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JB Special Review—Fundamental Roles of Glycans in Eukaryotes

The cytoplasmic peptide:*N*-glycanase (Ngly1)—basic science encounters a human genetic disorder

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Peptide:*N*-glycanase (PNGase) is a de-*N*-glycosylating enzyme that cleaves intact *N*-glycans from glycoproteins/glycopeptides. The activity of the cytoplasmic PNGase in several mammalian-derived cultured cells was first reported in 1993, and 7 years later, the gene encoding the enzyme was identified in budding yeast. Although the gene—*PNG1* in budding yeast and *NGLY1*/*Ngly1* in mammalian cells—appears to be well conserved throughout eukaryotes, the biological significance of this enzyme has remained elusive until recently. However, discovery of a human genetic disorder involving the *NGLY1* gene clearly indicates that this enzyme plays a critical role in human biology. This review summarizes the research history of cytoplasmic PNGase. The importance of curiosity-driven, pure 'basic science' will also be discussed.

Keywords: Ngly1/PNGase/ERAD/genetic disorder/basic science.

Abbreviations: ENGase, endo-β-*N*-acetylglucosaminidase; ER, endoplasmic reticulum; ERAD, ER-associated degradation; fOS, free oligosaccharide; GH, glycoside hydrolase; PAW, *P*NGases *a*nd other *w*orm; PNGase, peptide:*N*-glycanase; PUB, *P*NGase and *ub*iquitin; RTAΔ, ricin toxin A chain mutant; RTL, RTAΔ-transmembrane-Leu2; TGase, transglutaminase.

Progress in glycobiology over the past few decades has been explosive, and more and more researchers now appreciate the functional importance of glycan chains on glycoproteins. For example, the glycosylation of proteins has been shown to play critical roles in modulating the physiological/physicochemical properties of cognate proteins (*1*, *2*).

Peptide:*N*-glycanase (PNGase; peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidase; also referred to as glycoamidase or *N*-glycanase; EC 3.5.1.52) catalyzes the hydrolysis of the amide bond between the proximal *N*-acetylglucosamine (GlcNAc) residue and the asparagine residue of a protein, generating an aspartic acid-containing polypeptide chain and a 1-amino GlcNAc-containing oligosaccharide (Fig. 1). Ammonia is then spontaneously released from the 1-amino GlcNAc at physiological pH (<8), giving rise to a free oligosaccharide (fOS) with an *N,N*′-diacetylchitobiose structure at the reducing end. PNGase activity was first described in almonds by Dr Noriko Takahashi in 1977 (*3*), followed by the identification of PNGase F from bacteria (*4*). Since then, those PNGases from bacteria/plant origin have been extensively used for analysing structures/functions of *N*-glycans on cognate glycoproteins (*5*, *6*). In my opinion, it is not an overstatement to say that PNGase is a critical reagent for the advancement of glycobiology, as this enzyme greatly facilitates structure/function analyses of *N*-glycans because glycans can be released from glycoproteins under much milder conditions, compared with chemical deglycosylation, which may result in damaging the protein. Interestingly, however, the physiological function of PNGase itself had not been rigorously investigated in any organisms when I started my career as a researcher.

The Cytoplasmic PNGase—Early History

In 1991, I was an undergraduate student in the laboratory of Prof. Yasuo Inoue, then the Department of Biochemistry and Biophysics, Faculty of Science, the University of Tokyo. That was the monumental year for PNGase research as Dr Akira Seko, then a graduate student in Prof. Inoue's laboratory, discovered the first occurrence of PNGase activity of animal origin, which prefers an acidic pH for optimal activity, in Medaka fish embryos (*7*). My initial project therefore was to search for similar PNGase activity in mammalian cells. Unfortunately, we were not able to identify PNGase activity similar to that found in Medaka embryos, but after a year of struggle, we unexpectedly identified the PNGase activity with totally distinct enzymatic properties in various mammalian-derived cultured cells (*8*, *9*). The optimal activity of the typical mammalian PNGase is at a neutral pH, and a reducing reagent such as dithiothreitol is required for the *in vitro* detection of activity. The most striking (and puzzling) observation was that most of the enzyme activity was recovered from the cytosol fraction (*8*, *10*). Initially, this observation obscured the functional importance of this enzyme, as not many researchers believed that the *N*-glycoproteins, potential substrates for the enzyme, would be localized in the cytosol. Why would one expect PNGase to be localized in the cytosol, where no substrates are there?

Featured Article

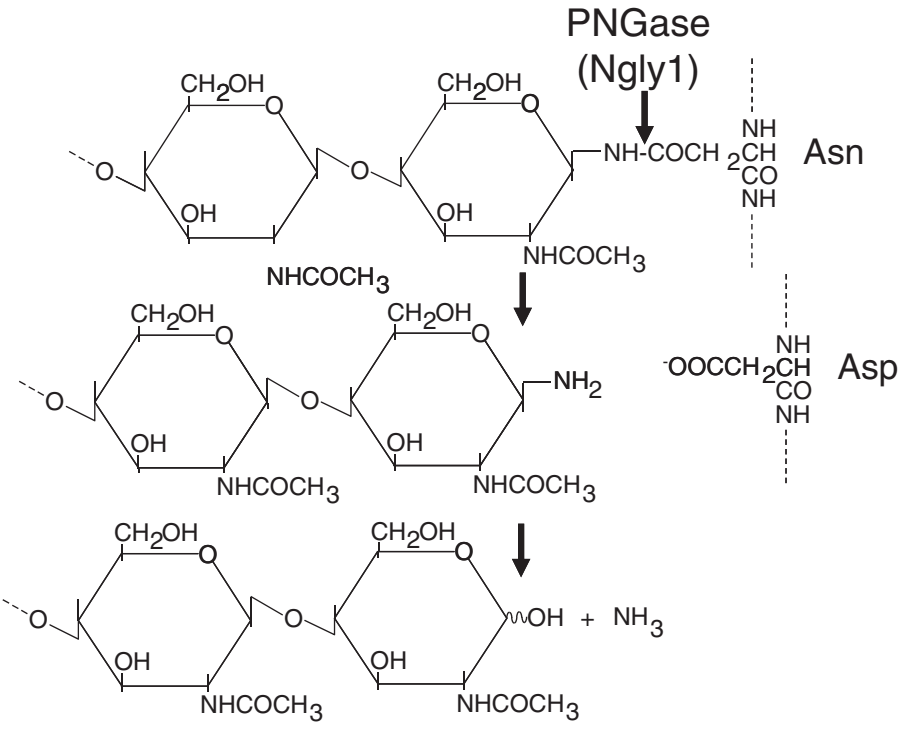

Fig. 1 Reaction scheme of PNGase. PNGase catalyzes the cleavage of the amide bond between the proximal GlcNAc residue and the asparagine residue, thereby producing a deglycosylated peptide in which the glycosylated asparagine is converted to aspartic acid and a free glycan with a 1-amino GlcNAc at the reducing end. The glycan is spontaneously hydrolyzed at physiological pH (<8) to generate ammonia and the corresponding free glycan with *N,N*-diacetylchitobiose at the reducing end.

Although we confirmed that the soluble PNGase activities occur ubiquitously in mammalian cells and tissues (*8*, *10*), the functional role for this enzyme was very uncertain. Accordingly, I conducted an extensive literature survey to look for a clue for the potential function of PNGase or other deglycosylation enzymes in various cellular events, and several possibilities arose (*11*, *12*). One of those hypotheses was that this enzyme could be involved in 'non-lysosomal' processing or the degradation of proteins (*12*). This was about the time when the emerging concept of 'endoplasmic reticulum (ER)-associated degradation' rocketed to fame.

ER-Associated Degradation–Cytosolic Connection

The ER functions to synthesize soluble/membrane-spanning proteins that pass through the secretory pathway in eukaryotic cells. Proteins that are translocated into the lumen of the ER are then co- and post-translationally modified in various ways, including *N*-glycosylation, and eventually exit the ER to be delivered for their respective destinations via vesicular transport. On the other hand, it has long been known that this organelle appears to retain a 'quality control' or 'homeostasis' system, so that especially non-functional or malfunctional proteins that fail to fold or oligomerize in a correct manner do not follow the similar transport pathway and are instead degraded rapidly. This degradation process was independent of lysosomal degradation and was initially assumed to occur in the ER (*13*). However, it was subsequently found that the cytoplasmic proteasome plays a central role in degrading such proteins (*14–20*). The process, now called ER-associated degradation or ERAD, constitutes one of the protein quality control systems in eukaryotes (*21*, *22*). In this system, misfolded glycoproteins in the lumenal side are released into the cytosol, a process referred to as retrotranslocation or dislocation, after which the cytoplasmic PNGase gains an access to the substrates, *N*-glycoproteins, during their proteasomal degradation (Fig. 2). Soon after the cytoplasmic connection associated with the ERAD process was revealed, several glycoproteins were proposed to be deglycosylated, presumably by the cytoplasmic PNGase, during the degradation process (*19*, *23–32*). These results prompted me to identify the gene encoding the cytoplasmic PNGase to gain additional insights into the functional importance of this enzyme.

Identification of Gene Encoding the Cytoplasmic PNGase

After completing the PhD degree in 1997, I moved to the laboratory of Prof. William J. Lennarz at the State University of New York at Stony Brook as a post-doctoral fellow. I started to work with budding yeast, *Saccharomyces cerevisiae*, and after successful detection of cytoplasmic PNGase activity with similar enzymatic properties (neutral optimal pH and the

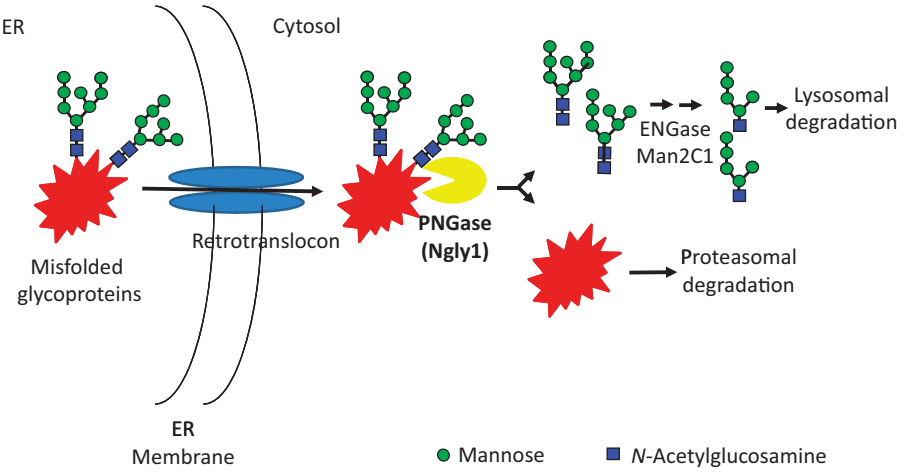

Fig. 2 The involvement of cytoplasmic PNGase in ERAD. The glycoproteins destined for degradation are translocated from the ER lumen to the cytosol. There, proteins are degraded mainly by proteasomes. PNGase acts on misfolded glycoproteins in the cytosol during the proteasomal degradation. This reaction is believed to enhance the efficiency of proteasomal degradation, while the precise order of the deglycosylation and proteasomal degradation under physiological conditions remains unclarified. Note that other protein modification reactions, such as ubiquitination, are omitted in this scheme for the sake of simplification.

requirement of a reducing reagent for an enzyme assay) (*33*), we finally identified the gene encoding the cytoplasmic PNGase, which is referred to as *PNG1* (*34*). The process involved in the identification of the gene was very unique; first, we screened over 400 of Hartwell's temperature-sensitive strain collections (*35*) and isolated strain no. 352 as the one that was defective in PNGase activity. Our initial expectation was that the PNGase might be an essential gene in yeast, and therefore mutants with defective PNGase would exhibit a signature phenotype, if isolated. To our disappointment, however, after several backcrosses with wild-type strains, no detectable phenotypes co-segregated with a defect of PNGase activity. We then made full use of the combination of classical genetics and the most recent genome information of *S. cerevisiae* to identify the causative gene responsible for the loss of enzyme activity. The gene, *PNG1*, was indeed the one encoding the cytoplasmic PNGase (*34*). The lack of significant phenotypes in *png1* mutant in budding yeast, however, prevented us from clarifying the biological significance of this enzyme. The mouse orthologue, referred to as *Ngly1* (*36*), was found to be widely expressed in all tissues examined, which is consistent with the results of an *in vitro* PNGase activity assay (*10*).

Structural Divergence of the Cytoplasmic PNGase—Evolutional Consequence?

When examined in the DNA database, it was found that the *PNG1* gene orthologue was widely distributed throughout eukaryotes (*34*, *37*). Consistent with this finding, the soluble PNGase activity with a neutral optimal pH has also been widely reported among eukaryotes (Table I). An interesting feature of the cytoplasmic PNGase is that, while the core transglutaminase (TGase) domain critical for the PNGase activity is conserved, additional domains can be found in the orthologues of higher eukaryotes (Fig. 3). These results most likely suggest that this protein acquired those additional domains as an evolutional consequence. For example, it is interesting to note that in *Caenorhabditis elegans*, the cytoplasmic PNGase is a dual functional enzyme as thioredoxin, an oxidoreductase, and PNGase (*38*, *39*). This domain structure may reflect the evolutional requirement linking redox potential and PNGase activity, especially as this enzyme has a catalytic Cys residue. The contribution of those domains outside the catalytic core to the function of the cytoplasmic PNGase represents a key issue that remains to be fully elucidated.

Among the additional domains found in the orthologues of higher eukaryotes, the PUB (*P*NGase- and *ub*iquitin related) domain was first identified through a bioinformatics analysis, as I noted that this domain was also observed in various ubiquitin–proteasome system-related proteins (*46*, *47*). Although it was initially hypothesized that it may serve as a protein–protein interaction domain (*46*), experimental evidence supporting the hypothesis is now accumulating (*48–51*). On the other hand, the C-terminal PAW domain (domain present in *P*NGases *a*nd other

Table I. Reported occurrence of cytoplasmic PNGase activity.

Source	References
Mammalian cells	(*8*, *10*, *40*)
Hen	(*28*)
Medaka Fish	(*41*)
C. elegans[a]	(*38*, *39*)
D. discoidium	(*42*)
S. cerevisiae	(*33*)
Schizosaccharomyces pombe[a]	(*43*)
A. thaliana[a]	(*44*, *45*)

[a]PNGase activity was found in the Png1-orthologue protein expressed in a heterologous system.

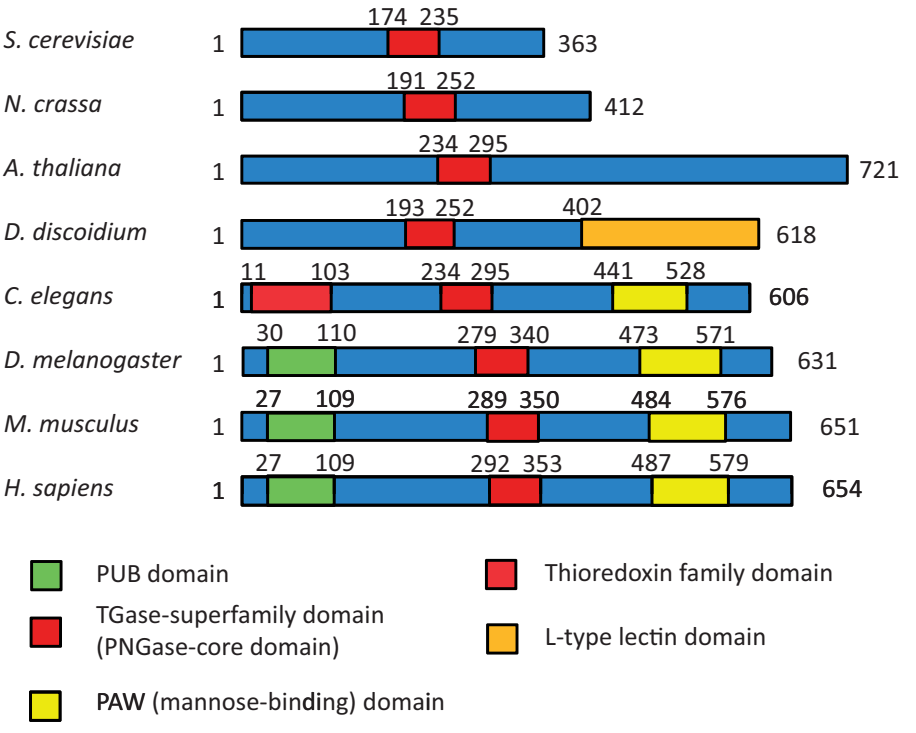

Fig. 3 Schematic representation of the primary structure of various eukaryotic PNGase orthologues. Accession numbers for the protein sequences in Universal Protein Resource (http://www.uniprot.org) are as follows: *S. cerevisiae*, Q02890; *N. crassa*, D1MY48; *A. thaliana*, Q9FGY9; *D. discoideum*, Q55FC8; *C. elegans*, Q9TW67; *Dr. melanogaster*, Q7KRR5; *Mus musculus*, Q9JI78; *Homo sapiens*, Q96IV0. The domain structure is based on the conserved domain database in National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/cdd) and common region aligned in all PNGase orthologues are shown: PUB domain (domain ID: cd10459; residue 11–93); TGase-superfamily domain (domain ID: pfam01841; residue 37–107); PAW (mannose binding) domain (domain ID: smart00613; residue 1–89); Thioredoxin-family domain (domain ID: cd02947; residue 2–92); L-type lectin domain (domain ID: cd01951; residue 11–223).

*w*orm proteins) (*47*) has now been shown to be involved in the oligosaccharide binding of PNGase (*52*). The cytoplasmic PNGase from mammalian cells was previously found to retain lectin-like properties (*53*) and it was later suggested that certain types of fOSs can inhibit the enzyme activity in a non-competitive manner (*54*). The PAW domain, as an additional carbohydrate-binding domain besides the catalytic site, may be involved in this interesting biochemical property found in mammalian cytoplasmic PNGases. With regard to the sugar-binding specificity of the PAW domain, it was reported that the domain preferably binds larger high-mannose glycans, especially with those retaining the intact C-arm and lack the terminal α1,2-linked mannose in B-arm (Fig. 4) (*55*). How these specificities correlate with the properties of the cytoplasmic PNGase remains to be investigated.

Besides the mannose-binding PAW domain, the core TGase domain has an intrinsic ability to bind to *N*, *N*-diacetylchitobiose-containing glycans (*54*, *56*, *57*). Haloacetamidyl derivatives of oligosaccharides containing an *N*,*N*-diacetylchitobiose structure are potent inhibitors as well as a labelling reagent of the cytoplasmic PNGase, as they specifically label the catalytic Cys residue (*56*, *58*). Those probes were shown to bind to other PNGase orthologues (*59*, *60*), indicating that *N*,*N*-diacetylchitobiose-binding properties are a common feature of the cytoplasmic PNGase. The *N*,*N*-diacetylchitobiose-modified yeast PNGase has been structurally characterized, and the residues responsible for carbohydrate binding have been identified (*57*). It was also shown that Z-VAD-fmk, a general caspase inhibitor, is a potent inhibitor of cytoplasmic PNGase (*61*, *62*). Although Z-VAD-fmk may be used as an inhibitor of cytoplasmic PNGase, caution may be taken in its use, since it has a broad inhibition profile.

Protein–Protein Interaction Involving the Cytoplasmic Peptide:*N*-glycanase

It should be noted that the cytoplasmic PNGase can participate in various protein–protein interactions with other proteins. In 2001, Rad23 was identified as a PNGase-binding protein through yeast two-hybrid screening (*63*). Although Rad23 was first identified as a protein involved in nucleotide excision repair (*64*), evidence shows that this protein binds to the 26S proteasome through the N-terminal ubiquitin-like domain (*65*) and also plays a role in proteasomal degradation (*66*, *67*). Although the interaction between Rad23 and the PNGase appears to be conserved, the complex interphase is fundamentally different between yeast and mouse proteins, as revealed by the structure analysis of the PNGase–Rad23 complex (*68*, *69*). It is also worth mentioning that Rad23 forms a complex with another DNA repair-related protein, Rad4, which has a similar core 'TGase fold' (*70*). It is therefore tempting to speculate that PNGase–Rad23 and

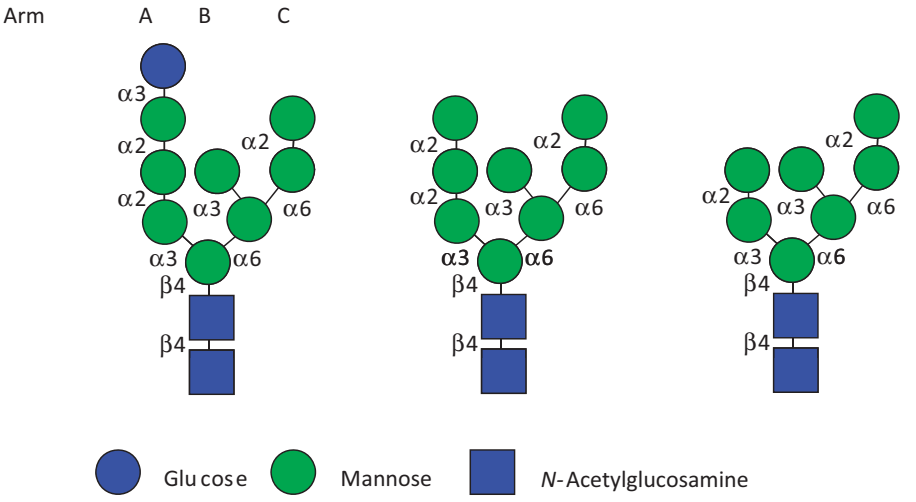

Fig. 4 Structures of *N*-glycans bound with relatively higher affinity ($Ka > 4.0 \times 10^4$) to the PAW domain of mouse Ngly1, as determined by frontal affinity chromatography analysis (55). The definition of the arms (A/B/C arm) described in the text is also indicated.

Rad4–Rad23 may have evolved from a common ancestor protein complex.

In addition to Rad23, various PNGase-binding proteins have been isolated for mouse Ngly1 protein (*71*). It is interesting to note that, among the proteins identified, most bind through the N-terminal region of Ngly1, which contains the PUB domain. One should also note that, upon subcellular fractionation, a minor fraction of the PNGase activity was associated with the membrane-bound fraction (*8*, *10*, *33*). Interestingly, the cytoplasmic PNGase in mammals is able to bind to p97/VCP/Cdc48, a key ATPases accosiated with diverse cellular activities (AAA) adenosine triphosphate (ATPase) for the ERAD pathway, as well as other ERAD or ubiquitin–proteasome pathway-related proteins, some of which are intrinsic membrane proteins (*71–75*). Those protein–protein interactions may also be mainly mediated by the PUB domain. Given the minor distribution of Ngly1 in the membrane fraction, however, binding to membrane proteins should be a sub-stoichiometric interaction. How such minor interactions contribute to the critical function of Ngly1 will need to be clarified via further studies. Recent findings also suggest that, at least *in vitro*, Fbs1, a glycoprotein-specific ubiquitin ligase (*76*, *77*), protects misfolded glycoproteins from the action of cytoplasmic PNGase (*78*). These protein–protein interactions may be critical for establishing complex protein networks and for regulating the sequential reactions in a concerted manner, as the formation of such so-called 'glycoprotein degradation complexes' may greatly enhance the efficiency of ERAD by carrying out a number of reactions, *i.e.* ubiquitination, deglycosylation, deubiquitination and proteolysis in one location (*79*, *80*).

PNGase-Dependent and -Independent Pathway for Non-Lysosomal Degradation of Free Oligosaccharides

In yeast cells, the absence of cytoplasmic PNGase (Png1) results in significant reduction of the levels of fOSs found in the cytosol, suggesting that the majority, if not all, of the fOSs in yeast are generated from misfolded glycoproteins in a PNGase-dependent manner (*81–83*). Therefore, these results indicate that analyses of the structure/amount of fOSs could serve as a readout of overall glycan processing during the ERAD process (*82*, *83*). Interestingly, this does not appear to be the case in mammalian cells, as the knockdown or knockout of the *Ngly1* gene has only a negligible effect on the structure/amount of fOSs (*84*) (Harada, Y. and Suzuki, T., unpublished data). These results clearly suggest that, in mammalian cells, the fOSs are derived from the cytoplasmic PNGase-independent reactions, the majority of which are, most likely, due to the hydrolytic activity of oligosaccharyltransferase (*85*). Therefore, one should evaluate the origin of fOSs carefully when analysed in different organisms (*86*). There are also cytosolic glycosidases involved in the catabolism of fOSs in mammals, such as endo-β-*N*-acetylglucosaminidase (ENGase) (*87*, *88*) or the cytoplasmic α-mannosidase, Man2C1 (*89*, *90*). For more details on the non-lysosomal process of glycan catabolism, readers are directed to review articles (*80*, *86*, *91*, *92*).

Functional Role of the Cytoplasmic PNGase in Various Organisms

Since the gene encoding the cytoplasmic PNGase was identified, much progress has been made in clarifying its role in the ERAD process. In budding yeast, a ricin toxin A chain mutant (RTAΔ) was identified as an ERAD substrate that is degraded in a Png1-dependent manner (*67*, *93*). Moreover, we recently established an *in vivo* system designed to evaluate the Png1-dependent ERAD using a new substrate, RTL (RTAΔ-transmembrane-Leu2), which consists of a lumenal RTAΔ and the Leu2 cytoplasmic protein that is involved in the biosynthesis of leucine connected with a transmembrane domain (*44*, *94*). RTL can be used to detect strains with defective ERAD by assessing the growth of yeast strains bearing a *leu2* mutation, as cells with

Table II. Reported phenotypes/pathological conditions caused by mutations in gene orthologues of cytoplasmic PNGase.

Source	Phenotypes observed	References
S. cerevisiae	Defect in ERAD; no growth/viability defects	(*34*, *67*, *94*)
N. crassa	Temperature-sensitive growth with strong polarity defects	(*60*, *105*, *106*)
A. thaliana	No obvious defect	(*45*)
D. discoideum	Slow growth and development; defect in cell aggregation during multicellular development	(*42*, *107*)
C. elegans	Abnormal axon branching of VC4/VC5 egg-laying neurons, egg-laying behaviour defect	(*108*)
Dr. melanogaster	Severe developmental delay	(*59*)
H. sapiens	Global developmental delay, movement disorder, hypotonia	(*109–111*)

defective RTL degradation, when RTL is expressed, can supply sufficient Leu2 (as a part of RTL protein) to survive in a media lacking leucine. It should be noted that the deglycosylation of CPY*, the best-studied glycoprotein ERAD substrate in yeast, was not observed in *png1Δ* cells, while a delay in degradation of CPY* was observed (*34*). Quite recently, however, we observed the PNGase-mediated deglycosylation of intact CPY* as well as its proteolytic degradation intermediates when proteasomal activity was compromised (Hosomi, A. and Suzuki, T., unpublished data). This result clearly indicates that Png1 is indeed involved in the degradation of CPY*. It was also suggested that degradation of 6Myc-Hmg2, an ERAD substrate with a lesion in the transmembrane domain (ERAD-M), was attenuated in *png1Δ* cells (*95*), while the presence of deglycosylated 6Myc-Hmg2 was not detected in the presence of Png1.

In sharp contrast to the case of yeast, the functional importance of the cytoplasmic PNGase in mammalian ERAD process remains relatively unknown, other than the proposed critical role for class I major histocompatibility complex antigen presentation (*29*, *96–100*). Initial attempts to inhibit PNGase activity by RNA interference or the use of inhibitors resulted in little or no effect on the efficiency of degradation of model substrates (*61*, *101*, *102*). However, recently quite a reliable assay system was developed for detecting *in vivo* PNGase activity, using a green fluorescent protein (GFP)-derived model ERAD substrate that exhibits fluorescence in a deglycosylation-dependent manner (*103*). This assay promises to be a very facile system for assessing *in vivo* PNGase activity in mammalian cells. More recently, it was also reported that EDEM1 protein, a key component of the glycoprotein ERAD process, was stabilized on the inhibition of cytoplasmic PNGase, implying that the EDEM1 may be an endogenous substrate for cytoplasmic PNGase (*104*). This result may also shed light on the functional importance of the cytoplasmic PNGase in mammalian cells. It should be noted that the inhibition of PNGase activity appeared to have little effect on the deglycosylation of EDEM1. Further studies will be needed to clarify the functional importance of PNGase on the stability of the EDEM1 protein.

Although the mutant of the gene orthologue for the cytoplasmic PNGase in budding yeast (*34*) or in *Arabidopsis thaliana* (*45*) did not show any significant phenotypic consequences, more recent studies suggest that it may play important physiological roles in other eukaryotes (Table II).

Deglycosylation-Dependent and -Independent Function of the Cytoplasmic PNGase: Which Is Important?

It was shown that mutants of PNG1 in *Neurospora crassa* showed temperature-sensitive growth with strong polarity defects (*105*) (Table II). Interestingly, the PNG1 in *N. crassa* contains intrinsic mutations in two of the three essential amino acids in the catalytic triad (*i.e.* Cys-to-Ala and His-to-Tyr mutations; (*60*)) that consists of Cys, His and Asp (Fig. 5) (*112*, *113*). It is therefore predicted that, at least in some organisms, PNGase orthologues will also have a deglycosylation-independent function. It is also interesting to note here that the phenotypes observed in *N. crassa* (hyphal growth defect) and *C. elegans* (abnormal axon branching) are both temperature sensitive (*105*, *108*). As hyphal growth and other cell migration processes in animal cells such as axon branching may share, at least in part, a common machinery, it will be important to see whether the phenotype observed in *C. elegans* may somehow be related to the deglycosylation-independent function of the PNGase orthologue found in *N. crassa*.

It should be noted here that cytoplasmic PNGase possesses two well-conserved CXXC motifs (Fig. 5). Initial mutagenesis study revealed that a mutation of the Cys residues in these CXXC motifs results in the loss of enzyme activity, suggesting that those motifs are critical for enzyme activity (*113*). As one of the CXXC motifs in *Drosophila melanogaster* PNGase orthologue (Pngl) is missing, and initial attempts to detect PNGase activity in the fly failed, it was proposed that the fly Pngl protein may also have an important enzyme-independent function (*59*). However, we now have evidence to show that Pngl also appears to have deglycosylating activity (Huang, C. and Suzuki, T., unpublished data), and those CXXC motifs are also missing in the *Dictyostelium discoideum* orthologue (Fig. 5), while having deglycosylating activity (*42*). These results imply that the CXXC motif may not be an absolute requirement for enzyme activity.

Quite recently, it was reported that, in filamentous fungi where no active cytoplasmic PNGase has been found, fungi-specific cytoplasmic ENGase appears to

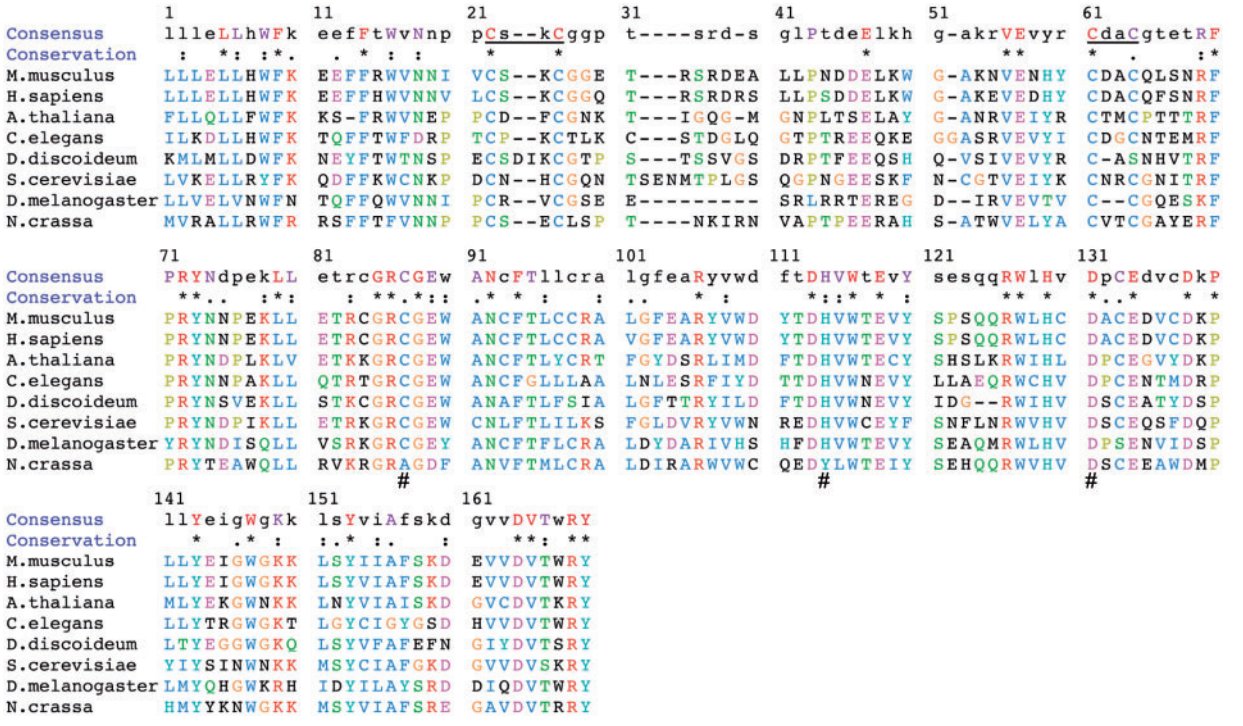

Fig. 5 Comparison of amino acid sequences around the catalytic residues between the cytoplasmic PNGase orthologues. The sequences of the TGase domain (Fig. 3) and its surrounding regions well conserved throughout the PNGase orthologues were aligned. The amino acid residues aligned are as follows; *M. musculus* (Q9JI78), 226–388; *H. sapiens* (Q96IV0), 229–391; *A. thaliana* (Q9FGY9), 173–333; *C. elegans* (Q9TW67), 170–333; *D. discoideum* (Q55FC8), 129–290; *S. cerevisiae* (Q02890), 108–273; *Dr. melanogaster* (Q7KRR5), 225–378; *N. crassa* (D1MY48), 129–290. The two CXXC motifs conserved in some orthologues are underlined. The catalytic residues (Cys, His and Asp) are indicated by number signs (#). Sequence alignment was carried out using ClustalW (*114*) and the alignment result is shown using UCSF Chimera software (*115*) (courtesy of Mr Masaki Kato, Structural Glycobiology Team, RIKEN). Note that two of the three catalytic residues are not conserved in the PNGase orthologue from *N. crassa*.

be present. This fungi ENGase belongs to the glycoside hydrolase (GH) family 18 in the CAZy database (*116*), which is different from the GH family 85 cytosolic ENGase ubiquitously found in other eukaryotes (*87*). It was found that the fungi-specific cytoplasmic ENGase can promote the degradation of RTL in budding yeast *png1*Δ cells (*117*). It is tempting to speculate that filamentous fungi may have acquired the fungi-specific GH 18 cytosolic ENGase to substitute for the deglycosylation function of the cytoplasmic PNGase.

Discovery of Human Patients of *NGLY1* Human Genetic Disorder

Recently, an exome analysis identified a human patient with mutations in the *NGLY1* gene (*109*) and an increasing number of the patients harbouring mutations in *NGLY1* alleles have been reported since then (*110*, *111*). The patients exhibited multiple symptoms that include global developmental delay, multifocal epilepsy, involuntary movement, abnormal liver function and the absence of tears (*109–111*). The mechanistic insight into these phenotypic consequences remains elusive. Whatever the mechanism, however, these observations clearly indicate the functional importance of the cytoplasmic PNGases in human biology. This rapidly developing *NGLY1* story has enjoyed exposure from major media such as CNN (http://edition.cnn.com/2014/03/20/health/ngly1-genetic-disorder/) or the New Yorker magazine (http://www.newyorker.com/reporting/2014/07/21/140721fa_fact_mnookin) because the research was actually not at all led by researchers; it was patients' families who were the driving force (*118*). I am hopeful that this example will serve as a new paradigm for conducting medical research, as we now know that concerted efforts among patients' families, clinicians and researchers can make a huge difference.

Beauty of 'Basic Science'

As I stated above, many unexpected, serendipitous findings have arisen during my 20-plus year scientific journey dealing with research on cytoplasmic PNGase (its discovery was already a completely unexpected result). Who would have thought that human genetic disorders could exist because of mutations in *NGLY1* gene, when no obvious phenotype is seen in mutants of the yeast orthologous gene? These unexpected findings continue as we analyse *Ngly1*–KO mice, on which we hope to report in the near future. The bottom line is that my research on PNGase/Ngly1 is all about curiosity-driven science. If one can only be allowed to carry out 'important science', we would not have been able to continue our studies of this enzyme.

In the beginning, I could not have cared less about how 'important' this enzyme will be. I just wanted to find out what this enzyme is doing, irrespective of whether it is important or not.

My scientific journey, which represents quite a turnaround from pure basic science to identify human genetic diseases, reminds me of the old words by Prof. Yasuo Inoue, my very first mentor; He always emphasized that both basic research and applied research are important; while everyone understands the importance of the latter, the former is sometimes not so much appreciated. Whichever the direction will be, however, there was one important motto on research (in the original words of Prof. Fujio Egami, Prof. Inoue's mentor):

> 'There is no ''important'' research subject.
> If there is, it must have already been made important by others.
> What the most important is to make our own research be important by ourselves!'

After all this time, I came to realize that I simply followed my mentor's (or my mentor's mentor's) words. In this connection, it has been just 20 years since we first wrote reviews describing the potential functions of deglycosylating enzymes in nature (*11*, *12*), some of which still remain as attractive hypotheses. I just feel fortunate that, in our research field, there are so many outstanding questions that still remain to be explored.

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Conflict of Interest

None declared.

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