incubated at 37°C, for 8 h. (B) ER retention and discharge signals as a percent of total glycan. Open bars: retention signals (G1M9 and M9), closed bars: discharge signals (M8, M7, M6 and M5). (C) Time course of GII activity. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 1 μM G1M9-BODIPY, 30 μg ER fraction (10 weeks old), 5 mM deoxymannojirimycin, 10 mM CaCl2, 0.6% Tween20 and 10 mM HEPES (pH 7.4) at 37 °C. (D) Time course of UGGT activity. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 1 μM G1M9-BODIPY, 30 μg ER fraction (10 weeks old), 5 mM deoxymannojirimycin, 5 mM deoxynojirimycin, 10 mM CaCl2, 0.6% Tween20 and 10 mM HEPES (pH 7.4) at 37 °C. (E) Time course of mannose trimming activities containing MI, EDEM1 and EDEM3. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 1 μM M9-BODIPY, 30 μg ER fraction (10 weeks old), 10 mM CaCl2, 0.6% Tween 20 and 10 mM HEPES (pH 7.4) at 37 °C. (F) M9/G1M9 of the CNX/CRT cycle. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 0.5 μM G1M9-BODIPY, 0.5 μM M9-BODIPY 30 μg ER fraction (10 weeks old), 5 mM UDP-Glc, 5 mM deoxynojirimycin, 10 mM CaCl2, 0.6% Tween20 and 10 mM HEPES (pH 7.4), and were incubated at 37°C for 8 h. Open bar: M9, closed bar: G1M9. (G) Western blot analysis of cell extracts from WT and GK rat livers (10 weeks old) was performed using anti-ER related enzymes/lectins (GII® subunit, UGGT, MI, EDEM1) and anti-β-Actin (house-keeping protein) antibodies. Analysis conditions for (A), (C), (D) and (E): TSK-gel Amide-80 5 μm column (4.6 mm Ø × 25 cm), mobile phase CH3CN/H2O, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL min⁻¹ at 40°C. Each data point represents the mean values of three experiments for three individuals with standard deviation.
and EDEM1 will mediate most of the Man trimming that take place in the ER. Therefore, enhancement of the Man-trimming activity in GK (Figure 4E) would be caused by an increase in the expression of these enzymes. Indeed, the expression level of MI in GK was much higher than that in WT, whereas the expression level of EDEM1 was similar between them. Since MI generates M8 glycan that is recognized by ERGIC53 (Figure 1) to transport to the Golgi apparatus, the increase in the amount of M8 generated in the diabetic model indicates that the enhancement of discharge signal in GK might be caused by up-regulation of the secretion signal.

Reconstructed glycan analysis of osteoporotic model mouse
To make clear generality of our approach, we have analyzed a model mouse of aging-related diseases, so-called senescence-accelerated mouse (SAM) (Takeda 1999). Here, we carried out disease evaluation of SAMP6 (Takahashi et al. 1994) as a model of osteoporosis, SAMP10 (Shimada et al. 1992) as a model of dementia and SAMR1 as a normal aging model by using the glycan profile reconstruction. Since the relationship between osteoporosis and abnormal protein biosynthesis has been pointed out (Murakami et al. 2009; Makareeva et al. 2011), SAMP6 was selected as a model of protein disorder. On the other hand, SAMP10 develops dementia via oxidative damage (Sasaki et al. 2008), and we selected this as a disease model with normal protein biosynthesis. Because of the reasons described above, we selected a liver as a target organ to obtain ER-enriched microsome by centrifugal fractionation (Berthet and De Duve 1951). Moreover, the fact that osteoporosis has been known to develop hepatopathy (Guanabens and Pares 2010) is also a reason to choose a liver.

Fig. 5. Analysis of aging-related diseases using reconstructed glycan profiles. (A) HPLC chromatograms of reconstructed ER glycan processing starting from G1M9-BODIPY for SAMR1 (control; R1), SAMP6 (osteoporosis; P6) and SAMP10 (dementia; P10). Assay conditions: The reaction mixture contained, in a total volume of 10 μL, 2.5 μM G1M9-BODIPY, 10 μg microsome fraction (10 weeks old), 2 mM UDP-Glc, 10 mM CaCl2, 0.6% Triton X100, 10 mM HEPES (pH 7.4), 37°C, 24 h. (B) Signal ratios for the M8 isomers. Closed bars: degradation signals (M8A and M8C), open bars: secretion signal (M8B). (C) Time course of GII activity. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 2.5 μM G1M9-BODIPY, 12 μg microsome fraction (10 weeks old), 4 mM deoxymannojirimycin, 10 mM CaCl2, 0.6% Triton X100, 10 mM HEPES (pH 7.4), 37°C. (D) Time course of UGGT activity. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 5 μM G1M9-BODIPY, 12 μg microsome fraction (10 weeks old), 4 mM UDP-Glc, 4 mM deoxynojirimycin, 4 mM deoxymannojirimycin, 10 mM CaCl2, 0.6% Triton X100, 10 mM HEPES (pH 7.4), 37°C. (E) Time course of mannose trimming activities containing MI, EDEM1 and EDEM3. Assay conditions: The reaction mixture contained, in a total volume of 30 μL, 2.5 μM M9-BODIPY, 90 μg microsome fraction (10 weeks old), 10 mM CaCl2, 0.6% Triton X100, 10 mM HEPES (pH 7.4), 37°C. (F) Western blot analysis of cell extracts from SAMR1, SAMP6 and SAMP10 liver (10 weeks old) were performed using anti-ER-related enzymes/lectins (GII, UGGT, MI, EDEM1, EDEM3, OS9, ERGIC53) and anti-β-actin (house-keeping protein) antibodies. Analysis conditions for (A), (C), (D) and (E): TSK-gel Amide-80 3 μm column (4.6 mm Φ × 15 cm), mobile phase CH3CN/H2O, linear gradient from 65:35 to 50:50 in 50 min, flow rate 1.0 mL min \(^{-1}\) at 40°C. Each data point represents the mean values of three experiments for three individuals with standard deviation.
Glycan-processing profiles from G1M9 to M5 were first reconstructed in a test tube by incubation of G1M9–BODIPY with the ER-enriched microsome fraction (Composition and purity, see Supplementary data, Figure S1) of SAMR1 (control), SAMP6 (osteoporosis) and SAMP10 (dementia). After incubation for 24 h, HPLC analysis of the processing products provided reconstructed glycan profiles of SAMR1, SAMP6 and SAMP10, each of which showed no significant difference in a rate of glycan processing (Figure 5A). However, when we focused on the generations of M8 isomers that can be separated by high-performance column (Tosoh TSK-gel Amide-80 3 μm) (Attribution of the HPLC peaks, see Supplementary data), the ratio of structural isomers was found to be different in SAMP6. Both M8A and M8C were major M8 isomers in osteoporotic mouse, whereas the major isomer in control and dementia mouse was M8B. Since MI and EDEM1 preferentially trim the B-arm and the C-arm, respectively (Hosokawa et al. 2010b), these findings suggest that our reaction system can reflect not only MI activity but also EDEM1 activity. M8A and M8C are degradation signals of glycoprotein in contrast to that M8B functions as a glycoprotein secretion signal (Figure 1). Redrawing the ratios of M8 isomers in Figure 5A to a graph as shown in Figure 5B makes it clear that the degradation signal in osteoporotic mouse was significantly increased. Taking into account that generation ratios of M8 isomers are important turning point for the decision of glycoprotein destiny, the composition change in M8 isomers in SAMP6 is interesting and may be used as a fingerprint of osteoporosis.

We next analyzed individual activity of glycan-processing enzymes to understand the change in the glycan profile among SAMR1, SAMP6 and SAMP10. Both glucose trimming activity of GII (Figure 5C) and glucose transfer activity of UGGT (Figure 5D) significantly decreased in SAMP6, whereas SAMP10 showed the same levels of activities to the control. Since expression levels of GII and UGGT, which affects interconversion of G1M9 and M9, were constant among each SAM mice (Figure 5F), specific activities of GII and UGGT in SAMP6 would decrease with changes in the folding status of these enzymes. Thus, abnormal glycan processing change of ER in osteoporotic mouse was first determined by our activity-based approach. On the other hand, mannose trimming activities mediated by MI, EDEM1 and EDEM3 were increased in SAMP6 and SAMP10 compared with SAMR1 (Figure 5E), although expression levels of these enzymes did not show significant changes to the control in the same way (Figure 5F). These results suggest that each activity of glucose trimming, glucose transfer and mannose trimming in osteoporotic mouse was changed, resulted in disease-specific glycan profiles.

We also compared the glycan trimming activities of the microsome fraction extracted from livers of SAMR1, SAMP6 and SAMP10 for each branch of high-mannose glycan (A-arm, B-arm and C-arm). When we incubated G1M9–BODIPY with the microsomes in the presence of glucosidase inhibitor, the resulting relative C-arm trimming activity to the B-arm trimming activity revealed that the C-arm trimming was accelerated in SAMP6 (Figure 6A). Moreover, comparison of A-arm trimming activities of the microsome fractions using G1M7-BODIPY (Synthesis of G1M7-BODIPY, see Supplementary data) as a substrate showed that the A-arm trimming activity of SAMP6 was lower than that of the control (Figure 6B). Namely, enhancement of the HPLC peak of M8A, C (tR = 13.5 min) in SAMP6 shown in Figure 5A seems to be derived from increase of M8C production. Since degradation-enhancing protein OS9 recognizes M8C glycoprotein (Alcock and Swanton 2009), the glycoform plays an important role for glycoprotein degradation. Therefore, the connected up-regulations of M8C glycan (Figure 5A) and OS9 (Figure 5F) in SAMP6 were reasonably understood. Decrease in the secretion signal M8B in SAMP6 (Figure 5A) was also connected with down-regulation of the cargo receptor ERGIC53 (Figure 5F), which can associate with M8B–glycoprotein to transport to the Golgi apparatus.

**Conclusion**

We have successfully developed the first quantitative procedure for estimation of the status of the ER glycoprotein quality control using a reconstructed glycan profile that can
detect an activity-based operational change in the protein-folding factory. Our approach has first provided direct evidence of correlation between operational balance of ER glycoprotein quality control and some diseases. Using this procedure, we have found that model animals of non-obese type 2 diabetes and osteoporosis, both concerning with disorder of protein biosynthesis and their folding, showed the disease-specific reconstructed glycan profiles that will directly connect with the operating status of glycoprotein quality control in the ER. Based on features of the resulting glycan profiles, precise analysis revealed the functional shift in glycoprotein quality control as follows: (i) Glycoprotein quality control in a model rat liver of non-obese type 2 diabetes was found to be regulated in such a way that it actively discharges accumulated glycoproteins from the ER to Golgi and cytosol, (ii) the quality control in a model mouse liver of osteoporosis was found to be regulated by increasing the production of degradation signal M8C and the corresponding receptor OS9 to reduce the accumulated glycoprotein. Although these findings are based on artificial glycoprobe, the differences in the reconstructed glycan profiles are expected to be useful as novel types of disease marker, and also may give a speculation to understand the abnormal operation of their glycoprotein quality control. Since glycan functions are diverse and complex, the volume of glycomic information will exceed that obtained using other “omics” analyses (Turnbull and Field 2007). We consider that the use of chemical probes for glyobiology studies (Timmer et al. 2007; Takeda et al. 2009; Kiessling and Splain 2010) will provide conclusive data that will facilitate decoding of the molecular basis of complex glycan functions. To this end, the proposed reconstruction approach of glycan profile can be widely applied to various cells and organs including other diseases related to protein biosynthesis. Further studies are in progress along this line and will be reported in due course.

Materials and methods

General
Reagents and solvents were purchased from standard suppliers and used without further purification. Anti-ERGIC53 (sc-66880), anti-MI (sc-104975) and anti-EDEM1 (sc-27391) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Alpha glucosidase II (ab69489), anti-BiP (ab21685) and anti-OS9 (ab19853) were from Abcam plc (Cambridge, UK). Anti-β-actin (#4968) was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Anti-UGGT (VAP-PT068) was from Stressgen-Gentaur Bvba (Kampenhout, Belgium). Anti-GII (β-subunit) was obtained from Novus Biologicals, LLC (Littleton, CO). Anti-GM130 (610822) was from BD Biosciences (San Jose, CA). Wistar rat, GK rat, SAMR1, SAMP6, SAMP10 livers were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). G1M9-BODIPY (Figure 3A) was synthesized based on a previously reported convergent approach (Totani et al. 2006). HPLC was performed on a JASCO LC-2000 with a TSK-GEL Amide-80 column (Tosoh). All experiments were performed at least three times.

Extraction of ER and microsome fractions
Rough ER pellets were extracted from Wistar or GK rat (male) livers (0.5 g) by using an Endoplasmic Reticulum Enrichment Kit (IMGENEX Co., San Diego, CA) essentially according to the manufacturer’s instructions. The rat liver (0.5 g) were pre-cut using operating scissors and suspended in a 1 × isosmotic homogenization buffer (2 mL) containing 100 × protease inhibitor cocktail (20 μL). The suspension was crushed in a motor-driven tight-fitting glass/Teflon Potter Elvehjem homogenizer (20 strokes, 4°C). The resulting smooth suspension was centrifuged (1000 × g) at 4°C for 10 min. The floating lipid layer was carefully removed. The recovered supernatant was then centrifuged (12,000 × g) at 4°C for 15 min. The recovered supernatant was transferred to a beaker. 1 × CaCl2 solution (15 times the volume of the supernatant) was added drop by drop for precipitation while the supernatant stirred at 4°C for 15 min. The recovered supernatant was centrifuged (8000 × g) at 4°C for 10 min. The resulting ER pellet (42 mg) was dissolved in solubilization buffer (427 μL) (0.25 M sucrose, 2 mM EDTA, 5 mM 2-mercaptoethanol, Protease inhibitor cocktail 1 tab./50 mL (Roche), 0.5% Tween 20, 10 mM HEPES, pH 7.4). After 2 h of being flipped upside down at 4°C, the solution was recovered as the ER fraction.

SAMR1, SAMP6 or SAMP10 mouse (male) livers (0.5 g) were pre-cut using operating scissors and suspended in a buffer (5 mL) containing 0.25 M sucrose, 2 mM EDTA, 10 mM HEPES and 5 mM 2-mercaptoethanol, and Protease inhibitor cocktail 1 tab./50 mL (Roche) (pH 7.4). The suspension was crushed in a blender (10 s × 3 times) and a motor-driven tight fitting glass/Teflon Potter Elvehjem homogenizer (3 strokes, 4°C). The resulting smooth suspension was centrifuged (900 × g) at 4°C for 10 min. The recovered supernatant was then centrifuged (5000 × g) at 4°C for 10 min. The recovered supernatant was then centrifuged (8000 × g) at 4°C for 10 min, before being recovered again and centrifuged (20,000 × g) at 4°C for 120 min. Finally, the pellet containing microsome was collected by removing the supernatant. The pellet was dissolved with solubilization buffer (400 μL) (0.25 M sucrose, 2 mM EDTA, 5 mM 2-mercaptoethanol, Protease inhibitor cocktail 1 tab./50 mL (Roche), 0.5% Tween 20, 10 mM HEPES, pH 7.4). After 2 h of being flipped upside down at 4°C, the solution was recovered as the microsome fraction.

Protein concentration of the solubilized ER or microsome fractions was determined using the bicinchoninic acid (BCA) protein assay. Composition of the each centrifugal fraction was analyzed by western blotting using anti-ER and anti-Golgi maker proteins (Supplementary data, Figure S1).

Synthesis of G1M9-PA
Synthetic G1M9 (Matsuo et al. 2006) (1.2 mg) were dissolved in coupling reagent (20 μL) containing 2-aminopyridine (55.2 mg) and AcOH (20 μL). After incubation at 90°C for 1 h, reducing reagent (75 μL) containing borane–dimethyloxide complex (115 μg) and AcOH (46 μL) and water (29 μL) was added. After incubation at 80°C for 35 min, the solution was mixed with 2.8 M ammonia aqueous solution (300 μL). After vortexing, the aqueous layer was twice washed with chloroform (400 μL). The aqueous layer was lyophilized to give
PA-labeled G1M9 (51%). The PA glycans were analyzed by HPLC (Tosoh TSK-gel Amide-80 5 μm column (4.6 mm (Ø) × 25 cm), mobile phase CH₃CN/3%AcOH–Et₃N (pH 7.3), linear gradient from 80:20 to 40:60 over 50 min, flow rate 1.0 mL min⁻¹ at 40°C, detection: excitation, 310 nm, emission, 380 nm).

Glycoprotein oligosaccharide analysis of rat liver
The rat liver ER fraction (1.1 mg) was suspended in 400 μL buffer A (0.25 M sucrose, 2 mM EDTA, 5 mM 2-mercaptoethanol, Protease inhibitor cocktail 1 tab./50 mL (Roche), 10 mM HEPES (pH 7.4)). Solution was sonicated by ultrasonic disintegrator (10 s × 3) and boiled at 100°C for 5 min. The solution was mixed with ethanol (EtOH) (600 μL) and centrifuged (14,600 × g) at 4°C for 20 min. The precipitate was suspended in a mixture of buffer A (400 μL) and EtOH (600 μL). The mixture was centrifuged (14,600 × g) at 4°C for 20 min. The precipitate was suspended in water (1 mL) and lyophilized. The lyophilized powder was dissolved in SDS buffer (0.1% SDS, 50 mM 2-mercaptoethanol, 50 mM phosphate buffer, pH 7.5) and boiled at 100°C for 5 min. 15% TritonX-100 (25 mL) and PNGaseF (769 μg, Sigma, G5166) was added. After incubation at 37°C for 72 h, the resulting free glycans were purified using LudgerClean™ E10 cartridge (Cosmo-bio), followed by lyophilization to give free glycans. The lyophilized glycans were dissolved in coupling reagent (40 μL) containing 2-aminopyridine (110.4 mg) and AcOH (40 μL). After incubation at 90°C for 1 h, reducing reagent (140 μL) containing borane–dimethylamine complex (215 μg) and AcOH (86 μL) and water (54 μL) was added. After incubation at 80°C for 35 min, the solution was mixed with 2.8 M ammonia aqueous solution (400 μL). After vortexing, the aqueous layer was twice washed with chloroform (400 μL). The aqueous layer was lyophilized to give PA-labeled glycans. The PA glycans were analyzed by HPLC (Tosoh TSK-gel Amide-80 5 μm column (4.6 mm (Ø) × 25 cm), mobile phase CH₃CN/3%AcOH–Et₃N (pH 7.3), linear gradient from 80:20 to 40:60 over 50 min, flow rate 1.0 mL min⁻¹ at 40°C, detection: excitation, 310 nm, emission, 380 nm).

Reconstruction of glycan-profiles (typical procedure for Figure 4A)
Reaction mixtures contained, in a total volume of 30 μL, 1 μM G1M9-BODIPY, 30 μg ER fraction, 0.6% Tween 20, 10 mM CaCl₂, 10 mM UDP–glucose, and 10 mM HEPES (pH 7.4). After 1–8 h at 37°C, 2.5 μL of the reaction mixture was removed by pipette and heated at 100°C for 1 min to stop the enzymatic reaction. The reconstructed glycan profile in each reaction was analyzed by HPLC (Tosoh TSK-gel Amide-80 5 μm column (4.6 mm (Ø) × 25 cm), mobile phase CH₃CN/H₂O, linear gradient from 65:35 to 50:50 over 50 min, flow rate 1.0 mL min⁻¹ at 40°C, detection: excitation, 504 nm, emission, 514 nm).

Analysis of glycan-processing activity (typical procedure for Figure 4C–F)
Reaction mixtures contained, in a total volume of 30 μL, 1 μM G1M9-BODIPY (for Figure 4C) or M9-BODIPY (for Figure 4D and E), 0.5 μM G1M9-BODIPY and 0.5 μM M9-BODIPY (for Figure 4F), 30 μg of ER fraction, 0.6% Tween 20, 10 mM CaCl₂, 5 mM deoxymannojirimycin (for Figure 4C, D and F), 5 mM deoxynojirimycin (for Figure 4D), 5 mM UDP–glucose (for Figure 4D) and 10 mM HEPES (pH 7.4). After 10 min to 8 h at 37°C, 2.5 μL of the reaction mixture was removed by pipette and heated at 100°C for 1 min to stop the enzymatic reaction. The reconstructed glycan profile in each reaction was analyzed by HPLC (conditions: refer to the preceding clause, detection: excitation, 504 nm, emission, 514 nm).

Western blot analysis
Livers (0.5 g) of model animals were pre-cut using operating scissors and suspended in the Isosmotic homogenization buffer (see manufacturer’s instruction of ER enrichment kit, IMGENEX Co.) (2 mL) containing Protease inhibitor cocktail 1 tab./50 mL (Roche), 1 mM Na₃VO₄, 10 mM NaF, 0.5% Triton-X100. The suspension was sonicated using ultrasonic disintegrator (10 s × 3 times). The resulting smooth suspension was centrifuged (10,000 × g) at 4°C for 10 min. Protein concentration of the supernatant was measured by BCA protein assay kit (Pierce) and was diluted with water to 4, 2 and 1 mg/mL, respectively. The each protein solution (20 μL) was added to 20 μL of soybean deoxymethyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1% SDS, 1% 2-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue, 50 mM Tris–HCl (pH 6.8)) and boiled at 100°C for 10 min. The treated protein samples were analyzed by SDS–PAGE, followed by transferring to polyvinylidene fluoride membrane using wet blotting apparatus (Bio-Rad, Hercules, CA). The transferred membrane was dunked in blocking buffer (10 mL) [3% Top Block/Sigma in Tris-buffered saline with Tween-20 (TBS-T)] with gently shaking for 1 h. After removal of the blocking buffer, the membrane was washed by water (20 mL × 2). The washed membrane was incubated with primary antibody solution with gently shaking for 12 h. After washing of the membrane by TBS-T (20 mL × 3), the washed membrane was incubated with secondary antibody solution (Anti-Rabbit IgG (Goat), HRP-labeled, Perkin Elmer, Waltham, MA) with gently shaking for 1 h. Finally, the membrane was washed by TBS-T (20 mL × 3) and water (20 mL × 2), followed by infiltration of chemiluminescent reagent (Western lightning ultra, Perkin Elmer) to the membrane to give detectable band of the target protein.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest
None declared.

Abbreviations
AcOH, acetic acid; BCA, bicinchoninic acid; BODIPY, boron-dipyrromethene; CNX, calnexin; CRT, calreticulin; n-Man, mannose; EDEM, ER degradation enhancing α-mannosidase-like protein; ER, endoplasmic reticulum; ERGIC53, ER–Golgi intermediate compartment protein of 53 kDa; Glc, α-glucose; GII, glucosidase II; GK, Goto-Kakizaki; GlcNAc, N-acetyl-α-glucosamine; HPLC, high-performance liquid chromatography; MI, mannosidase I; OS9, osteosacroma amplified 9; PA, pyridylamino; PNGase F, peptide N-glycosidase F; SAM, senescence-accelerated mouse; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline with Tween-20; UDP, uridine diphosphate; UGGT, UDP-Glc:glycoprotein glucosyltransferase; VIPL, vesicular integral-membrane protein of 36 kDa-like protein; WT, Wistar; XTP3B, XTP3-transactivated gene B phosphate; UGGT, UDP-Glc:glycoprotein glucosyltransferase; dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, SAM, senescence-accelerated mouse; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline with Tween-20; UDP, uridine diphosphate; UGGT, UDP-Glc:glycoprotein glucosyltransferase; VIPL, vesicular integral-membrane protein of 36 kDa-like protein; WT, Wistar; XTP3B, XTP3-transactivated gene B protein.

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