Review: Gene

The cytoplasmic peptide:*N*-glycanase (NGLY1); structure, expression and cellular functions

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Abstract

NGLY1/Ngly1 is a cytosolic peptide:N-glycanase, *i.e.* de-*N*-glycosylating enzyme acting on *N*-glycoproteins in mammals, generating free, unconjugated *N*-glycans and deglycosylated peptides in which the *N*-glycosylated asparagine residues are converted to aspartates. This enzyme is known to be involved in the quality control system for the newly synthesized glycoproteins in the endoplasmic reticulum (ER). In this system, misfolded (glyco)proteins are retrotranslocated to the cytosol, where the 26S proteasomes play a central role in degrading the proteins: a process referred to as ER-associated degradation or ERAD in short. PNGase-mediated deglycosylation is believed to facilitate the efficient degradation of some misfolded glycoproteins. Human patients harboring mutations of *NGLY1* gene (*NGLY1*-deficiency) have recently been discovered, clearly indicating the functional importance of this enzyme. This review summarizes the current state of our knowledge on *NGLY1* and its gene product in mammalian cells.

1. Introduction

Cellular metabolism involves not only the handling of small molecules but also the biosynthesis and catabolism of macromolecules such as proteins, glycans and lipids. Compared with their biosynthesis, our knowledge of catabolic aspect of the macromolecules is limited. For instance, *N*-glycosylation of proteins occur in all domains of life (eukaryotes, bacteria or archaea) and is one of the most common modifications of proteins (Schwarz and Aebersold, 2011). While the biosynthetic pathway for *N*-glycosylation has been well clarified in mammals and yeast, many issues remain to be clarified in terms of their catabolism, even in this “post-genome” era. It is well known that the degradation of *N*-glycoproteins occurs predominantly in lysosomes (Winchester, 2005; Suzuki, 2009), whereas the occurrence of a “non-lysosomal” degradation pathway has also been revealed during the past decade (Suzuki, 2007; Suzuki and Harada, 2014; Harada et al., 2015a).

The cytosolic PNGase (peptide:*N*-glycanase; NGLY1/Ngly1 in human/mice) is a well-conserved deglycosylating enzyme that is involved in the non-lysosomal degradation of *N*-glycoproteins. PNGase releases intact *N*-glycans from glycoproteins and is involved in the clearance of misfolded glycoproteins from the cytosol. The recent discovery of a *NGLY1*-deficiency, a human genetic disorder, clearly indicates that this enzyme is critical for the normal development of mammals (Need et al., 2012). This review focuses on mammalian gene orthologues and their products. For more detailed reviews on the cytoplasmic PNGase, readers

are directed to the following articles (Suzuki et al., 2002a; Suzuki and Lennarz, 2003; Suzuki, 2007; Hirayama et al., 2015; Suzuki, 2015).

2. The cytoplasmic PNGase – distribution and enzymatic characteristics

PNGase catalyzes the hydrolysis of the amide bond between the innermost *N*-acetylglucosamine (GlcNAc) and an Asn residue on an *N*-glycoprotein, generating a de-*N*-glycosylated protein, in which the *N*-glycosylated Asn residue is converted to Asp, and a 1-amino-GlcNAc-containing free oligosaccharide (Figure 1). Ammonia is then spontaneously released from the reducing end at physiological pH (Isbell and Frush, 1950; Makino et al., 1968) resulting in the production of a free oligosaccharide with an *N,N'*-diacetylchitobiose at the reducing end (Figure 1).

Occurrence of cytoplasmic PNGase activity in mammalian cells was first reported in several cultured cells (Suzuki et al., 1993). This enzyme differs from other “reagent” PNGases from plants (glycoamidase/PNGase A) or bacteria (*N*-glycanase/PNGase F) in several enzymatic properties, including the requirement of a reducing reagent for activity and a neutral pH for optimal activity (Suzuki et al., 1993; Suzuki et al., 1994b; Kitajima et al., 1995). In addition to its enzyme activity, Ngly1 has an intrinsic carbohydrate-binding property (Suzuki et al., 1994a; Suzuki et al., 1995). The catalytic core in the primary structure of the PNGase molecule contains a core GlcNAc₂-binding site (Suzuki et al., 2006; Zhao et al., 2009), and an additional C-terminal PAW domain (a domain present in PNGases and other worm proteins)

(Doerks et al., 2002) was reported to serve as a carbohydrate-binding domain for high mannose-type glycans (Zhou et al., 2006; Kamiya et al., 2009) (Figure 2). On the other hand, an N-terminal PUB domain (a domain present in *PNGase/UBA* or *UBX*-containing proteins) (Suzuki et al., 2001) have been demonstrated to serve as a p97 binding module (Allen et al., 2006).

The gene encoding the cytoplasmic PNGase was first identified in budding yeast, *Saccharomyces cerevisiae* (Suzuki et al., 2000) and gene orthologues have since been found in wide variety of eukaryotes including mammals (Suzuki et al., 2000; Suzuki et al., 2002a). In terms of the tissue distribution of the mouse cytoplasmic PNGase, a 2.6 kb *Ngly1* mRNA was found to be widespread in all tissues examined, with the highest level in the testis (Suzuki et al., 2003). An additional 2.4 kb transcript was also found in the testis. Regarding enzyme activity in female ICR mice, PNGase activity with similar enzymatic properties was found in all tissues and organs tested (Kitajima et al., 1995). On the other hand, such activity was not found in sera, suggesting that this enzyme is not secreted. Among the organs tested, the liver had the highest total activity, while the thymus showed the highest specific activity (Kitajima et al., 1995).

3. Function of Ngly1-a link to ERAD

In terms of the function of Ngly1, it has been shown that the enzyme is involved in the ER-associated degradation (ERAD) process. In the late 1990's several ERAD substrates were reported to be deglycosylated by the cytoplasmic PNGase during their degradation (Wiertz et

al., 1996a; Wiertz et al., 1996b; Halaban et al., 1997; Hughes et al., 1997; Huppa and Ploegh, 1997; Suzuki et al., 1997; Yu et al., 1997; Bebok et al., 1998; de Virgilio et al., 1998; Johnston et al., 1998; Mosse et al., 1998). More recently, *in vivo* Ngly1 based-deglycosylation has been detected using a sophisticated assay system based on the deglycosylation-mediated fluorescence (Figure 3) (Grotzke et al., 2013; He et al., 2015).

Despite all the evidence pointing to the role of Ngly1 in the ERAD process, the critical role of Ngly1-mediated deglycosylation in the degradation of the ERAD substrate remains unclear. For example, when Ngly1 expression is suppressed, the deglycosylation of the TCR α subunit and the MHC class I heavy chain, both of which are well-studied glycoprotein ERAD substrates, is reduced whereas no significant delay in degradation was observed (Hirsch et al., 2003; Blom et al., 2004). Moreover, the inhibition of Ngly1 activity had no effect on the degradation of the MHC class I heavy chain (Misaghi et al., 2004). Indeed, it has been shown that the removal of *N*-glycans is not a prerequisite for proteasomal degradation and proteasomes can degrade several glycoproteins efficiently *in vitro* (Kario et al., 2008). However, some examples of Ngly1-dependent degradation have recently been reported; Park et al. showed that EDEM1, a key component of the glycoprotein ERAD, was stabilized upon the inhibition of Ngly1 (Park et al., 2014). More recently, RTA Δ m, a model ERAD substrate originating from a toxic plant protein, was also shown to be stabilized in *Ngly1*-KO mice embryonic fibroblasts (Huang et al., 2015). Interestingly, in both cases, deglycosylation of the substrates were still observed even

though there was low/no Ngly1 activity. It was reported that, in the case of RTA Δ m, the unconventional deglycosylation was catalyzed by a cytosolic endo- β -*N*-acetylglucosaminidase (ENGase) (Huang et al., 2015), which also occurs ubiquitously in eukaryotes (Suzuki et al., 2002b). Surprisingly, no delay in RTA Δ m degradation was observed for *Ngly1/Engase* double KO cells (Huang et al., 2015). These collective results suggest that the action of ENGase on misfolded glycoproteins could cause the production of excess *N*-GlcNAc proteins (ENGase products), which could potentially lead to the delayed degradation of a subset of misfolded glycoproteins. Such a degradation defect could have detrimental effects for cells, by creating intrinsic toxicity as aggregates and/or the impairment of intracellular signaling via the *O*-GlcNAc (*N*-GlcNAc hypothesis; Figure 4) (Huang et al., 2015). This could explain, at least to some extent, the pathology associated with *NGLY1* deficiency, a newly found human genetic disorder (see below). If this is indeed the case, an inhibitor specific for the cytosolic ENGase would be an attractive drug candidate for the treatment of an *NGLY1*-deficiency. At this moment, however, whether the excess formation/accumulation of *N*-GlcNAc proteins can explain the pathophysiological conditions of an *NGLY1*-deficiency remains to be clarified.

4. Role of Ngly1 in MHC class I-mediated antigen presentation

While the biological significance of Ngly1-mediated deglycosylation *in vivo* is not presently fully understood, it has been well documented that it plays a critical role in MHC class I-mediated antigen presentation. First, when the natural peptide target for melanoma-

reactive cytotoxic T-cell clones was examined, it was found that an Asn residue at a potential *N*-glycosylation site was converted to Asp in a peptide derived from tyrosinase, a key enzyme for melanin production (Skipper et al., 1996). These results raised the possibility that Ngly1 may be the responsible enzyme for this Asn-to-Asp conversion. For the efficient presentation of the tyrosinase-derived peptides to MHC class I molecules, it was suggested that tyrosinase needed to be first translocated into the ER to receive the *N*-glycans (Mosse et al., 1998). It was subsequently found that unglycosylated tyrosinase is rapidly degraded and therefore failed to be efficiently presented on MHC class I molecules (Ostankovitch et al., 2009). Ngly1 activity was also shown to affect the efficiency of antigen presentation, pointing to the functional importance of Ngly1-mediated deglycosylation in the processing of antigen peptides (Altrich-VanLith et al., 2006; Kario et al., 2008). The Ngly1-mediated (*N*-glycosylated-)Asn-to-Asp deamidation, together with other reactions such as transpeptidation, constitutes unconventional post-translational modifications for antigenic peptides that are presented by MHC class I molecules (Dalet et al., 2011).

5. Role of Ngly1 in formation of cytosolic free oligosaccharides

The action of PNGase results in free oligosaccharides (free *N*-glycans or FNGs) being released in the cytosol (Figure 4). In mammalian cells, the ablation of both Ngly1 and ENGase had no effect on the total amount of FNGs (Harada et al., 2015b). It has been suggested that, in mammalian cells, the hydrolysis of dolichol-linked oligosaccharides, a donor substrate for

protein *N*-glycosylation, is the predominant source for FNG formation (Harada, et al., 2015b).

The hydrolysis reaction is most likely catalyzed by an oligosaccharyltransferase, an *N*-glycosylating enzyme complex in the ER (Harada, et al., 2013; Harada, et al., 2015b). The functional importance of the hydrolysis of dolichol-linked oligosaccharides in mammalian cells remains unclarified. This situation is in sharp contrast to the case with *S. cerevisiae*, where >95% of the FNGs were released by the cytosolic PNGase during the ERAD process (Hirayama et al., 2010; Harada et al., 2013). These results clearly indicate that the mechanisms responsible for the formation of FNGs are quite distinct among organisms.

6. Ngly1-interacting proteins

Through yeast two-hybrid screening, it has been shown that Ngly1 proteins can bind to several proteins, mostly through the N-terminal part that includes the PUB domain (Park et al., 2001). *In vivo* and *in vitro* interactions between Ngly1 and several ERAD-related proteins have subsequently been confirmed (Table 1). In some cases, other molecules such as ubiquitin E3 ligase AMFR (*autocrine motility factor receptor*; also known as gp78) or SAKS (a *stress-activated protein kinase substrate*) have been shown to bind to Ngly1 through their interaction with p97 (McNeill et al., 2004; Li et al., 2005; Li et al., 2006). It should be noted that the interaction of p97 with Ngly1 is through its terminal 10 amino acid residues, and the phosphorylation of Tyr806 of p97 in the binding motif could modulate the binding between two proteins (Zhao et al., 2007). More recently, an *in silico* network analysis using the BioGRID

database of physical and genetic interactions (Stark et al., 2006) identified the FAF1 (fas-associated factor 1) protein as a possible binding protein through the PUB domain, while experimental validation remains to be seen (Tickotsky-Moskovitz, 2015).

While the importance of those protein-protein interactions to Ngly1 functions remain to be clarified, it can be assumed that such interactions may be advantageous for an efficient ERAD process (Suzuki and Lennarz, 2003).

7. *NGLY1*-deficiency

In 2012, a human genetic disorder involving mutations in the *NGLY1* gene locus was identified through an exome analysis (Need et al., 2012). As of now, the clinical features of 12 patients have been reported (Enns et al., 2014; Caglayan et al., 2015; Heeley and Shinawi, 2015; Bosch et al., 2015). The symptoms of these patients include global developmental delay, movement disorder, hypotonia, abnormalities in electroencephalogram and the absence of tears. These observations clearly demonstrate the functional importance of the cytosolic PNGase. Details of the molecular mechanism responsible for the pathology of the *NGLY1* deficiency, however, remains poorly understood.

Among the *NGLY1*-deficiency patients examined to date, a non-sense mutation (c.1201A>T (p.R401X)) is the most commonly detected allele (Table 2). This p.R401X allele has been observed in 2 out of 8,598 chromosomes of European ancestry reported in the Exome Variant Server resource (Enns et al., 2014). It has been suggested that the homozygous mutation

associated with this allele shows more severe phenotypes compared to patients bearing other alleles (Enns et al., 2014). Further studies will clearly be required to understand how this mutation contributes to the more severe symptoms.

All patients so far reported are of Caucasian ethnic origin (Enns et al., 2014; Bosch et al., 2015; Caglayan et al., 2015; Heeley and Shinawi, 2015), implying that the frequency of this genetic disorder may be different between ethnic groups.

8. Concluding remarks

Ngly1 is a de-*N*-glycosylating enzyme that is widely expressed in the organs and tissues of mammals, and this protein is also evolutionary conserved throughout eukaryotes. Much remains to be clarified, however, in terms of the mysteries regarding this enzyme. For example, the *NGLY1* gene orthologue of *Neurospora crassa* (*PNG1*) encodes a catalytically-inactive protein as an enzyme due to the intrinsic mutations of catalytic residues, yet the strains bearing mutations on this gene exhibited severe temperature-sensitive defects in polar growth (Seiler and Plamann, 2003; Maerz et al., 2010). These results clearly indicate that, at least for fungi orthologues, the *NGLY1*-orthologue possesses an enzyme-independent function. The nature of the enzyme-independent function of this protein remains unknown, and accordingly, the issue as to whether it is conserved in mammals is yet to be clarified. Moreover, a single nucleotide polymorphism of a locus 105 kb upstream of *NGLY1* has been recently identified as one of the potential loci susceptible for Parkinson's disease through a multi-locus association analysis of

genome-wide SNP data (Shriner and Vaughan, 2011), while the biological relevance of this finding remains unclarified. Finally, *NGLY1* was identified as a gene that is differentially expressed in a number of mitochondrial diseases (Zhang and Falk, 2014). Why a mitochondrial dysfunction would lead to the dysregulation of the expression of the *NGLY1* gene, encoding a non-mitochondrial (cytosolic) enzyme, remains completely unknown. The question of how the expression of *NGLY1* can be correlated with mitochondria functions therefore is a subject worthy of being investigated. Further studies by more researchers with diverse research backgrounds should be critical for unveiling the functional details of this intriguing protein.

Acknowledgements

This review and the corresponding Gene Wiki article are written as part of the Gene Wiki Review series--a series resulting from a collaboration between the journal GENE and the Gene Wiki Initiative. The Gene Wiki Initiative is supported by National Institutes of Health (GM089820). Additional support for Gene Wiki Reviews is provided by Elsevier, the publisher of GENE. We thank the members of the Glycometabolome Team (RIKEN) for fruitful discussions. The work on Ngly1/PNGase in Suzuki laboratory was supported in part by PRESTO/CREST, Japan Science and Technology Agency (JST), Global Center of Excellence Program, Osaka University, Mizutani Foundation for Glycosciences, Yamada Science Foundation, Mochida Memorial Foundation for Medical and Pharmaceutical Research, Toray

Science Foundation, Grace Wilsey Foundation, and Grant-in-Aids for Scientific Research (grant no. 15770081, 17687009, and 26110725) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to TS), and a grant for Incentive Research Project, RIKEN (to HF). We also would like to express sincere gratitude to Mr. Hiroshi Mikitani (Rakuten Inc.; Tokyo, Japan) for his financial support for *NglyI* research. CH is supported by the RIKEN Foreign Postdoctoral Researcher Program.

Table 1 List of Ngly1-interacting proteins

Proteins	Role in ERAD	References
HR23B	Ubiquitin receptor	(Park et al., 2001)
		(Katiyar et al., 2004)
		(Kamiya et al., 2012)
S4	Subunit of Proteasome	(Katiyar et al., 2004)
		(Li et al., 2006)
Derlin-1	Predicted to be a part of retrotranslocation complex	(Katiyar et al., 2005)
Cdc48/p97/VCP	AAA-ATPase playing a central role to extract ERAD substrates from the ER to the cytosol	(McNeill et al., 2004)
		(Li et al., 2005)
		(Li et al., 2006)
		(Zhao et al., 2007)

Table 2 List of mutations reported in patients with an *NGLY1* deficiency

		Mutations
Enns et al., 2014	Patient 1	c. C1891del(p. Q631S)/ c. 1201A>T (p. R401X)
	Patient 2	c. 1370dupG (p. R458fs)/ c. 1370dupG (p. R458fs)
	Patient 3	c. 1205_12-7del (p. 402_403del)/ c. 1570C>T (p. R524X)
	Patient 4	c. 1201A>T (p. R401X)/ c. 1201A>T (p. R401X)
	Patient 5	c. 1201A>T (p. R401X)/ c. 1201A>T (p. R401X)
	Patient 6	c. 1201A>T (p. R401X)/ c. 1201A>T (p. R401X)
	Patient 7	c. 1201A>Y (p. R401X)/ c. 1201A>T (p. R401X)
	Patient 8	c. 1201A>T (p. R401X)/ c. 1201A>T (p. R401X)
Caglayan et al., 2015	Patient 1	c. 1533_1536delTCAA (p. N511KfsX51)/ c. 1533_1536delTCAA (p. N511KfsX51)
	Patient 2	c. 1533_1536delTCAA (p. N511KfsX51)/ c. 1533_1536delTCAA (p. N511KfsX51)
Heeley and Shinawi, 2015	Patient 1	c. 347C>G (p. S116X)/ c. 881+5G (p. IVS5+5G>T)
Bosch et al., 2015	Patient 1	c. 1201A>T (p. R401X)/ c. 1201A>T (p. R401X)

Abbreviations

ER, endoplasmic reticulum; ERAD, ER-associated degradation; ENGase, endo- β -*N*-acetylglucosaminidase; FNGs, free *N*-glycans; GlcNAc, *N*-acetylglucosamine; MHC, major histocompatibility complex; PNGase, peptide:*N*-glycanase; PAW domain, a domain found in PNGases and other worm proteins; PUB domain, a domain found in PNGase/UBA or UBX-containing protein; RTA Δ m, a monoglycosylated non-toxic mutant of ricin a subunit; SNPs, single nucleotide polymorphisms; TCR α , T cell receptor α chain

Figure 1: Reaction scheme for peptide:N-glycanase (Ngly1)

Figure 2: Schematic representation of the domain structure of PNGase (Ngly1) from human, mouse, yeast, and fungi.

Gray bar in the scheme is a putative zinc-binding site (Lee et al., 2005). PUB: a domain present in PNGase and UBA- or UBX-containing proteins (p97 binding motif), TG: transglutaminase domain (catalytic domain), PAW: a domain present in PNGase and other worm protein domains (high mannose-type glycan-binding domain). These domains were based on SMART (Simple Molecular Architecture Research Tool) program (<http://smart.embl-heidelberg.de/>). X indicates the sites for mutations found in *NGLY1*-deficiency patients.

Figure 3: Principle of Ngly1 activity assay using a split Venus system (Grotzke et al., 2013)

In this system, the C-terminal Venus fragment is expressed in the cytosol, while the N-terminus fragment with an *N*-glycosylation site is expressed in the ER to undergo *N*-glycosylation. The N-terminal fragment is recognized by the ER quality control system and is retrotranslocated into the cytosol. The N- and C-terminal fragment forms a complex, and only when the *N*-glycan on N-terminal fragment is removed by Ngly1, does the fluorescence increase. One can therefore measure the *in vivo* activity of Ngly1 through the fluorescence of the Venus protein.

Figure 4: Scheme of *N*-GlcNAc hypothesis (Huang et al., 2015)

Under normal conditions, Ngly1 preferentially acts on misfolded glycoproteins during their proteasomal degradation, while ENGase acts on free oligosaccharides for non-lysosomal (cytoplasmic) glycan catabolism (Suzuki, 2007; Suzuki and Harada, 2014; Harada et al., 2015a).

In the case of the absence of Ngly1, however, ENGase stochastically acts on misfolded glycoproteins. Reaction of ENGase with misfolded glycoproteins results in the formation of *N*-GlcNAc proteins. The formation of excessive amounts of *N*-GlcNAc proteins in the cytosol may cause (1) the formation of toxic protein aggregates and/or (2) impairment of proper *O*-GlcNAc signaling in the cytosol/nucleus.

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Figure 1

Figure 2

Figure 3

Cytoplasm

Figure 4