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GLYCOPINION MINI-REVIEW

N-Glycosylation/deglycosylation as a mechanism for the post-translational modification/remodification of proteins

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Abbreviations: PNGase, peptide-N4-(N-acetyl-b-d-glucosaminyl) asparagine amidase or peptide:N-glycanase; endo-b-GlcNAc'ase, endo-b-N-acetyl-d-glucosaminidase; GPP, glycophosphoproteins; L-929 PNGase, PNGase isolated from C3H mouse-derived fibroblast cells, L-929; UNGs, unconjugated N-glycans; Xyl, xylose; Gal, galactose; Ara, arabinose; Con A, concanavalin A; RER, rough endoplasmic reticulum; Man5GlcNAc2-pyrophosphoryl dolichol; pentamannosyl N,N′-diacetylchitobiosyl pyrophosphoryl dolichol.

Introduction

Glycoproteins are major components of eukaryotic cells and although their biological or physiological functions are known in most cases, the biological roles of their glycan chains remain mostly undefined. However, various hypotheses and much circumstantial evidence have been reported regarding both the biological and chemical roles of the prosthetic glycan chains [1–3].

Peptide:N-glycanase (PNGase), an enzyme catalysing cleavage of the amide bond between the proximal GlcNAc and the linkage amino acid Asn residue, has been widely accepted as a powerful and useful 'tool reagent' in structural and functional studies of N-linked glycan chains of a wide variety of glycoproteins. However, little attention has been given to the physiological significance of this enzyme in living organisms. This class of enzyme had been thought to occur only in plant cells [4, 5] and in a bacterium, *Flavobacterium meningosepticum* [6], until we demonstrated for the first time the occurrence of PNGase activity in the early embryos of Medaka fish, *Oryzias latipes* [7]. Subsequently, we have been successful in the identification of PNGase activities in various animal cells [8–13]. These findings permitted us to postulate a potential biological significance for *in vivo* de-N-glycosylation due to the action of PNGase, i.e. the non-lysosomal regulation of various physiological and/or physicochemical properties of certain target protein(s) [7–15].

In this paper, we extend our hypothesis for biological roles of PNGase to other deglycosylating enzymes, i.e. endo-β-N-acetyl-D-glucosaminidase (endo-β-GlcNAc'ase), and discuss the possibility of the widespread occurrence of 'non-lysosomal' deglycosylation reactions as a novel processing mechanism for N-glycosyl glycoproteins.

Deglycosylating enzymes: occurrence in nature

(a) PNGase

The occurrence of peptide:N-glycanase [peptide-N^4-(N-acetyl-β-D-glucosaminyl) asparagine amidase, EC 3.5.1.52; trivial name, PNGase], which catalyses the scission of the bond between the proximal GlcNAc residue and the linkage asparagine residue in glycoproteins (Fig. 1), was first reported in the seeds of almond by Takahashi in 1977 [4], and since then this enzyme was proved to exist widely in plant cells [5, 16] as well as in a bacterium [6]. However, the occurrence of PNGase in animal cells was not reported until recently, as described below. Our initial study of PNGase from animal sources came from the finding of free oligosaccharides in oocytes and early embryos of several species of fish [14, 17–21]. These free

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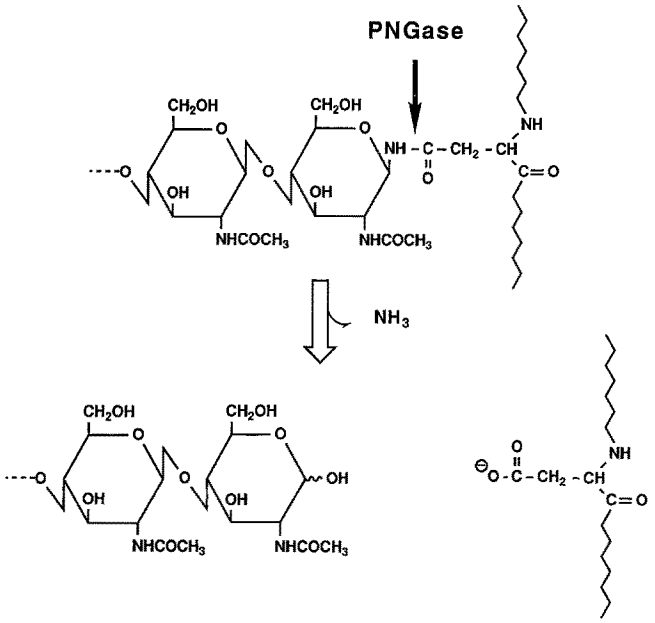

Figure 1. – Stoichiometric representation of peptide:N-glycanase (PNGase)-catalysed action on an N-linked glycoprotein. PNGase hydrolyses the asparaginyl amide bond to give an aspartic acid-containing polypeptide chain and 1-amino-*N*-acetylglucosaminyl oligosaccharide; the latter is subsequently hydrolysed spontaneously to *N*-acetylglucosaminyl oligosaccharide and ammonia.

oligosaccharides were divided into two categories based on their progenitor proteins. The first type was complex-type sialooligosaccharides which accumulated in relatively large quantities in oocytes [17–19] and was proved to originate from glycophosphoproteins (GPP) [19]. GPP is an abundant protein in fish eggs, and it was considered to be one of the molecular species of phosvitin, which was derived from vitellogenin. The second type of free oligosaccharides had multibranched multiantennary structures [20, 21] and was shown to originate from a cortical alveolar-derived glycopolyprotein, hyosophorin [20–24], and to be liberated during early embryogenesis [20, 21].

The interesting feature of these free glycans is that they have the N,N′-diacetylchitobiosyl structure at their reducing termini, which led us to propose that the putative peptide:N-glycanase was expressed in oocytes and embryos of fish and had a biologically significant function [17]. And indeed, we identified PNGase activities in the early embryos of Medaka fish (*Oryzias latipes*) [7, 8]. More recently, we searched for PNGase activities in several mammalian-derived cultured cells to investigate how widespread the occurrence of this enzyme was, and showed that all mouse and human cell lines so far examined exhibited PNGase activities [9]. The occurrence of PNGase in organs and tissues from BALB/c and ICR mice was also proven, although the level of the activities differs among tissues [10, 12]. Furthermore, we purified soluble

Table 1. Comparison of properties of L-929 PNGase with those of PNGase F from *F. meningosepticum*.

	L-929 PNGase	*PNGase F*
Molecular Weight (K)	212 [13][a]	34.8 [105]
pH Optimum	7.0 [13]	8.5 [6]
K_m (m*M*)[b]	0.114 [13]	0.525 [25]
Subunit	Dimeric [13]	Monomeric [105]
Hydrophobicity	High [13]	High [106]
Requirement of –SH Groups(s) for enzyme activity	Yes [13]	No [107]
Carbohydrate binding property	Yes [25]	No [25]

[a] Numbers in parentheses are references.

[b] Values for calf serum fetuin-derived glycopeptide I [Leu-Asn($Man_3Gal_3GlcNAc_5Sia_3$)-Asp-Ser-Arg].

Table 2. Reported occurrence of PNGase in nature.

	Source	*Reference*
Animal	Medaka (*Oryzias latipes*)	7, 8
	Cultured cells from human and mouse	9–11, 13
	Mouse	10–12
	Chicken	11
Plant	Almond emulsin	4, 108
	Jack bean	69, 70
	Various plant seeds	5
	Cultured cells from white campion	16
Bacteria	*Flavobacterium meningosepticum*	6, 105, 109

(cytosolic) PNGase to homogeneity from C3H mouse-derived fibroblast cells, L-929, designated L-929 PNGase, and characterized several unique enzymatic properties [13, 25] (Table 1). Soluble PNGases in brain, liver, kidney, and spleen of ICR mice were partially purified and characterized. It was shown that high hydrophobicity, a neutral pH optimum, inhibition by Cu^{2+}, Zn^{2+}, and Fe^{3+} and sensitivity to thiol-modification reagents were shared by all mammalian soluble PNGases thus far examined [12]. PNGase activities were also observed in membranous fractions of various organs and tissues of ICR mice [12]. Most recently, we have shown that the developmental expression of PNGase activity in chick brains during embryogenesis was temporally regulated [11]. The reported occurrence of PNGase in nature is summarized in Table 2.

(b) Endo-β-GlcNAc'ase

Endo-β-N-acetyl-D-glucosaminidase (endo-β-GlcNAc'ase; EC 3.2.1.96) hydrolytically cleaves the glycosidic linkage in the

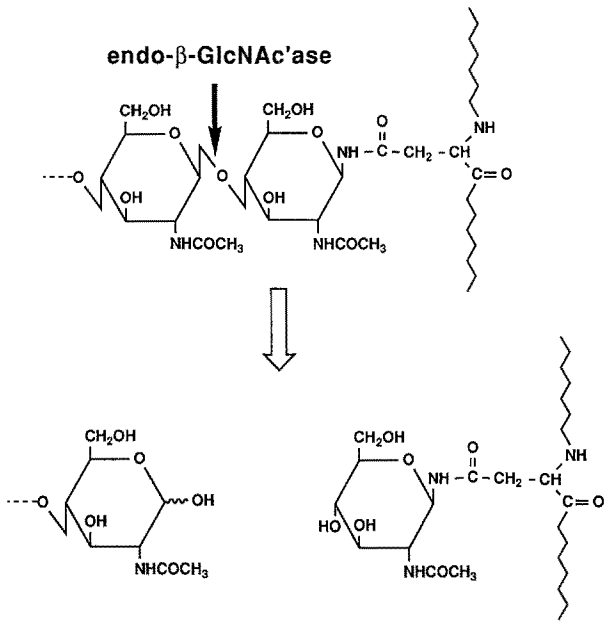

Figure 2. Endo-β-N-acetylglucosaminidase(endo-β-GlcNAc'ase)-catalysed action on the N-linked oligosaccharide chains of glycoproteins.

N, N′-diacetylchitobiose moiety of the core region of N-linked oligosaccharides (Fig. 2). This enzyme was first found in the culture fluid of *Diplococcus pneumoniae* by Muramatsu in 1971 [26]. Following this finding, endo–β–GlcNAc'ases have been found in various sources from bacteria to man as shown in Table 3.

Studies of endo-β-GlcNAc'ase in animals were first directed towards identification of enzyme activities involved in lysosomal diseases. The occurrence of the enzyme in vertebrates was reported in human kidney [27, 28], human brain, spleen and liver [27], fibroblasts derived from a fucosidosis patient [29], human urine [30], human saliva [31], rat liver [32–37], rat kidney and spleen [32] , porcine liver and kidney [32], mouse-derived L-929 cells [13], calf thyroid [38], rabbit serum [39], and hen oviduct [40]. Recently, we have also detected endo-β-GlcNAc'ase activities in soluble fractions of brain, liver, kidney, and spleen of ICR mice [Suzuki T. *et al.*, unpublished results]. Properties of these enzyme activities were not investigated in detail, but they appear to have several common characteristics except for human saliva and calf thyroid enzymes: (i) they are localized in the cytosol; (ii) their optimal pH is near neutral pH (6.0–7.0); and (iii) they favour substrates having glycan chains of the oligomannose type [27, 29, 32–37, 41]. Endo-β-GlcNAc'ase is obviously different from 'chitobiase' [42, 43], which is an exoglycosidase acting upon the reducing terminal GlcNAc residue of the *N*, N′-diacetylchitobiosyl structure [42–45]. Chitobiase is localized within lysosomes and is believed to be the enzyme involved in the lysosomal degradative pathway [28, 37, 42–45].

Possible biological roles of deglycosylating enzymes

At present, our knowledge of the physiological roles of PNGase and endo-β-GlcNAc'ase is limited. Nevertheless, some interesting phenomena relevant to the biological significance of these enzymes can be found in the literature as compiled below.

(a) Modulation of receptor-ligand interaction

We have identified two distinct PNGases in the early embryos of Medaka fish, designated 'acid PNGase' and 'alkaline PNGase' depending on their pH optima [7, 8]. The target protein for the acid PNGase was considered to be glycophosphoprotein, GPP [8, 19; Seko A. *et al.*, in preparation]. The ability to release free glycan from GPP due to the action of this PNGase during oogenesis in fish was thought to be important in vitellogenesis [14, 15]: De-N-glycosylation of GPP may be related to the recycling of the vitellogenin receptor on the egg surface. Vitellogenin incorporation is known to occur via receptor-mediated endocytosis [46, 47] (Fig. 3), and dissociation of the ligand vitellogenin from the receptor may be a prerequisite for recycling of the receptor incorporated into the cell. De-N-glycosylation appears to be necessary for the dissociation [14, 15]. Alternatively, de-N-glycosylation may be the rate-limiting step for the subsequent proteolysis of vitellogenin [14, 15].

When plants are attacked by potentially pathogenic microorganisms, they generally display an active defence response [48–50], which includes the induction of enzymes like phenylalanine ammonia-lyase, modification of cell walls by deposition of callose, lignin, and related cell wall bound phenolics, induction of lytic enzymes, and a rapid formation of the plant hormone ethylene. Studies with intact plants and plant cell cultures have shown that chemical stimuli derived from fungi, so-called elicitors [51], can induce the active defence response [48–51]. In tomato cells, glycopeptides prepared from yeast, which have a fungus-specific α-1,6-linked mannose residue in their glycan moieties (Fig. 4), were reported to have strong elicitor activity [52– 54]. Interestingly, the elicitor activity of these glycopeptides was abolished almost completely by treatment with endo-β-GlcNAc'ase or PNGase. Furthermore, the oligosaccharides released from such elicitor-active glycopeptides by PNGase or endo-β-GlcNAc'ase were shown to inhibit competitively the elicitor activity of the glycopeptides [52-54]. The presence of a specific elicitor binding site in tomato cell membranes was also suggested [54]. These results collectively suggested that glycopeptides and free glycans from microorganisms could act as agonists and antagonists, respectively, for the induction of the stress response of plants by competing for the binding site(s) in plant cell membranes. Although this is a matter of pure speculation, one can safely say that a putative deglycosylating enzyme from such microorganisms (or plants) could inhibit the plant's defence system. This hypothetical process deserves further investigation. Identification of the relevant enzyme activity in fungi (or plants) may be the next logical step for clarification of this hypothesis.

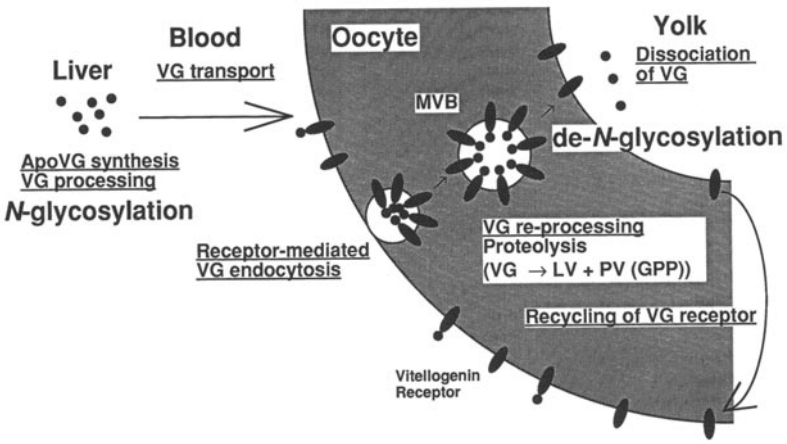

Figure 3. Schematic representation of vitellogenesis and vitellogenin incorporation into oocyte. Vitellogenin (VG) is synthesized in hepatocytes and transported through the blood stream to oocytes. VG is incorporated into the oocyte by receptor-mediated endocytosis [46, 47]. Cytoplasmic migration in the oocyte is accompanied by the hydrolytic processing of VG to yolk proteins such as lipovitellin (LV), phosvitin (PV), and glycophosphoprotein (GPP). This process is considered to occur within multivesicular bodies [104].

(b) Formation of bioactive molecules

H-Hyosophorin, a high molecular weight form of hyosophorin, is a glycopolyprotein localized in the cortical alveoli of unfertilized fish eggs and the apo-protein is made up of tandem-repetition of the identical oligopeptide sequence [24]. Upon fertilization, cortical alveolar exocytosis occurs and H-hyosophorin is discharged from the vesicles to the perivitelline space [55, 56] concomitantly with depolymerization into the repeating unit, L-hyosophorin, by a unique protease, hyosophorinase [57]. During early embryogenesis, a part of L-hyosophorin is further processed by the action of alkaline PNGase, which was found in the blastodisc of Medaka embryos, to give rise to deglycosylated peptide and free glycan chain [7, 8, 20, 21; Seko A. *et al.*, in preparation] (Fig. 5). Accumulation of hyosophorin-derived free glycan was stage-specific [21], indicating that the de-N-glycosylation of L-hyosophorin in early embryos of fish could be an essential event required for the conversion of L-hyosophorin into functional peptide or oligosaccharide [14, 15]. We have observed that polyclonal antibodies against the deglycosylated peptide formed by the action of PNGase on L-hyosophorin, but not against L-hyosophorin itself or free glycan from hyosophorin, was able to block cell migration during gastrulation, suggesting that the deglycosylated peptide from L-hyosophorin has hormonal activity required for the normal development of the embryos by an as yet undefined mechanism [Seko A. *et al.*, unpublished result].

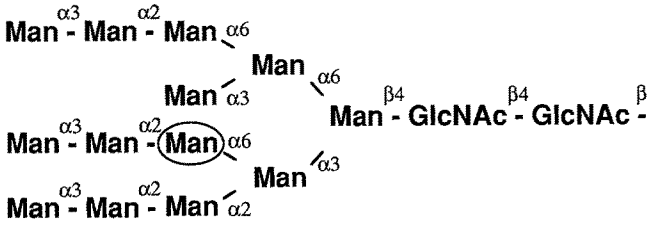

Figure 4. Oligosaccharide structure of elicitor-active glycopeptide obtained from yeast. The oligosaccharide structure is that from a typical elicitor-active glycopeptide [52]. Linkage types are indicated by the lines connecting Man or GlcNAc (e.g. α3 corresponds to an α1→3 linkage). Encircled Man residue is a fungus-specific α1→6-residue which is critical for the elicitor activity.

Recently, it was reported that free oligosaccharides, called 'unconjugated N-glycans' (UNGs) [58], were isolated from the extracellular medium of a plant-cell suspension of white campion [16, 59] as well as from the tomato fruit pericarp [60]. The UNGs were classified into two subclasses according to their structures [16, 58–60]: one is the oligomannose type glycans, which are composed of Man and GlcNAc residues and have only one GlcNAc residue at their reducing termini, and the other is the complex-type glycans, which contain other sugars including Xyl, Gal, Ara and Fuc α1-3 attached to the proximal GlcNAc residue in addition to Man and GlcNAc. Both types of UNGs can be generated from glycoproteins by the action of specific deglycosylating enzymes. PNGase activity was detected in both culture medium and cell extracts of white campion suspension cultures [16]. The authors argued that the PNGase activity was possibly responsible for the formation of UNGs [16]. The oligosaccharides having only one GlcNAc residue at the reducing termini could be derived due to the action of a chitobiase-like enzyme acting on free N-glycans released by the action of PNGase [16, 59], although the activity has not yet been identified. Since the free glycan concentration increased on carbon deprivation, it was hypothesized that the UNGs may possibly arise from carbon starvation [16]. Indeed, a positive correlation between sucrose starvation and de-N-glycosylating activities in white champion cells was

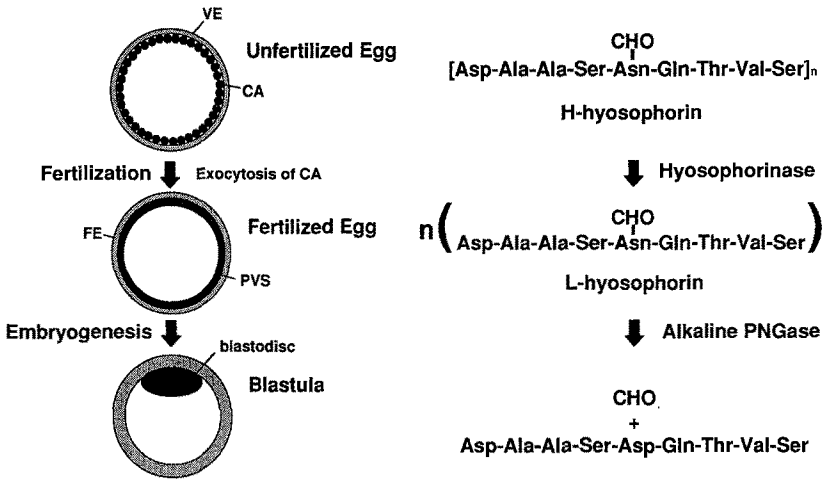

Figure 5.– Morphological changes of an egg upon fertilization and during early embryogenesis together with the accompanied structural changes of hyosophorin of Medaka fish. VE, vitelline envelope; CA, cortical alveolus; FE, fertilization envelope; PVS, perivitelline space; CHO, N-linked oligosaccharide.

observed [58]. In tomato fruit, the amount of UNGs in the total oligosaccharidic extract was shown to increase during maturation [58, 60].

UNGs are capable of influencing both growth [61] and senescence [62–64]. These effects were dose-dependent, suggesting that these free glycans can be regarded as a kind of plant hormone factor [58, 62–64]. The deglycosylating enzyme, most likely PNGase, may thus be responsible for generating such hormone factors in plants. The target physiological protein(s) of the enzyme in plant cells remain to be determined.

Concanavalin A (Con A), a lectin from jack bean, is known to be synthesized as a glycosylated precursor while the mature protein is not glycosylated [65, 66] (Fig. 6). Recently, the removal of glycan during maturation of Con A was shown to be critical for its lectin activity: deglycosylation of the precursor form of Con A, but not the subsequent proteolysis, is essential for the expression of its lectin activity [67, 68], although the involvement of PNGase or endo-β-GlcNAc'ase which are present in jack bean [69, 70; see Tables 2 and 3], remains to be elucidated.

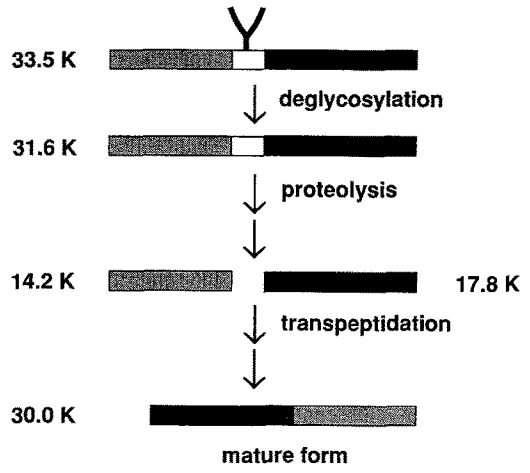

Figure 6.– Diagrammatic representation of post-translational processing of Con A according to Bowles *et al.* [66].

Table 3. Reported occurrence of endo-β-GlcNAc'ase in nature.

	Source	*Reference*
Animal	Human	27–31
	Rat	32–37
	Pig	32
	Mouse	13
	Rabbit	39
	Hen	40
	Calf	38
Plant	Jack bean	70
	Fig	110, 111
Fungi	*Aspargillus oryzae*	112
	Dictyostellium discoidium	113
	Fusarium oxysporum	114
	Mucor hiemalis	115, 116
	Sporotrium dimorphosporum	117
Bacteria	*Arthrobacter protophormiae*	118
	Bacillus alvei	119
	Bacillus circulans	120
	Clostoridium perfrigence	121
	Diplococcus pneumoniae	26, 122
	Flavobacterium meningosepticum	123–126
	Flavobacterium sp.	127, 128
	Pseudomonus sp.	129
	Streptomyces plicatus	130–133

Table 4. Reported molecular multiplicity assumed to be generated by deglycosylation catalysed by PNGase and/or endo-β -GlcNAc'ase.

	Protein	*Reported difference between glycosylated and deglycosylated form on bioactivity and stability.*	*Reference*
PNGase (-Asn(CHO)- → -Asp-)	Ricin D A chain	Not known	7, 13, 14, 134–136
	Bonito gonadotropin II α subunit	Not known	15, 137
endo-β-GlcNAc'ase (-Asn(CHO)- → -Asn(GlcNAc)-)	Human salivary α-amylase	Similar in specific activity; exhibit the same thermal stability	31, 138
	Porcine spleen cathepsin D	Similar in enzyme activities (pH optimum, response to inhibitors) but differ in terms of efficiency of enzyme activity (specific activity)	139, 140
	Aspergillus oryzae Taka-amylase A	Almost the same in amylase activity but different slightly in phenyl maltosidase activity	112, 141
	Fusarium oxysporum α-fucosidase	Exhibit similar enzyme activity and thermal stability; deglycosylated enzyme was slightly unstable in the acidic pH range compared with the glycosylated form	114

(c) Regulation of proteolytic processing

Deglycosylation of Con A precursor can also be interpreted as the process regulating maturation of Con A, since the removal of the glycan chain could alter the accessibility of a processing protease [13] (Fig. 6). Recently, a plausible model was proposed to explain the possible biological roles of deglycosylation in maturation of *Graminae* lectins, where deglycosylation of proprotein was rate-limiting and preceded proteolytic processing [71], although the enzyme activity responsible for the detachment of the glycan has not yet been identified. Interestingly, several examples have been reported for plant lectins with a potential N-glycosylation site in the processing region of proproteins [65, 66, 71–75], indicating that deglycosylation may possibly be a more common basic reaction in the processing of certain plant lectins than is presently recognized. A model similar to this has been proposed for maturation of honeybee venom hyaluronidase [15].

(d) Generation of structural polymorphism

Prolactin is known to have various molecular forms [76], through which the hormone is presumed to be capable of playing various functions within the multiple target organs. It is known to be glycosylated in various animals [77–83] and charge isomers have been described for nonglycosylated prolactin [81, 84] Current interpretation of the occurrence of such isomers in terms of 'nonenzymatic deamidation' [84] may not necessarily be correct. As an alternative possible mechanism, de-N-glycosylation of the glycosylated form by PNGase activity should be considered since PNGase converts the glycan-linked Asn residue into an Asp residue. The differential glycosylation pattern of prolactin was shown to be of key importance in its metabolic clearance [85], and the receptor-mediated incorporation of prolactin into different target organs was shown to be apparently specific to the carbohydrate structure in this hormone [85]. Noteworthy was the fact that the glycosylated form of prolactin was less bioactive compared with the nonglycosylated form [77, 85, 86]. These results raise the questions: How can the less active glycosylated prolactin be effective in the target organs and is there any particular mechanism by which the less active glycosylated form of prolactin can be converted into the more active nonglycosylated form? Our postulated answer is PNGase-catalysed transformation of the less active form to the more active form.

Some examples indicating the presence of 'deglycosylated' variant forms, presumably generated by PNGase or endo-β-GlcNAc'ase, are listed in Table 4, although critical roles, if any, for deglycosylation have not yet been documented.

(e) Proof of correct folding and/or multimeric formation

It has been reported [38, 87–90] that liberation of oligosaccharides takes place in tissues and cells. Recently, the observed accumulation of free oligomannose type oligosaccharides having a single residue of GlcNAc at their reducing termini was ascribed to degradation of newly synthesized glycoproteins by a putative endo-β-GlcNAc'ase in B3F7 cells, a glycosylation mutant cell line of Chinese Hamster Ovary cells, although identification of the enzyme activity was not carried out [89]. The authors interpreted the observed release of the free glycan chains as having taken place within the rough endoplasmic reticulum (RER) or related vesicles [89].

Recent studies demonstrated that the ER is a site where newly synthesized proteins undergo degradation by proteinases when they are misfolded or when subunits of a given oligomeric complex are over-expressed [91–93]. By analogy,

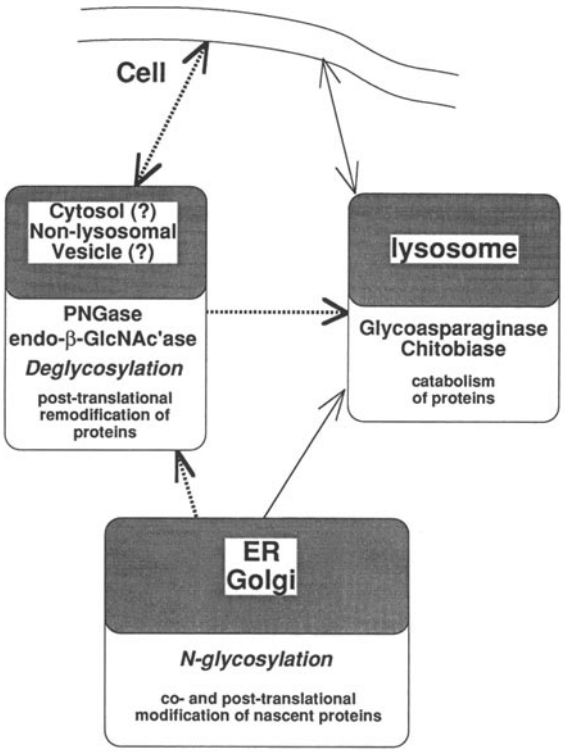

Figure 7.– Proposed intracellular sorting pathways and nonlysosomal processing of glycoproteins in mammalian cells. Dashed arrows show the proposed post-translational remodification processes of proteins.

endo-β-GlcNAc'ase may be considered to act as a probing quality control enzyme which diagnoses correct folding or oligomerization of glycoproteins. Anumula and Spiro [38] detected neutral endo-β-GlcNAc'ase, but not PNGase, which is possibly responsible for the formation of these free oligosaccharides in the calf thyroid microsomal fraction.

The N-glycosylation/deglycosylation system

(a) A novel catabolic pathway for N-glycosyl glycoproteins

We have proposed that an 'N-glycosylation/de-N-glycosylation system' is one of the post-translational remodifications of proteins [15, 19]. Following co-translational N-glycosylation of the primary translation products in the RER, certain glycoproteins may be modified in a site-specific manner by PNGase (de-N-glycosylation), thereby introducing a negative charge into the core protein by converting **glycosylated Asn** residue into **Asp** as well as by removing rather bulky N-linked glycan chains from the protein. These drastic changes may serve to regulate such physicochemical properties as stability and solubility and such physiological properties as bioactivities, susceptibility towards proteases and affinity to receptors. This system may thus be a biologically important mechanism of post-translational remodification of certain proteins and may occur more ubiquitously than previously anticipated.

It seems reasonable to extend our hypothesis of 'N-glycosylation/de-N-glycosylation' to other related reactions such as endo-β-GlcNAc'ase-catalysed modification of functional glycoproteins. Endo-β-GlcNAc'ases are 'deglycosylating enzymes', which are known to occur widely in living organisms and which can alter the physiological and/or physicochemical properties of proteins by converting **R-GlcNAcβ1-4GlcNAcβ1-protein** into R-GlcNAc-OH + **GlcNAcβ1-protein**. Increasing numbers of papers have appeared reporting the occurrence of PNGases (Table 2) and endo-β-GlcNAc'ases (Table 3) in mammalian systems. They share common enzymatic properties, e.g. neutral optimal pH, indicating that they are probably not involved in lysosomal degradation of N-linked glycoproteins but may function as **post-translational remodifying enzymes** (Fig. 7) though this is still a matter of conjecture.

(b) How can soluble deglycosylating enzymes be accessible to their endogenous substrates?

Concerning the biological roles of deglycosylating enzymes, the most important question would be: What are the physiological substrates for these enzymes? This problem is unresolved in mammalian systems in spite of the wide occurrence of the deglycosylating enzymes (Tables 2 and 3). As the first logical step to gain insight into the biological function of deglycosylation catalysed by PNGase and/or endo-β-GlcNAc'ase in cellular processes, the subcellular localization of these enzymes should be clarified.

In mammalian systems, both deglycosylating enzymes are assumed to be localized mainly, if not completely, in the cytosol as judged by fractionation studies [9, 25, 33, 34, 36, 37], while the localization of N-linked glycoproteins is believed not to be topologically the same as that of cytosolic proteins [94, 95]. This apparently contradictory situation, however, has been partly overturned by the fact that a number of N-linked glycoproteins have recently been shown to be in the cytoplasm and nuclei [37, 96–99].

In 1990, Pedemonte *et al.* showed that the Na^+-pump α subunit is an N-linked glycoprotein having PNGase F-susceptible glycan chains oriented to the cytoplasmic surface of the ER membrane [96]. Later, the glycan structure was shown to be a single GlcNAc residue attached to the asparagine residue [97]. These results led us to suggest that endo-β-GlcNAc'ase may be involved in formation of the Asn-linked GlcNAc residue. A fundamental question raised here is how the N-linked glycan chains can be oriented towards the cytosol. Reasoning by analogy with a 'consensus' model for the translocation of $Man_5GlcNAc_2$-pyrophosphoryldolichol across the RER membrane via a flip-flop mechanism, which is presumed to be protein-mediated [100, 101], we suggest detachment of the glycan units of newly synthesized glycoproteins in the cytoplasmic space after translocation from the RER lumen to the cytoplasmic side of the membrane through a similar mechanism. It should be noted that translocation of such hydrophilic

polysialyl chains across a membrane was recently reported to occur in a prokaryotic system [102].

It is also interesting to note that chondroitin sulfate proteoglycan, known to contain an N-linked glycan chain [103] in addition to a vast number of O-linked glycosaminoglycan chains, is present in the extracellular matrix of the early postnatal rat brain, but after this stage of development it changes its localization to the cytosol of neurons and astrocytes [98, 99]. It is not known how such translocation of the proteoglycan occurs during development.

Since there is no clear evidence that PNGase or endo-β-GlcNAc'ase exists in the cytosol, one cannot preclude the possibility that endo-β-GlcNAc'ases and/or PNGases found in mammalian cells may be localized within certain non-lysosomal vesicles and may act upon endogenous N-glycosylated glycoprotein substrates colocalized within such vesicles. Subcellular localization of these enzymes may be clarified when specific antibodies to these enzymes or cDNA encoding them become available. Studies aimed at eliciting antibodies to mammalian PNGases and determining their primary structure are currently underway.

Finally, pertinent to the possible biological roles of cytosolic endo-β-GlcNAc'ases, it should be noted that cytosolic endo-β-GlcNAc'ase was recently reported to react with glycosylpyrophosphoryl dolichols [37]. Haeuw *et al.* [37] proposed that this cytosolic enzyme may be involved in controlling the biosynthesis of N-linked glycoproteins by eliminating unfinished or immature glycan chains on dolicholpyrophosphate [37].

Concluding remarks

The majority of functional proteins of eukaryotic organisms is known to be covalently glycosylated, and early suggestions were made that glycosylation serves mainly to effect the export of proteins from cells or to protect the proteins from proteolysis. Although many other biological functions for the carbohydrate moieties of glycoproteins have since been suggested, our basic understanding of the function of protein glycosylation is still currently an area of most intense investigation in 'glycobiology'.

There is a large body of evidence which supports the concept that protein-linked carbohydrate chains play a key role in the regulation of complex biological events in multicellular organisms, and it is now generally accepted that protein glycosylation is critical for maintenance of normal life and normal reproductive processes [1–3]. Therefore, it is most important to elucidate the role of the carbohydrate moiety in the basic biological functions of individual glycoproteins at the molecular level.

Not only the assembly, processing, and elongation of N-linked carbohydrate chains but also the metabolism of the carbohydrate chains of glycoproteins are important for understanding the *in vivo* functions of N-linked glycoproteins. However, it has been generally considered that protein-linked oligosaccharide chains are not detached until the glycoproteins are no longer required, and it has been established that the N-linked glycan chains of glycoproteins are not separated from the protein cores in the lysosomal compartment until extensive degradation of the core proteins by lysosomal proteinases has occurred [42–45] (Fig. 7).

In 1989, we discovered the accumulation of large amounts of N-linked glycoprotein-derived free oligosaccharides in the eggs of a freshwater trout, *Plecoglossus* altivelis ('ayu' in Japanese) [17]. Contrary to the prevailing concept, we suggested that the free glycans represent the products of 'nonlysosomal' deglycosylation pathways of N-linked glycoproteins. Since then systematic investigations have been carried out to answer the following major questions [7–15, 17–21,25]: how general is the de-N-glycosylation in cellular processes and what is the physiological significance of de-N-glycosylation? As a result of our studies, it became apparent that the unique free oligosaccharides are detached from glycoprotein during fish oogenesis and embryogenesis by the action of specific peptide:N-glycanases on their respective progenitor glycoproteins and glycopeptides. It was suggested by structural analysis of the liberated glycan chains and identification of PNGase activities that the conversion of these progenitors into structurally different forms (glycan-linked Asn → Asp) may have functionally important roles [7, 8, 14, 15, 17–21] (see Figs 3 and 5).

N-Glycosylation occurs on nascent chains as a post-translational modification of proteins, whereas the subsequent de-N-glycosylation takes place later, when necessary, as a new post-translational remodification of proteins to express their function. Thus, we have postulated that an N-glycosylation/de-N-deglycosylation system could explain certain biological processes at the molecular level in eukaryotic cells [7–15, 17–21, 25]. As an extended form of this hypothesis, it should be emphasized that, at least at certain cellular levels, a part of a given N-linked glycoprotein, before being transported to lysosomes for degradation, could undergo a 'nonlysosomal type' of cleavage of the glycan chain(s) by non-lysosomal enzymes, PNGase or endo-β-GlcNAc'ase, to give rise to forms which are biologically active either in intracellular transportation/intermolecular recognition events or molecular conformation/solubility/susceptibility to proteases (Fig. 7).

Although a large body of circumstantial evidence has been accumulated to support the rather wide occurrence of deglycosylation in living organisms, a general role of deglycosylation for *de novo* glycoprotein functioning still remains to be elucidated. A framework for future systematic investigations necessary for validation of our hypothesis should include (a) reexamination of the intracellular localization of PNGase and endo-β-GlcNAc'ase which are known to occur widely in mammals and (b) identification of the target glycoprotein substrates within the cells for each case.

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