Glycobiology, 2022, vol. 32, no. 2, 110–122
https://doi.org/10.1093/glycob/cwab115
Advance Access Publication Date: 10 November 2021
Original Article

OXFORD

Glycan Metabolism

A method for assaying peptide: *N*-glycanase/*N*-glycanase 1 activities in crude extracts using an *N*-glycosylated cyclopeptide

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Received 6 September 2021; Revised 9 October 2021; Accepted 31 October 2021

Abstract

Cytosolic peptide: *N*-glycanase (PNGase; NGLY1), an enzyme responsible for de-glycosylation of *N*-glycans on glycoproteins, is known to play pivotal roles in a variety of biological processes. In 2012, NGLY1 deficiency, a rare genetic disorder, was reported and since then, more than 100 patients have now been identified worldwide. Patients with this disease exhibit several common symptoms that are caused by the dysfunction of NGLY1. However, correlation between the severity of patient symptoms and the extent of the reduction in NGLY1 activity in these patients remains to be clarified, mainly due to the absence of a facile quantitative assay system for this enzyme, especially in a crude extract as an enzyme source. In this study, a quantitative, non-radioisotope (RI)-based assay method for measuring recombinant NGLY1 activity was established using a BODIPY-labeled asialoglycopeptide (BODIPY-ASGP) derived from hen eggs. With this assay, the activities of 27 recombinant NGLY1 mutants that are associated with the deficiency were examined. It was found that the activities of three (R469X, R458fs and H494fs) out of the 27 recombinant mutant proteins were 30–70% of the activities of wild-type NGLY1. We further developed a method for measuring endogenous NGLY1 activity in crude extracts derived from cultured cells, patients' fibroblasts, iPS cells or peripheral blood mononuclear cells (PBMCs), using a glycosylated cyclopeptide (GCP) that exhibited resistance to the endogenous proteases in the extract. Our methods will not only provide new insights into the molecular mechanism responsible for this disease but also promises to be applicable for its diagnosis.

Key words: cyclopeptide, deglycosylation, NGLY1, NGLY1-defeciency, peptide: *N*-glycanase

Introduction

N-Glycosylation is one of the major co- and post- translational modifications of proteins. *N*-Glycans on proteins not only enhance the stability of the accepter proteins but also play pivotal roles in a variety of biological processes such as protein quality control, cell development, cell differentiation, cell–cell interactions, signal transduction and cancer metastasis (Varki 1993, 2017). In the area of protein quality control, it is well known that *N*-glycans on nascent proteins in the endoplasmic reticulum (ER) are recognized as a tag that dictates the folding status of carrier proteins. In the subsequent recognition of the misfolded proteins by the ER quality control system such as molecular chaperones (BiP) and ER-resident lectins (OS9 and XTP3), misfolded glycoproteins are retro-translocated from the ER into the cytosol for proteasomal degradation. These disposal

systems for misfolded proteins in the ER are generally referred to as ER-associated degradation (ERAD) (Vembar and Brodsky 2008; Xu and Ng 2015). It is also widely accepted that misfolded glycoproteins are deglycosylated by a cytosolic peptide: *N*-glycanase (PNGase, NGLY1 in humans) during their proteasome-dependent degradation, and the removal of such "bulky" glycans on misfolded proteins, at least in some cases, facilitates efficient degradation of misfolded glycoproteins in the proteasome (Suzuki et al. 2002a, 2016).

NGLY1, a widely conserved enzyme throughout eukaryotes, catalyzes the deamidation on the side chain of *N*-glycosylated asparagines and can therefore be defined as an asparagine amidase (E.C., 3.5.1.52) (Suzuki et al. 2002a). In this reaction, *N*-glycans on the consensus sequence (NxT/S, x; any amino acid except proline) of the substrate proteins are removed by hydrolysis, resulting in (i) the formation of deglycosylated proteins in which glycosylated asparagine are converted into aspartic acid (Hirayama et al. 2015; Suzuki et al. 2016) and (ii) the generation of free-forms of *N*-glycans (free *N*-glycans) that undergo further catabolism in the cytosol and lysosomes (Harada et al. 2015; Hirayama 2018). It was recently reported that, at least in some cases, the conversion of glycosylated asparagines to aspartic acids in NxS/T sites is essential for the activation of an ER-resident transcription factor (NFE2L1/NRF1 in mammals and SKN-1A in *Caenorhabditis elegans*), which plays pivotal roles in the alleviation of several types of cellular stress (Lehrbach and Ruvkun 2016; Fukushige et al. 2017; Tomlin et al. 2017; Lehrbach et al. 2019). NFE2L1 is synthesized as an *N*-glycosylated protein in the ER lumen, followed by, under non-stressed circumstances, constant retrotranslocation into the cytosol for proteasomal degradation. On the other hand, certain types of cellular stress including the inhibition of proteasome activity lead to the accumulation of the NGLY1-deglycosylated form of NFE2L1, which is further processed by a protease DDI2 (Koizumi et al. 2016; Lehrbach and Ruvkun 2016). It should also be noted that the active NFE2L1 protein thus formed is translocated into the nucleus for the transcriptional activation of genes that have an antioxidant responsive element (ARE) in their promoter region. These include 26S proteasome subunits, resulting in a "bounce-back" or recovery of proteasome activity (Radhakrishnan et al. 2010; Steffen et al. 2010). It therefore appears that NGLY1 is critical, not only for the efficient degradation of misfolded glycoproteins by removing *N*-glycans but also for the activation of some glycoproteins by converting glycosylated asparagine into aspartic acid.

In 2012, a rare genetic disorder caused by a defective mutation in NGLY1, designated as NGLY1 deficiency or NGLY1-CDDG, was reported and, since then, more than 100 patients have thus far been identified worldwide (Need et al. 2012; Enns et al. 2014; Lam et al. 2017; van Keulen et al. 2019; Ge et al. 2020; Lipari Pinto et al. 2020; Kariminejad et al. 2021) (personal communication, Mr. Matt Wilsey (Grace Science Foundation)). Although the spectrum of the symptoms among patients are quite diverse, patients with this disease commonly exhibit several general symptoms that include global developmental delay, movement disorders, liver dysfunction, alacrimia, abnormal electroencephalograms and epilepsy (Enns et al. 2014; Lam et al. 2017; van Keulen et al. 2019; Ge et al. 2020; Lipari Pinto et al. 2020; Kariminejad et al. 2021). However, the molecular mechanisms responsible for these symptoms in this disease are poorly understood. It was reported that NGLY1 knock out cells show an impaired BMP4/Dpp signaling (Galeone et al. 2020), resulting in the absence of acidification of the midgut as well as a defect in the formation of mesoderm during developmental processes in *Drosophila* (Galeone et al. 2017) and that a mutant of *png-1*, a worm homolog of NGLY1, exhibits larval growth arrest due to the defective activation of SKN-1A under conditions of proteasomal stress (Lehrbach and Ruvkun 2016). In a more recent study, it was also reported that NFE2L1 is involved in the regulation of mitophagy and that NGLY1 regulates homeostasis in mitochondria and mitophagy through NFE2L1 (Yang et al. 2018). These facts are consistent with a previous study that reported that both embryonic fibroblasts derived from NGLY1-knock out mice and human fibroblasts derived from patients with an NGLY1-deficiency exhibit phenotypes of mitochondrial dysfunction such as an impaired mitochondrial membrane potential, an increase in mitochondrial matrix oxidant burden and a reduced cellular respiratory capacity (Kong et al. 2018). These reports therefore indicate that there are defects in diverse biological processes in NGLY1-deficient cells/organisms, including development, signal transduction, stress responses and mitochondria homeostasis, and that these defects appear to be tightly correlated with the general symptoms observed in the human subjects.

Despite the wealth of information regarding the detailed phenotypes of NGLY1-deficient cells, correlations between the severity of the patient symptoms and the extent of the reduction of NGLY1 activity remain to be clarified, due to the absence of a facile quantitative NGLY1 assay that can be used to assay the enzyme activity in crude extracts. We therefore sought to establish a non-RI-based, quantitative assay for measuring this enzyme activity using fluorescence-labeled glycopeptides derived from hen egg yolk, namely, a BODIPY-labeled asialoglycopeptide (BODIPY-KVAN(CHO)KT, CHO; glycan) designated as BODIPY-ASGP, and examined the enzymatic activities of 27 recombinant NGLY1 mutants that have been found in patients' alleles as of July 2018 (personal communication with the Grace Science Foundation) (Enns et al. 2014; He et al. 2015; Lam et al. 2017). It was found that three mutants, R469X, R458fs and H494fs, out of the 27 mutants tested, exhibited reduced, yet significant activities when compared with wild-type NGLY1. For further analysis of intrinsic NGLY1 activity in cells derived from the patients, we also established a method for measuring endogenous NGLY1 activity in crude extracts using a novel glycosylated cyclopeptide (GCP) that is resistant to digestion by endogenous proteases after deglycosylation, which allowed us to measure NGLY1 activities in fibroblast cells as well as iPS cells derived from actual patients. It was found that no endogenous NGLY1 activity was observed in fibroblasts carrying NGLY1$^{R458fs/R458fs}$ despite the fact that the enzyme activity can be potentially retained in the allele, possibly due to the premature decay of NGLY1 mRNA in the cells. On the other hand, low but detectable NGLY1 activity could be found in iPS cells from patients carrying the exon skip mutations NGLY1$^{c.930C > T/p.Q208}$ in the NGLY1 locus, consistent with the assumption that these patients can in theory express wild-type NGLY1. We also succeeded in measuring endogenous NGLY1 activity in peripheral blood mononuclear cells (PBMCs) derived from blood from a healthy human using the GCP. Our results demonstrate the novel assay can be successfully used to measure NGLY1 activity, and that this method will be useful for the diagnosis and evaluation of endogenous NGLY1 activity in samples derived from the subjects with NGLY1 deficiency.

Results

Establishment of an NGLY1 activity assay using a fluorescence-labeled glycopeptide

As of this writing, various mutant alleles have been reported for NGLY1 deficiency patients, while it remains to be clarified whether or not some of these NGLY1 mutants still possess the residual enzyme activity, because of the lack of a non-RI-based quantitative

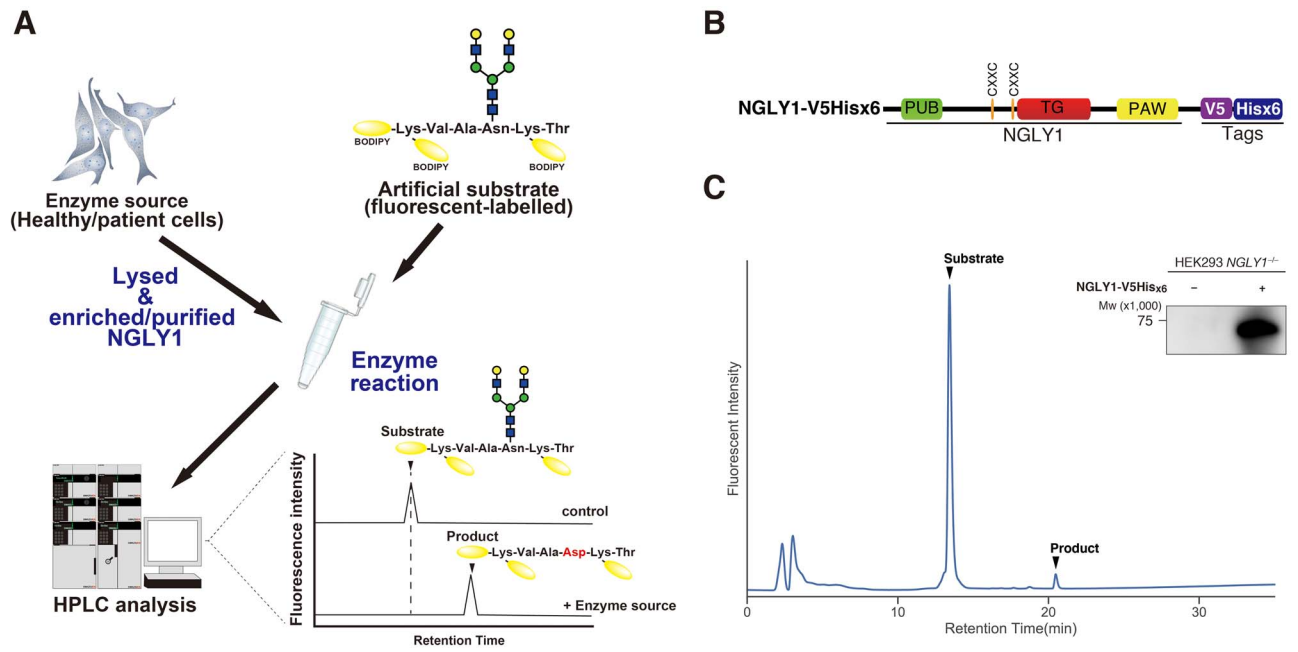

Fig. 1. Fluorescence-based method for quantitative analysis of enzyme activity of purified NGLY1. (A) Schematic representation of the method for measuring NGLY1 activity using a fluorescence-labeled glycopeptide (BODIPY-ASGP) as a substrate. Yellow circle, green circle and blue square in the schematic structure of the artificial substrate represent galactose, mannose and GlcNAc, respectively. (B) Primary structure of V5 and Hisx6 tagged NGLY1. PUB, CxxC, TG and PAW represent PNGase- and ubiquitin-related domain, a putative zinc-binding motif, transglutaminase domain and present in PNGase and other worm proteins domain, respectively. (C) Analysis of the protein expression and the enzyme activity of NGLY1 in NGLY1$^{-/-}$ HEK293 cells. Immunoblot analysis shows the expression level of NGLY1-V5Hisx6 that had been purified using anti-V5 magnetic beads. The HPLC profile shows the separation and quantification of the reaction product (i.e. deglycosylated BODIPY-ASGP). Elution positions of the substrate and the product were confirmed using chemically synthesized authentic standards.

assay system for NGLY1. To overcome this problem, we chose a commercially available glycopeptide derived from hen's egg as a substrate, and their amino groups were conjugated with fluorescence by amine-reactive BODIPY-succinimidyl esters (Figure 1A). After incubating the artificial substrate with various enzyme sources, the substrate and reaction product can be separated and quantified by reversed-phase HPLC (Figure 1A). Using this assay system, we measured the activity of affinity-purified V5/Hisx6-tagged NGLY1 (NGLY1-V5Hisx6) (Figure 1B) that is transiently expressed in NGLY1$^{-/-}$ HEK293 cells. As shown in Figure 1C, the reaction product (BODIPY-KVADKT), co-migrated with the chemically synthesized BODIPY-KVADKT, was clearly detected by HPLC, confirming that this fluorescence-labeled substrate can be used to measure the activity of purified NGLY1.

Enzyme activities of NGLY1 mutants that are transiently expressed in NGLY1$^{-/-}$ HEK293 cells

We next attempted to examine the enzyme activity of recombinant NGLY1 proteins that have mutations associated with this deficiency. For this purpose, expression plasmids of 27 NGLY1 mutants so far identified (as of July 2018) with C-terminal tandem affinity tags (V5 and Hisx6) were constructed and transfected into NGLY1$^{-/-}$ HEK293 cells (Figure 2A). Most of the NGLY1 mutants were stably expressed, whereas the expression of C283W mutants was not observed (Figure 2B). Since immunoblot analysis showed that GFP proteins that are co-expressed with C283W in NGLY1$^{-/-}$ HEK293 cells were stably expressed (data not shown), we assumed that the C283W mutant would be rapidly degraded by the ubiquitin proteasome system due to its instability. To further examine the enzyme properties of these mutants, the NGLY1 activity of each mutant was examined. As shown in Figure 2C, two mutants, R469X and R458fs, out of the total 27 mutants exhibited reduced NGLY1 activity when compared with the wild-type enzyme. This result indicates that the majority of the NGLY1 mutants found in NGLY1 deficiency patients do not retain NGLY1 activity.

Analysis of the enzyme properties of the recombinant NGLY1 mutants synthesized by a wheat germ cell-free system

While we were able to evaluate the enzyme activity of most of the NGLY1 mutants expressed in NGLY1$^{-/-}$ HEK293 cells, the assay conditions were not optimal since the proteins were only detected at the immunoblotting-level, and 3 h are needed to detect the activity using this system. We therefore attempted to establish a more robust expression system for NGLY1 in order to characterize enzymatic properties of the recombinant NGLY1 in greater detail. For the expression of mutant NGLY1, we chose a wheat germ cell-free protein expression system. As shown in Figure 3A, the expressed NGLY1-V5Hisx6 in the cell-free system can be easily purified from the extract by affinity chromatography using Ni-agarose. The purified protein could be readily visualized by staining with Coomassie Brilliant Blue (CBB), and the time course of the enzyme assay for the purified recombinant NGLY1 showed that the reaction reached a plateau after ~60 min (Figure 3B). The reaction curve is similar to that in a previous report (Wang et al. 2009). Having established a more robust assay system, we re-evaluated the enzyme activity of 20 mutants (eight missense mutants, seven nonsense mutants and five frame shift mutants) that contain the entire catalytic domain using this expression system. Again, most of the mutants were expressed well with an exception of C283W, which could not be detected (Figure 3C). In addition, two mutants, R469X and R458fs, exhibited significant activity (Figure 3D), consistent with the observation when

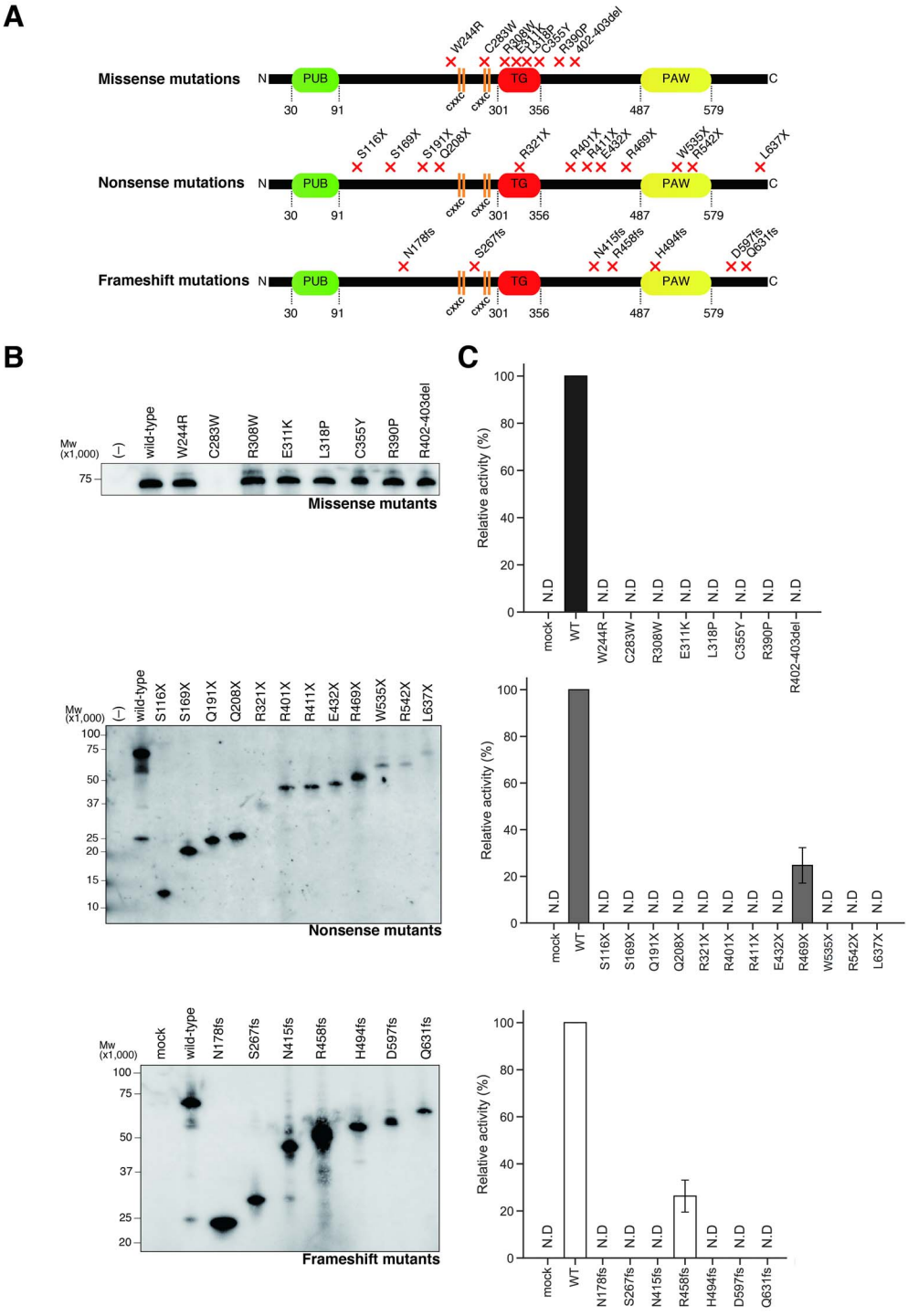

Fig. 2. Activity assay of NGLY1 mutants expressed in NGLY1$^{-/-}$ HEK293 cells. (A) The primary structure of NGLY1 proteins and mutations associated with NGLY1 deficiency. (B) Expression analysis of immunoprecipitated NGLY1-V5Hisx6 mutants by anti-V5 antibody. (C) Measurement of relative activities of NGLY1-V5Hisx6 mutants after incubation with BODIPY-ASGP for 3 h at 37°C. N.D., not detected. Error bars, S.D. from three biological replicates.

NGLY1$^{-/-}$ HEK293 cells were used (Figure 2C). Moreover, H494fs also exhibited ~30% of the activity as compared with the wild-type, while the other mutants exhibiting a < 10% activity when compared with wild-type NGLY1 (Figure 3D). This result clearly suggests that this wheat germ cell-free protein expression system is more sensitive than HEK293 cells for detecting the residual enzyme activity of NGLY1 mutants. To further dissect the properties of these active mutants, i.e. R469X, R458fs and H494fs, the kinetic parameters of the mutants were determined (Figure 3E, Table I). As expected, the three mutants exhibited a reduced affinity toward the substrate (increase of K_m values), as well as turnover number (k_{cat}) (Table I).

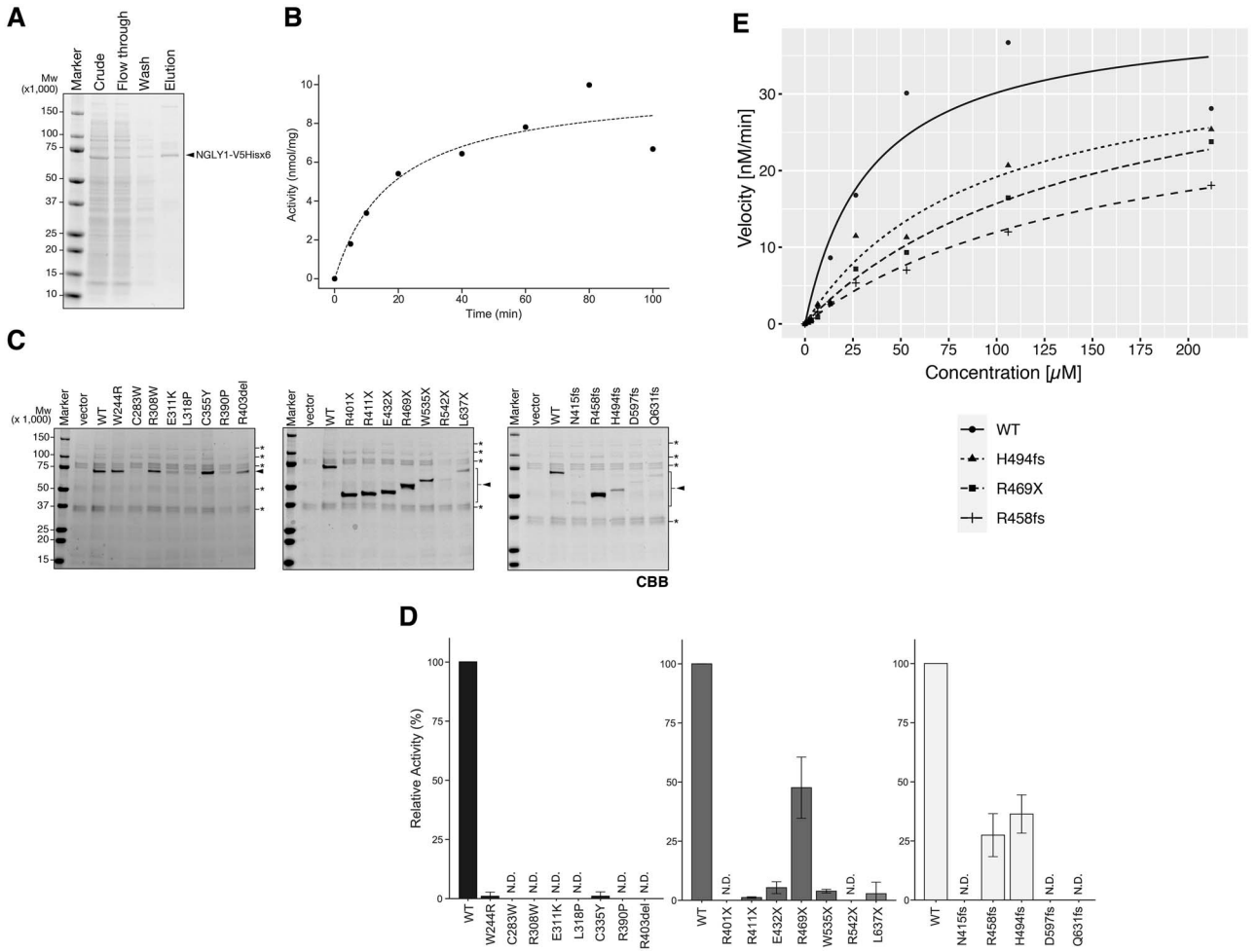

Fig. 3. Expression and activity assay of NGLY1 mutants prepared using the cell-free expression systems. (A) NGLY1-V5Hisx6 purified by a Ni-Sepharose column. An aliquot (10 μL) of each fraction was separated by 10% SDS–PAGE, and the gel was stained with CBB. (B) Time course analysis of the activity of the purified NGLY1-V5Hisx6. (C) CBB staining of a fraction containing purified NGLY1-V5Hisx6 after SDS-PAGE separation. *Band derived from non-specific proteins. (D) Measurement of relative activities of NGLY1-V5Hisx6 mutants after incubation with BODIPY-ASGP for 1 h at 37°C. NGLY1 activity of each mutant was normalized by the band intensity of the NGLY1 protein in Figure 3C. (E) Nonlinear regression curve fit (Michaelis–Menten) for NGLY1 activity in three mutants (R469X, R458fs and H494fs). The reactions were monitored by quantifying deglycosylated BODIPY-ASGP in NGLY1 buffer at 37°C for 10 min, as described in the Materials and Methods N.D., not detected. Error bars, S.D. from three independent experiments.

Table I. kinetic analysis of NGLY1 mutants (R458fs, H494fs and R469X)

NGLY1	K_m (μM)	V_{max} (nM·min^{-1})	k_{cat} (min^{-1})
wild-type	34.4	40.5	27.7
H494fs	90.7	36.6	15.0
R469X	144	38.3	9.67
R458fs	162	31.4	8.05

Establishment of an activity assay for the measurement of the endogenous NGLY1 activity

Our next challenge was to detect endogenous NGLY1 activity in the crude extracts of samples such as patient-derived fibroblasts. To this end, we needed to develop a new substrate, since glycopeptides such as BODIPY-ASGP are prone to be degraded by endogenous proteases, especially after the glycans are removed by NGLY1, when the substrate is incubated with crude cell extracts, as reported previously (Kitajima et al. 1995). We therefore sought to overcome this obstacle using a 5FAM-labeled glycosylated-cyclohepta peptide (5FAM-GCP), which, we assumed, is potentially resistant to proteolytic activity (Figure 4A). As shown in Figure 4B, deglycosylated 5FAM-CGP, which is eluted at the same position as the authentic product produced by bacterial PNGase F, was detected, when incubated with a HEK293 cell lysate. On the other hand, no proteolytic product was detected during the incubation. It should also be noted that another product, 5FAM-GCP modified with *N*-GlcNAc, was also generated, probably due to the presence of cytosolic endo-β-*N*-acetylglucosaminidase (ENGase) in the cell lysate (Figure 4B, a peak eluting at around 8 min) (Suzuki et al. 2002b; Huang et al. 2015), indicating that 5FAM-GCP is a substrate suitable for the measurement of not only endogenous NGLY1 activity but also endogenous ENGase activity without interference from endogenous proteases. The activity could be monitored for periods of up to 12 h without endogenous proteases having any influence (Figure 4C). In addition, using 5FAM-GCP, endogenous NGLY1 activities could be successfully detected without the proteolytic degradation of the substrate in other types of enzyme sources such as

cell lysates of HeLa cells and PBMCs derived from a healthy subject (Figure 4D).

We then sought to address endogenous NGLY1 activity in fibroblasts derived from NGLY1 deficiency patients carrying the mutations that have a residual activity (e.g., R469X, R458fs and H494fs) using 5FAM-GCP as a substrate. Unfortunately, however, fibroblast cells carrying R469X or H494fs were not available. We therefore examined endogenous activity in a fibroblast carrying R458fs. As shown in Figure 5A, no residual enzyme activity was detected in, not only fibroblasts carrying NGLY1$^{R401X/R401X}$ (considered NGLY1-null), but also the one carrying NGLY1$^{R458fs/R458fs}$, despite the fact that R458fs exhibited a reduced NGLY1 activity (Figures 2C and 3D). The transcription of *NGLY1* was also significantly compromised in fibroblasts derived from these patients (Figure 5B), suggesting that a lack of NGLY1 activity in fibroblasts carrying NGLY1$^{R458fs/R458fs}$ may, at least in part, be due to the premature decay of NGLY1 mRNA.

Study of the transcription pattern and the endogenous activity in iPS cells from patients carrying a splice mutation

Having an assay method compatible with crude extract in hand, we further examined if a mutation in the NGLY1 locus, i.e. c.930C > T (He et al. 2015; Lam et al. 2017), which would be expected to generate an incorrect splicing site, has an effect on the enzyme activity. To this end, we focused on a family carrying c.930C > T and a nonsense mutation (p.Q208X) lacking entire catalytic domain (Figures 2A and 6A) and sought to determine if a c.930C > T mutation generates an incorrect splicing-donor site and results in the deletion of a 75 bp fragment in exon 6 by reverse transcription polymerase chain reaction (RT-PCR). We designed a primer set, which can amplify and distinguish a normally spliced mRNA fragment (435 bp) from a mis-spliced mRNA fragment (360 bp) (Figure 6B). As shown in Figure 6C, an RT-PCR analysis of mRNA isolated from iPS cells derived from parent 1 showed the generation of the full-length mRNA of NGLY1. On the other hand, parent 2, carrying a heterozygous mutation of c.930C > T, showed the presence of both 435 bp and 360 bp fragments, indicating that a c.930 C > T mutation is responsible for the mis-splicing of NGLY1 mRNA. It should be noted that mRNA derived from p.Q208X could not be detected in this assay, because p.Q208X is located outside of exon 6 (in exon 4). Furthermore, the generation of full length NGLY1 mRNA was observed in iPS cells derived from two patients carrying NGLY1$^{c.930C > T/p.Q208X}$, suggesting that cells carrying a c.930C > T mutation have the potential to transcribe reduced amounts of full length NGLY1 mRNA (Figure 6C, lanes 3–6). Consistent with this observation, weak but significant endogenous NGLY1 activity was observed in these two patients using 5FAM-GCP as a substrate (Figure 6D). It is noteworthy that the deletion-mutant proteins, resulting from the same exon skip mutation, exhibited no NGLY1 activity (Figure S1). Taken together, these results show that, using our assay system, it is possible to detect the wild-type NGLY1 protein at low but detectable levels in patients carrying c.930C > T.

Discussion

It was established that the deglycosylation-dependent fluorescent protein (ddVenus) is a useful probe for detecting intracellular activity of NGLY1 *in vivo* (Grotzke et al. 2013; He et al. 2015). On the other hand, for a quantitative assay of PNGase/NGLY1 activity *in vitro*, a ^{14}C-labeled glycopeptide, which was prepared from proteolytically digested fetuin, was historically used as a substrate when PNGase activities were identified from diverse origins over two decades ago (Seko et al. 1991; Suzuki et al. 1993, 1998; Kitajima et al. 1995). After incubation of the substrate with the enzyme source, the substrate and de-glycosylated product are separated and quantitated by paper chromatography as well as paper electrophoresis. However, the preparation of the ^{14}C-labeled substrate and use of radioactive molecules make it difficult to perform this assay in a regular laboratory, especially under the tight regulations for the use of radioactive isotopes in Japan. On the other hand, another convenient assay for measuring PNGase activity using a glycoprotein, namely, *S*-alkylated RNaseB, as a substrate was reported (Tanabe et al. 2006). In this assay, substrates and product can be separated by SDS-PAGE and quantitated by CBB stain or immunoblotting. This method, however, suffers from being insensitive and also not compatible with use for crude extracts with high levels of contaminating proteolytic activities. It has therefore become an urgent issue to establish an alternative, facile assay method (i.e. non-RI-based, sensitive and quantitative method) for use with crude extracts as an enzyme source. In this article, we initially achieved an assay method for NGLY1 using a fluorescence-labeled glycopeptide, ASGP, using reversed-phase HPLC for the separation and quantification of the reaction product (Figure 1). We also established a method for the preparation of NGLY1 proteins by utilizing a wheat germ cell-free protein expression system. This system is particularly useful for the expression of NGLY1 mutants because the extract including NGLY1 can be completed in two steps within 12 h (overnight). With this protein expression system, we detected reduced enzyme activities in some mutant proteins found in NGLY1 deficiency patients. While fluorescence-labeled ASGP is a powerful substrate when measuring the enzyme activity of purified NGLY1, it is not suitable for measuring activity when a crude extract is used as an enzyme source, since deglycosylated ASGP is prone to be degraded by endogenous proteases (data not shown). This fact is critical for the quantification of endogenous NGLY1 activity in cells/tissues. To overcome this problem, GCP was introduced as a substrate, and it turned out to be the ideal substrate for measuring endogenous NGLY1 activity in crude extracts. We also demonstrated that GCP can be used to assess endogenous NGLY1 activity in cells derived from patients such as fibroblast cells and iPS cells.

It has been reported that yeast PNGase (Png1), which essentially has only a catalytic domain for deglycosylation activity (i.e. transglutaminase domain), can be efficiently produced in *Escherichia coli* (Suzuki et al. 2000). We also attempted to optimize the culture conditions that would permit recombinant human NGLY1 to be expressed in *E. coli*. A recombinant MBP-human NGLY1 fusion protein was obtained as the soluble cellular fraction in *E. coli*. However, we were not able to detect activity in the recombinant human NGLY1 expressed in *E. coli*, presumably due to the incorrect folding of human NGLY1. NGLY1 is a large and multi-domain protein composed of a PUB domain, a transglutaminase domain and a PAW domain (Figure 1B) (Suzuki et al. 2002a), which may render this protein problematic in terms of acquiring the correct folding to exert enzyme activity. This intrinsic difficulty concerning the correct folding of NGLY1 may have something to do with the fact that, when NGLY1 activity was tested, only three mutant proteins, R469X, R458fs and H494fs, out of 27 mutant proteins exhibited significant NGLY1 activity (30–70%; Figures 2C and 3D). Among those with no enzyme activity, eight missense mutations identified from the patients were found to be on the highly conserved residue in eukaryotes (from

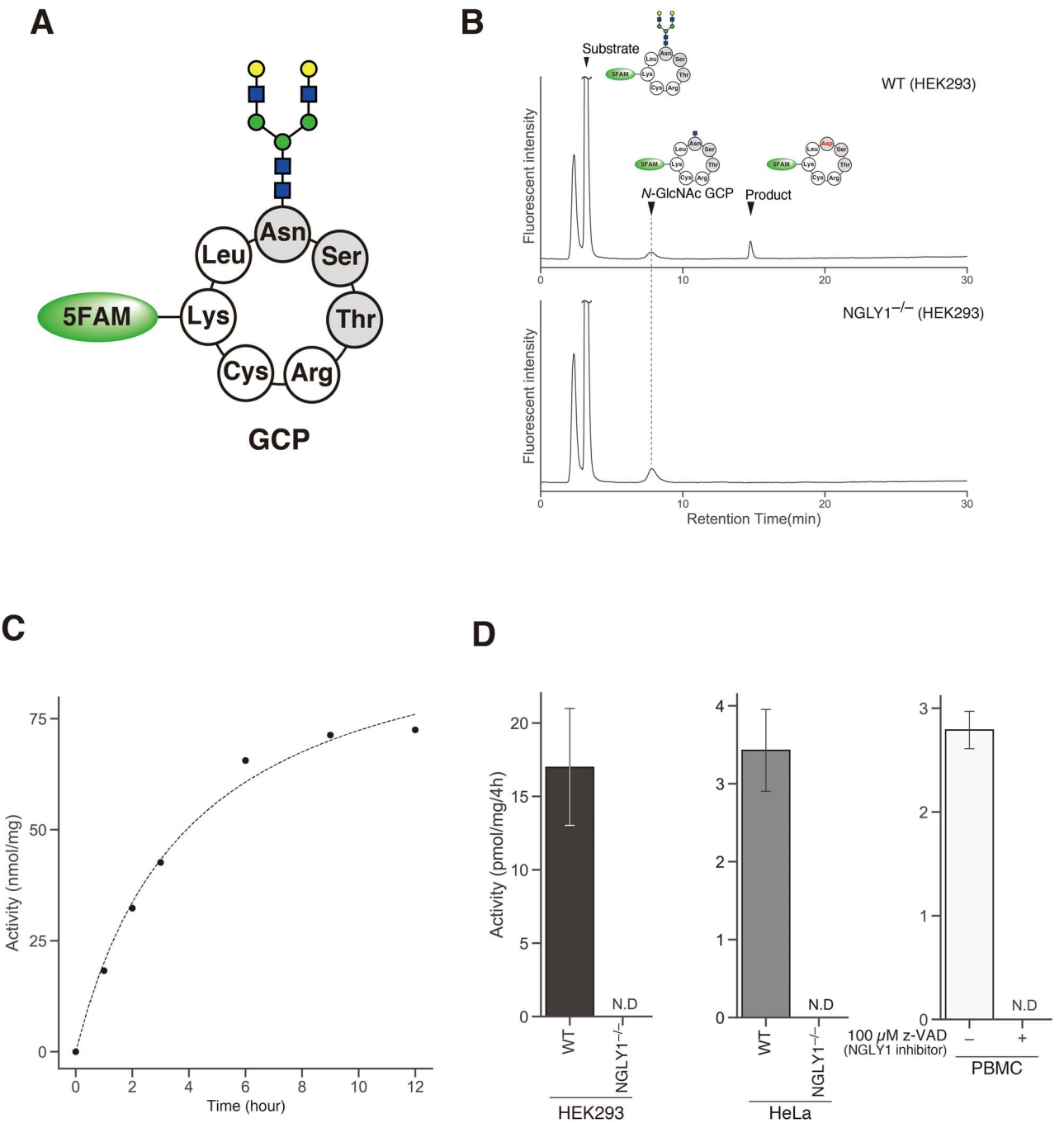

Fig. 4. Activity assay of the endogenous NGLY1 in cell lysates using 5FAM-GCP. (A) Schematic structure of 5FAM-labeled GCP. (B) Separation of 5FAM-GCP and deglycosylated GCP by HPLC. Elution position of deglycsylated 5FAM-GCP and *N*-GlcNAc modified 5FAM-GCP was determined by comparing their elution positions with those of authentic standards. (C) Time course analysis of the endogenous activity of NGLY1 in HEK293 cells. (D) Endogenous activity of NGLY1 in various cell lysates. Z-VAD represents the caspase inhibitor z-VAD-fmk, which is also a potent inhibitor of PNGase (Misaghi et al. 2004). N.D., not detected. Error bars, S.D. from three biological replicates.

fungi to humans) (Figure S2), and therefore it is reasonable to assume that these eight residues are critical for protein stability and/or function. It was also found that the most of the nonsense mutants and frameshift mutants bearing an intact catalytic domain still had no NGLY1 activity. On the other hand, the purified NGLY1 mutant proteins lacking a C-terminal domain showed enzyme activity (Park et al. 2001). This seeming discrepancy may represent the intrinsic nature associated with the difficulties related to the folding of this protein. We also studied endogenous NGLY1 activity in fibroblasts carrying NGLY1$^{R458fs/R458fs}$, which is one of the mutation alleles exhibiting enzyme activity, and found the absence of endogenous NGLY1 activity as well as a significant reduction in the mRNA levels of NGLY1 $^{R458fs/R458fs}$. Collectively, the absence of endogenous activity of NGLY1 in fibroblasts carrying NGLY1$^{R458fs/R458fs}$ not

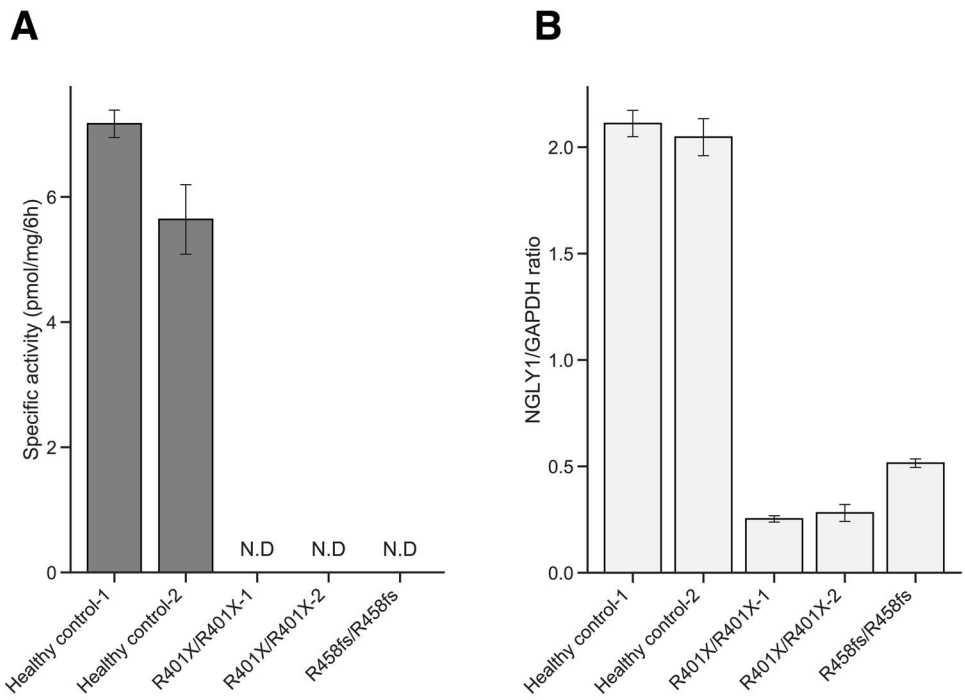

Fig. 5. Expression level of NGLY1 in patient-derived fibroblasts. (A) Endogenous activity of NGLY1 in patient-derived fibroblasts. For the analysis of fibroblasts from healthy controls and patients carrying homozygous R401X, cells derived from two independent individuals were used. (B) Quantitative PCR analysis of NGLY1 mRNA in patient-derived fibroblasts. The expression level of NGLY1 was calculated as a relative expression of NGLY1 compared with that of GAPDH. N.D., not detected. Error bars, S.D. from three biological replicates.

only arise from a reduced level of NGLY1 activity, but also the degradation of NGLY1 mRNA by a clearance system of mRNA carrying mutations such as nonsense-mediated mRNA decay (NMD) (Kurosaki and Maquat 2016). Although the issue of whether patients carrying R499X or H494fs have enzyme activity is unclear, cells from patients carrying these mutations would lose NGLY1 activity because most of the mRNA carrying nonsense/frameshift mutations tend to be degraded by NMD as is the case with fibroblasts carrying $NGLY1^{R458fs/R458fs}$ as well as $NGLY1^{R401X/R401X}$ (Figure 5B). While the exact relationship between NGLY1 enzyme activity and the symptoms of patients remains unclarified, it is quite possible that enzyme activity contributes significantly to the severity of the symptoms (see below). Accordingly, for patients bearing mutations with some activity being retained, stabilization of the mRNA may serve as a feasible therapeutic approach.

It is also noteworthy that cells of patients carrying $NGLY1^{c.930\ C > T/p.Q208X}$ have weak NGLY1 activity (Figure 6D), but neither the recombinant Q208X protein nor the deletion mutant formed by the exon skipping has NGLY1 activity (Figure 2 and S1), suggesting that patients carrying c.930 C > T are capable of transcribing low levels of the full length NGLY1 protein. However, we failed to detect NGLY1 activity in cells differentiated to endoderm, mesoderm or ectoderm from iPSC or in fibroblasts derived from patients carrying $NGLY1^{c.930\ C > T/p.Q208X}$ (unpublished data). Therefore, at present, the possibility that no endogenous NGLY1 exists in the differentiated cells and fibroblasts derived from these patients cannot be completely excluded. Our results, nevertheless, are consistent with the fact that the symptoms of patients bearing this mutation are far milder when compared with other reported patients (Lam et al. 2017). It is quite possible that the low level of wild-type NGLY1 provides a reduced level of enzyme activity and that this could contribute to the milder symptoms. It should also be noted that exon-skip mutations should be the target of potential therapeutic approaches using antisense-oligonucleotides (ASO) as inhibitors of exon-skipping, as has been previously established and approved as ASO therapies for several diseases such as Spinal Muscular Atrophy or Duchenne muscular dystrophy (Chiriboga et al. 2016; Aartsma-Rus and Krieg 2017; Roshmi and Yokota 2019; Heo 2020; Aoki and Wood 2021). Finally, we demonstrated that our assay using GCP is capable of measuring endogenous NGLY1 activity in 2.5×10^5 of PBMC, and that the results can be obtained from less than 5 mL of human blood. This method promises to be a prototype for methods for the diagnosis of NGLY1 deficiencies as well as for evaluating the efficacy of therapies of this disease (e.g., enzyme replacement therapy or gene therapy etc.) in the near future.

Materials and methods

Vector construction

Plasmid vectors and primers used in this study are listed in Tables SI and SII, respectively.

Construction of a plasmid for CRISPR/Cas9-mediated NGLY1 knockout (pAX458-NGLY1del and pAX459-NGLY1del)

pSpCas9(BB)-2A-GFP (pAX458) and pCas9(BB)2A-puro (pAX459) were purchased from Addgene (Plasmid #48138 and #48139, respectively). For the generation of NGLY1-knockout HEK293 cells, pAX458 was digested with *Bbs*I, after which 5′-phosphorylated complement target sequence, NGLY1-sgRNA1-fwd and NGLY1-sgRNA1-rvs, was annealed and ligated into linearized pAX458 to generate pAX458-NGLY1del. For the generation of NGLY1-

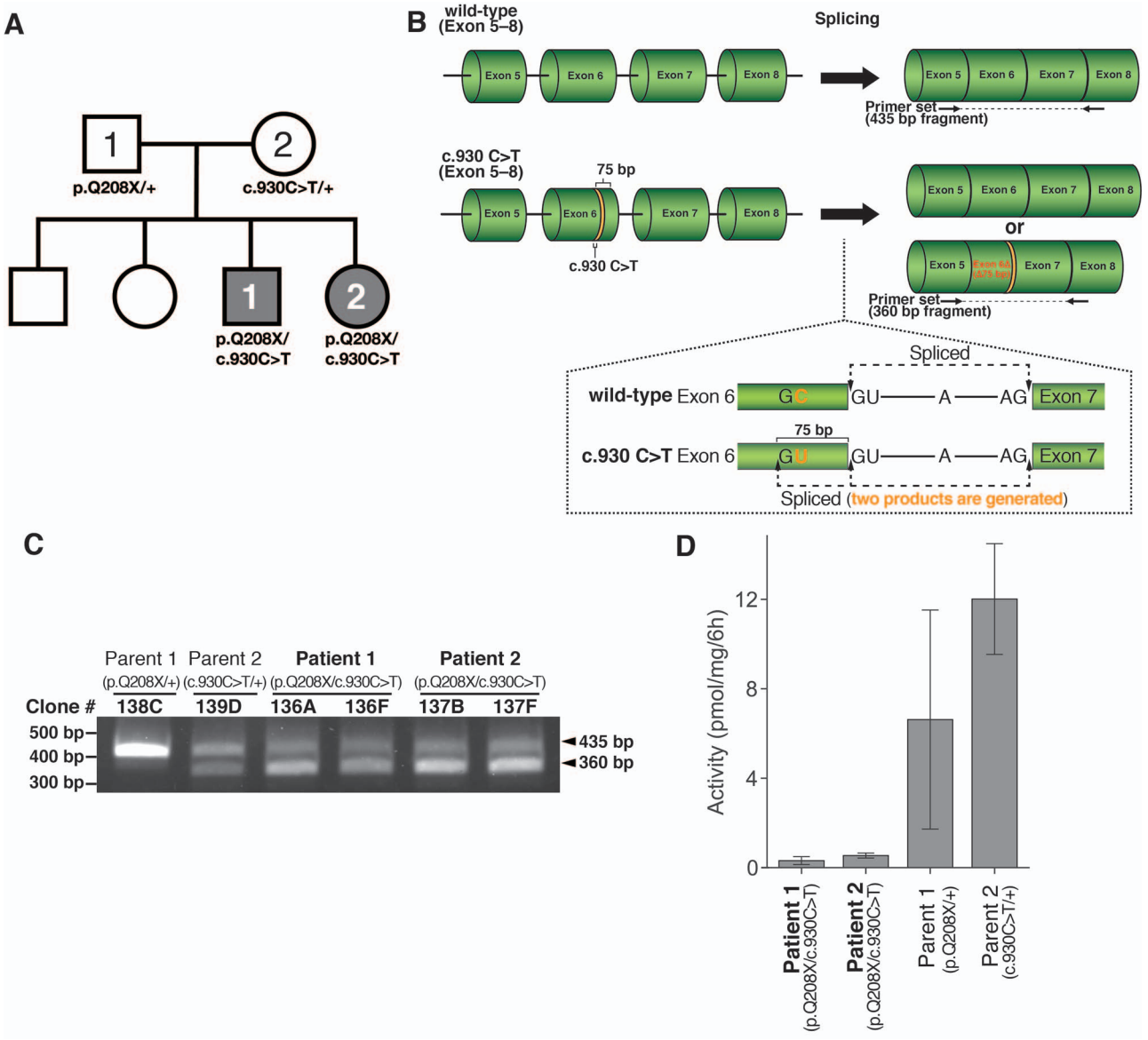

Fig. 6. NGLY1 activity in iPS cells derived from a family of a patient carrying a splicing mutation (c.930C > T). (A) Pedigrees of the family of a patient carrying c.930C > T and p.Q208X. (B) Schematic representation of splicing site in wild-type NGLY1 and c.930C > T mutant of NGLY1. The bases indicated by orange color in exon 6 represent mutation point of c.930C > T. Consensus sequences, GU, A and AG, in intron6 represent splice donor site, branched site and splice accepter cite, respectively. (C) Reverse transcription-PCR analysis of the splicing of NGLY1 in iPS cells derived from the family. For this analysis in patient 1 and patient 2, iPS cells derived from two independent clones were used (patient 1; 136A and 136F, patient 2; 137B and 137F). (D) Endogenous NGLY1 activity in the patients' family-derived iPS cells. Clone 136A and clone 137B were used in this experiment as the sample of patient 1 and patient 2, respectively. Error bars, S.D. from three biological replicates.

knockout HeLa cells, pAX459 was digested with *Bbs*I and the 5′-phosphorylated complement target sequence, NGLY1-sgRNA2-fwd and NGLY1-sgRNA2-rvs, was then annealed and ligated into the linearized pAX459 to generate pAX459-NGLY1del. The sequence of the primers used is shown in Table SII. The target sequences adjacent to the protospacer adjacent motif site (NGG) was designed using the E-CRISP website (http://www.e-crisp.org/E-CRISP/) (Heigwer et al. 2014).

Construction of pNGLY1-V5Hisx6

The cDNA sequence of NGLY1 was amplified by PCR using pCMV6-huPNGase-WT, kindly provided from Dr. Chengcheng Huang (Glycometabolic Biochemistry Laboratory, RIKEN) as a template and primers, pIRES-EcoRI-huNGLY1-F113 and v5His6-tga-SalI-pIRES-R114 (the sequence of the primers is shown in Table SII). The resulting NGLY1-V5Hisx6 fragment was cloned into pIRES-DsRed2-Express2 digested with *Eco*RI and *Sal*I using In-Fusion HD cloning kit (TaKaRa, Kyoto, Japan) according to the manufacturer's protocol.

Construction of vectors of missense mutants of NGLY1

Introduction of mutations on the *NGLY1* gene was carried out using QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Inc., Tokyo, Japan) according to the manufacturer's protocol using pIRES-DsRed2-Express2-NGLY1-V5Hisx6 as a template. Primer sets used for mutagenesis are shown in Table SII.

Construction of vectors of NGLY1 nonsense mutants

Nonsense mutants' sequences were amplified by PCR using the forward primer, pIRES-EcoRI-huNGLY1-F113, and reverse primers for each nonsense mutant (Table SII), and pIRES-DsRed2-Express2-NGLY1-V5Hisx6 as a template. Each fragment was cloned into pIRES-DsRed2-Express2, digested with *Eco*RI and *Sal*I, using In-Fusion HD cloning kit.

Construction of vectors of NGLY1 frameshift mutants

Double strands of mutant sequences containing V5 and Hisx6 coding sequences just before stop codon was chemically synthesized by GENEWIZ (Saitama, Japan). The sequences of the mutants that were synthesized are shown in Figure S4. Each fragment was cloned into pIRES-DsRed2-Express2, digested with *Eco*RI and *Sal*I, using In-Fusion HD cloning kit.

Construction of expression vectors of NGLY1 mutants for cell-free systems

Fragments of mutant sequences cloned to pIRES-DsRed2-Express2 were isolated by digestion with *Xho*I and *Bam*HI and purified. The purified fragments were ligated into equivalent sites of pEU-E01-MCS (CellFree Science, Ehime, Japan).

Cell culture and transfection

iPS cells generated from patients' PBMCs were obtained from the Center for iPS Cell Research and Application (CiRA), Kyoto University. Clones of iPS cells were cultured on iMatrix-511 (Nippi, Tokyo, Japan)-coated plates with StemFit-AK03N medium (Ajinomoto, Tokyo, Japan). PBMCs were purchased from HemaCare Corporation (Van Nuys, CA). Primary fibroblasts of healthy human subjects (GM01652 and GM03652), patients carrying R401X/R401X (GM26584 and GM26607) and patients carrying R458fs/R458fs (GM26590) were purchased from the Coriell Institute for Medical Research. Experiments with iPS cells, PBMC and patient fibroblasts were approved by the ethics committee of Takeda Pharmaceutical Company Limited (under permission number GEN-00100012) and RIKEN (under permission number Wako3 2019-23(3)). All cell lines used in this study (HeLa, HEK293 and fibroblasts) were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Wako, Japan) supplemented with 10% FBS and antibiotics (Wako, Japan) at 37°C in humidified incubator containing 5% of CO_2 gas. For transfection of HEK293 cells with plasmids, Fugene® HD (Promega, WI) was used according to manufacturer's instruction.

Generation of CRISPR/Cas9-mediated NGLY1 knockout in HEK293 and HeLa cell line

To generate NGLY1 knockout HEK293 cell lines by the CRISPR/Cas9 system, HEK293 cells were transfected with pAX458-NGLY1del. The sorting and collection of GFP-positive cells were then performed with a cell sorter, FACS Aria (BD Biosciences) at the Support Unit for Bio-Material Analysis in RIKEN CBS, Research Resources Division. The sorted cells were further cultured for 10 days. For generation of NGLY1 knockout HeLa cell line by CRISPR/Cas9 system, HeLa cells were transfected with pAX459-NGLY1del. The cells were selected by DMEM supplemented with 10% FBS and 3 μg/mL of puromycin. To obtain clonal cell lines, the bulk-selected populations were cloned by limiting dilution and further plated onto 96-well plates. Each clone was further cultured for 12 days, and genomic DNA was isolated from each clone for the identification of *NGLY1*$^{-/-}$ clones. Amplified target site by PCR was analyzed by DNA sequencing (sequence information will be provided upon request). The knockout of *NGLY1* gene was further confirmed by measuring NGLY1 activities in vitro.

Construction of fluorescence-based substrates, BODIPY-asialoglycopeptide

Labeling of amine group in side chain of lysine residues as well as N-terminus of the glycopeptide KVAN(CHO)KT and KVADKT by BODIPY-N-Hydroxysuccinimide (BODIPY-NHS) was performed according to manufacturer's instructions. Briefly, 50 μl of BODIPY™ FL NHS Ester (Thermo, Fisher Scientific, CA) dissolved in DMSO (1 mg/mL) was slowly added to 50 μL of 100 mM Sodium bicarbonate (pH 8.2) containing 0.5 mg of KVAN(CHO)KT (purchased from Fushimi Pharmaceutical Ltd (Kagawa, Japan)) or KVADKT (chemically synthesized by the Support Unit for Bio-Material Analysis in RIKEN CBS, Research Resources Division). After incubation for 2 h at room temperature, 100 μL of 1.6 M hydroxylamine was added to the reaction mixture and incubated for another 1 h at room temperature to terminate the reaction. For removing unreacted BODIPY-NHS and byproducts, reaction mixture was separated by reversed-phase HPLC using InertSustain C18, 5 μm, 7.6 × 150 mm (GL science, Tokyo, Japan). Then, peaks derived from BODIPY- KVAN(CHO)KT, designated as BODIPY-ASGP, and BODIPY-KVADKT were manually collected. The structures of purified BODIPY-ASGP and BODIPY-KVADKT were confirmed by ESI-MS (QSTAR Elite (AB SCIEX)) at the Support Unit for Bio-Material Analysis in RIKEN CBS, Research Resources Division (Figure S3). For calculating the molar extinction coefficient of BODIPY-ASGP, the concentration was measured using Quantitative Colorimetric Peptide Assay (Thermo) according to the manufacturer's instructions.

RT-PCR

The splicing of NGLY1 in the iPS cells carrying the c.930C > T mutation was assayed using RT-PCR. Briefly, iPS cells carrying c.930C > T and/or p.Q208X were collected, and total RNA was extracted using the RNeasy minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was generated from 1 μg of total mRNA using the PrimeSTAR Max (TaKaRa). Full length NGLY1 (435 bp) and exon7 skipped NGLY1 (360 bp) were amplified by PCR using template cDNA and the primers NGLY1-RT-fwd, and NGLY1-RT-rvs (Table SII). Amplicons were analyzed by electrophoresis on 2% agarose gel.

Real-time PCR

mRNA levels of NGLY1 in patients' fibroblast were quantified by quantitative real-time PCR. Total RNA was extracted from patients' fibroblasts using RNeasy Mini Kit (Qiagen) and treated with RNase-Free DNase (Qiagen) to avoid contamination of genomic DNA. The cDNA was then generated from 1 μg of total RNA using SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The cDNAs thus obtained were amplified with 200 nM of NGLY1-qPCR-fwd, 200 nM of NGLY1-qPCR-rvs (Table SII), 200 nM of FAM-labeled TaqMan probe for

NGLY1 (5′- TGCCGAGCTGTAGGGT −3′) and 2 × TaqMan Gene Expression Master Mix (Applied Biosystems). The expression levels of the target gene were measured in triplicate and normalized to the corresponding GAPDH expression levels.

Immunoblotting

For immunoblotting, cultured cells in 6 cm dishes were harvested 48 h after transfection. Harvested cells were washed twice with 1 mL of PBS and lysed by 500 μL of RIPA buffer (5 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholic acid and 0.1% SDS). The resulting cell extract was then sonicated using a probe sonicator UD-21P (TOMMY, Tokyo, Japan) for shearing genomic DNA and centrifuged at 20,000 × *g* for 5 min at 4°C for removing insoluble materials, after which 6× sample buffer (0.35 M Tris–HCl (pH 6.8), 10% SDS, 38% glycerol, 0.02% BPB and 0.6 M DTT) was added to the supernatant, and the samples were boiled at 98°C for 5 min. A total of 5 μL of samples were subjected to SDS-PAGE analysis using 4–20% Mini-PROTEAN® TGX™ Precast Gels. The separated proteins were transferred to PVDF membrane, and the membrane was blocked with TTBS (25 mM Tris–HCl (pH 7.4), 150 mM NaCl and 0.1% (v/v) Tween 20) containing 0.5% (w/v) skim milk. The blot was incubated with anti-V5 mouse antibody, 1:5,000 (6F5) (Fujifilm, Tokyo, Japan), followed by incubation with anti-mouse IgG goat antibody conjugated with starlight blue 700, 1:5,000 (Bio-Rad, Hercules, CA). Bands were detected using ChemiDoc Touch MP (Bio-Rad).

Purification of NGLY1-V5Hisx6 from HEK293 cells

NGLY1$^{-/-}$ HEK293 cells cultured in 10 cm dishes were harvested at 48 h after the transfection of pIRES-NGLY1-V5Hisx6. The harvested cells were washed with PBS and lysed by IP-lysis buffer (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% (w/v) TritonX-100 and 5% (w/v) glycerol, 1× cOmplete™ protease inhibitor cocktail (Sigma-Aldrich Japan, Tokyo, Japan)). The cell extract was sonicated for shearing genomic DNA and centrifuged at 20,000 × *g* for 5 min at 4°C to remove insoluble materials. The supernatants were incubated with an anti-V5 monoclonal antibody (OZA3)-conjugated magnetic beads (MBL, Nagoya, Japan) with end-over-end rotation for 1 h at 4°C. The beads with NGLY1-V5Hisx6 bound to them were washed three times with PBS and half amount of the beads were used for NGLY1 activity assay. Rest of the enzyme-binding beads were used for measuring relative amount of NGLY1-V5Hisx6 by immunoblotting.

Activity assay of NGLY1-V5Hisx6 purified from NGLY1$^{-/-}$ HEK293 cells using BODIPY-ASGP

In this assay, anti-V5 magnetic beads-bound NGLY1-V5Hisx6 was used as an enzyme source. A total of 25 μL of NGLY1-binding beads were incubated with 50 pmol of BODIPY-ASGP in 50 μL of NGLY1 reaction buffer (100 mM Tris–HCl (pH 7.5), 500 mM sucrose, 5 mM EDTA, 1 mM DTT, 1 mM Pefabloc® SC, cOmplete™ protease inhibitor cocktail) for 3 h at 37°C. The reaction was terminated by adding 100 μL of 100% EtOH and centrifugation at 20,000 × *g* for 5 min. The resultant supernatant was collected, evaporated to dryness in a Speed-Vac concentrator, and subjected to HPLC analysis.

Protein expression by cell-free system

Protein expression was carried out using an ENDEXT®Technology Protein Research Kit (H) (Cell Free Science (Ehime, Japan)), according to the manufacture's protocol. Briefly, for the transcription of NGLY1 mutant genes, a mixture of 2 μg of the expression plasmid and transcription buffer LM premix were incubated for 1 h at 37°C. The transcribed mRNA and wheat germ extract were then mixed. The mixture was transferred to the bottom of translation buffer to form a bi-layer with the mixture of wheat germ extract and mRNA (lower layer) and the translation buffer (upper layer). The bilayer solution was incubated for 24 h at 15°C for the translation reaction to proceed.

Purification of NGLY1-V5Hisx6 from germ wheat extract

For the affinity purification of NGLY1-V5Hisx6, 15 μL of Ni-Sepharose resin (GE Healthcare Life Sciences, Piscataway, NJ) was added to the translated reaction mixture which was then incubated for 1 h at 4°C with rotation. After washing the NGLY1-V5Hisx6-bound Ni-Sepharose resin with 400 μL of washing buffer (20 mM Na-phosphate (pH 7.5), 300 mM NaCl and 20 mM Imidazole), NGLY1-V5Hisx6 proteins were eluted by 400 μL of elution buffer (20 mM Na-phosphate (pH 7.5), 300 mM NaCl and 500 mM Imidazole). The concentration of the sample, as well as removing imidazole, was carried out using Amicon Ultra-0.5 mL centrifugal filters with a molecular weight cutoff of 30 kDa (30 k MWCO) (Millipore, Bedford, MA).

Activity assay of purified NGLY1 from germ wheat extract

An aliquot (10 μL) of purified NGLY1 was incubated with 50 pmol of BODIPY-ASGP in 40 μL of NGLY1 reaction buffer (50 mM Tris–HCl (pH 7.5), 10 mM sucrose 5 mM EDTA, 1 mM DTT, 1 mM Pefabloc® SC, 1× cOmplete™ protease inhibitor cocktail) for 5–120 min at 37°C. The reaction was terminated by adding 100 μL of 100% EtOH and then centrifuged at 20,000 × *g* for 5 min. The resulting supernatant was collected, evaporated to dryness in a Speed-Vac concentrator, and subjected to HPLC analysis. Enzyme activities were normalized by the total amount of NGLY1 proteins based on the intensity of the bands stained by CBB.

Measuring endogenous NGLY1 activity in cell lysates

For the analysis of PBMCs, 1.25 × 10^6 cells were used as an enzyme source. For the analysis of cultured cell lines or iPS cells, cells were grown to semi confluence in 6 cm dishes and harvested by a means of a cell scraper. The harvested cells were washed with PBS and lysed by a probe sonicator UD-21P (TOMMY) in NGLY1 buffer (50 mM Tris–HCl (pH 7.0), 10 mM sucrose, 5 mM EDTA, 1 mM DTT, 1 mM Pefabloc® SC, 1× cOmplete™ protease inhibitor cocktail). The insoluble materials were then removed by centrifugation at 20,000 × *g* for 5 min at 4°C. An aliquot (100 pmol) of 5FAM-labeled GCP, which was chemically synthesized by Glytech Inc. (Kyoto, Japan), was incubated with 50 μL of cell extract at 30°C. Structure of 5FAM-labeled GCP was shown in Figure 4A, and the cyclization of the glycopeptide was achieved by condensation reaction between the chloroacetamide group at the N-terminal and the thiol group of the cysteine at the C-terminal. The reaction was terminated by adding 100 μL of 100% EtOH and centrifugation at 20,000 × *g* for 5 min. The resultant supernatant was collected, evaporated to dryness in a Speed-Vac concentrator, and subjected to HPLC analysis.

Separation and quantification of the reaction products by HPLC

Substrate and reaction products were separated by HPLC using InertSustain C18 HP (3 μm, 3.0 × 150 mm, GL Science). For the separation of BODIPY-ASGP, the elution condition was as follows: eluent A, DW containing 0.1% Trifluoroacetic Acid (TFA); eluent B, 100% acetonitrile containing 0.1% TFA. The column was equilibrated with eluent A/eluent B (60/40) at a flow rate of 0.45 mL/min. After injecting a sample, the concentration of eluent B was increased linearly from 40 to 95% over 25 min. BODIPY-ASGP and its reaction products were detected by measuring fluorescence (λ excitation 503 nm, λ emission 512 nm). For the separation of 5FAM-GCP, the elution condition was as follows: The column was equilibrated with eluent A/eluent B (75/25) at a flow rate of 0.45 mL/min. After injecting a sample, the concentration of eluent B was increased linearly from 25 to 50% over 25 min. 5FAM-GCP and its reaction products were detected by measuring fluorescence (λ excitation 494 nm, λ emission 518 nm).

Calculation of Michaelis–Menten constants (K_m, V_{max} and k_{cat})

The Michaelis–Menten constants (K_m) and maximum velocity (V_{max}) values for the purified NGLY1 were determined using BODIPY-ASGP as a substrate. NGLY1 proteins were incubated at 37°C for 10 min with various concentrations of the substrate (1.6–212 μM) in NGLY1 buffer. Curve fitting and calculation of the constants were carried out using an R package, Drc. For measuring the concentration of NGLY1-mutant proteins for the k_{cat} calculation, standard wild-type NGLY1 proteins, which are produced in Expi293 cells (Thermo) and purified by affinity chromatography, or mutant NGLY1 proteins were separated by SDS-PAGE and stained with SYPRO™ Ruby Protein Gel Stain (Thermo). The concentrations of NGLY1 mutant proteins were then calculated by comparison to the standard curve, plotting the concentrations and the band intensities of serially diluted wild-type NGLY1 proteins.

Supplementary data

Supplementary data are available at *Glycobiology* online.

Acknowledgement

We are grateful to the Center for iPS Cell Research and Application (CiRA), Kyoto University for providing the iPS cells generated from PBMC from patients. We also wish to thank Dr. Takayuki Kamei, Dr. Ryuichi Tozawa, Dr. Hiroshi Yukitake and the members of NGLY1 deficiency project (T-CiRA) for fruitful discussions and Dr. Haruhiko Fujihira (RIKEN) and Dr. Yoichiro Harada (Osaka International Cancer Institute) for critical reading of the manuscript. The research in Glycometabolic Biochemistry Laboratory is supported by RIKEN Pioneering Research Project (Glyco-lipidologue Initiative) and AMED-CREST (JP21gm14100003) to T.S. The image of cells in Figure 1A was provided by DBCLS Togo Picture Gallery (© 2016 DBCLS TogoTV; https://togotv.dbcls.jp/pics.html).

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