Glycoconjugate Journal (1994) **11**:469–476

Does an animal peptide: *N*-glycanase have the dual role as an enzyme and a carbohydrate-binding protein?

TADASHI SUZUKI[1], KEN KITAJIMA[1], SADAKO INOUE[2] and YASUO INOUE[1]*

[1] *Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo-7, Tokyo 113, Japan*

[2] *School of Pharmaceutical Sciences, Showa University, Hatanodai-1, Tokyo 142, Japan*

Received 19 May 1994, revised 6 July 1994

Recently, we have reported purification and characterization of a de-*N*-glycosylating enzyme, peptide: *N*-glycanase (PNGase) found in C3H mouse fibroblast L-929 cells, and designated L-929 PNGase [Suzuki T, Seko A, Kitajima K, Inoue Y, Inoue S (1994) *J Biol Chem* **269**, 17611–18]. The unique properties of L-929 PNGase are that the enzyme had a high affinity to the substrate glycopeptide (e.g. K_m = 114 μM for fetuin derived glycopentapeptide) and that the PNGase-catalysed reaction is strongly inhibited by the released free oligosaccharides but not by the free peptides formed, suggesting that L-929 PNGase is able to bind to a certain type of carbohydrate chain. In this study, we report the new findings of the mannan-binding property of L-929 PNGase: the de-*N*-glycosylating enzyme activity of L-929 PNGase was inhibited by yeast mannan and triomannose, Manα1 → 3(Manα1 → 6)Man, but not by mannose and α-methyl-D-mannoside. Furthermore, L-929 PNGase was revealed to bind to the glycan moiety of yeast mannan by using mannan-conjugated Sepharose 4B gel as a ligand, suggesting that L-929 PNGase could serve not only as an enzyme but also as a carbohydrate recognition protein *in vivo*. Such 'dual' properties found for animal-derived L-929 PNGase are unique and are not shared with other previously characterized plant- and bacterial-origin PNGases – PNGase A and PNGase F, respectively.

Keywords: animal PNGase, mouse L-929 cell, mannan-binding

Abbreviations: GLC, gas liquid chromatography; GlcNAc-Asn, 2-acetamido-1-β-(L-aspartamido)-1,2-dideoxy-D-glucose; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose; triomannose, Manα1 → 3(Manα1 → 6)Man; MES, 2-(*N*-morphorino)ethanesulfonic acid: NeuAc, *N*-acetyl-neuraminic acid; PNGase, peptide: N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (peptide: *N*-glycanase, *EC* 3.5.1.52); PNP, *p*-nitrophenyl.

Introduction

Remarkable progress in the field of animal lectins during the last decade suggests that membrane-incorporated lectins play important roles in the carbohydrate-recognition events in animal cells [1–3]. Lectins are generally defined as neither enzymatic nor immune proteins but di- or multivalent carbohydrate-binding proteins which are grouped together because they agglutinate cells [4, 5]. However, several lines of evidence indicate that membrane-associated glycosidases as well as cell surface glycosyltransferases could function as lectin-like (carbohydrate-binding) molecules and be involved in intercellular recognition or cell-cell adhesion during fertilization and embryogenesis [6–12]. The possible biological significance of the binding properties of such surface located enzymes (glycosyltransferases and glycosidases) has been documented [6–12].

Peptide: *N*-glycanase (PNGase) is the enzyme responsible for the detachment of *N*-linked glycan chains from glycopeptides or glycoproteins by hydrolyzing the β-aspartylglucosamine linkage. PNGase had been considered to occur only in a variety of plant seeds [13, 14] and a bacterium, *Flavobacterium meningosepticum* [15] until our demonstration of the occurrence of the enzyme in early embryos of *Oryzias latipes* (Medaka fish) [16], which was the first example of PNGase from an animal origin. Subsequently, we also found PNGase activities in mammalian cultured cells as well as in different organs of BALB/c mouse [17, 18]. These findings showed the widespread occurrence of PNGase and raised the question of the importance of the

* To whom correspondence should be addressed.

de-*N*-glycosylating enzyme in biological processes *in vivo*. As the next step of our studies in this direction, we have purified cytosolic PNGase from C3H mouse-derived fibroblast L-929 cells to homogeneity and characterized the physical and enzymatic properties of the purified enzyme designated L-929 PNGase [19]. L-929 PNGase has unique and intriguing properties, e.g. it is inhibited by free oligosaccharides released from glycopeptides or glycoproteins in the PNGase reaction. This result suggests that in addition to a catalytic role of L-929 PNGase in de-*N*-glycosylation, such specific carbohydrate-binding may also be involved in intracellular interactions. The relevance of this finding is as yet unknown in the context of the theory of cell adhesions involving surface-bound glycosyltransferases [20]. To understand the intrinsic properties of animal-origin PNGases, we have in this study examined the carbohydrate binding activity of L-929 PNGase.

L-929 PNGase activity towards fetuin glycopentapeptide I was markedly inhibited by yeast mannan and triomannose, Manα1 → 3(Manα1 → 6)Man, but not by mannose and α-methyl mannoside, indicating that the enzyme specifically recognized larger oligomannose (at least triomannose) sequences rather than mono- or disaccharide residues. These enzymatic properties of the L-929 PNGase were compared with those of the known PNGases including PNGase A from almond and PNGase F from *F. meningosepticum*, and we found that the inhibitory effects of these free glycans were solely limited to L-929 PNGase and that PNGase A and PNGase F activities were not inhibited even by higher levels of free glycans. The binding experiment also demonstrated that L-929 PNGase has a marked carbohydrate-binding activity with asialofetuin-derived oligosaccharide alditol. Thus, the general properties of animal-derived PNGase described in this paper are contrasted with those of the commonly used PNGases and, in particular, the catalytic and carbohydrate-binding properties of L-929 PNGase appear to reflect the dual role of this molecule as a de-*N*-glycosylating enzyme and a carbohydrate-recognition (lectin-like) molecule.

Material and methods

Assay for PNGase activities

For determination of PNGase activity, the substrate used was fetuin asialoglycopeptide I [Leu-Asn($Man_3Gal_3GlcNAc_5$)-Asp-Ser-Arg], which was prepared from fetal calf serum fetuin (Nacalai Tesque Co., Japan) as previously described [19]. Fetuin asialoglycopeptide I was ^{14}C-labelled at the amino terminal residue by reductive methylation as previously described [16]. ^{14}C-labelling experiments were performed at the Radioisotope Centre, University of Tokyo. Specific radioactivity for fetuin [^{14}C]asialoglycopeptide I was 7.0×10^4 cpm nmol^{-1}.

PNGase activity was assayed by the method previously reported [19]. Briefly, the reaction mixture containing the enzyme fraction in a total volume of 10 µl of 100 mM MES buffer (pH 7.0), 0.25 M sucrose and 10 mM DTT together with 210 pmol of fetuin [^{14}C]asialoglycopeptide I (15 000 cpm) was incubated in a polypropylene microtube at 25 °C for 12 or 24 h. Reaction products were separated by paper chromatography and paper electrophoresis followed by quantitation of the radioactivities by a Bio-Imaging analyser (Fujix BAS 2000) as previously described [19]. One unit of L-929 PNGase activity was defined as the amount of enzyme that catalyses the release of 1 µmol of fetuin [^{14}C]peptide under the assay conditions at 25 °C for 24 h.

PNGase A from almond and PNGase F from *F. meningosepticum* were purchased from Seikagaku Kogyo Co., Tokyo and Takara Shuzo Co., Kyoto, respectively. PNGase A (0.77 µU) was incubated in 10 µl of 50 mM sodium citrate-phosphate buffer (pH 5.0) with 210 pmol of fetuin [^{14}C]asialoglycopeptide I at 25 °C for 15 min. The reaction was terminated by addition of 1 µl of 1 N NaOH followed by heating at 100 °C for 1 min. As for PNGase F, 0.37 µU of enzyme was incubated in 10 µl of 50 mM sodium phosphate buffer (pH 8.5) with 210 pmol of fetuin [^{14}C]asialoglycopeotide I at 25 °C for 20 min. The reaction was terminated by the addition of 1 µl of 0.1 M $MnCl_2$ followed by boiling at 100 °C for 1 min. For PNGase A and PNGase F, one unit of the enzyme activity is defined as the amount of enzyme required to hydrolyse 1 µmol min^{-1} of fetuin [^{14}C]asialoglycopeptide I to release fetuin [^{14}C]peptide under the respective assay conditions described above at 25 °C.

Endo- and exo-glycosidase assay

For endo-β-*N*-acetylglucosaminidase activity, ovalbumin-derived glycoasparagine GP-IVD ($Man_6GlcNAc_2Asn$) was used as the substrate after being ^{14}C-labelled as previously described [16]. GP-IVD was prepared and provided by Dr H Nomoto [21]. Specific radioactivity for the substrate was 1.3×10^5 cpm nmol^{-1}. Incubation and product determination was carried out as previously described [19].

Activities for β-galactosidase, β-*N*-acetylglucosaminidase, and α-mannosidase were assayed by the methods of Li and Li [22] using the appropriate *p*-nitrophenyl (PNP) glycosides, Galβ1 → PNP, GlcNAcβ1 → PNP, and Manα1 → PNP (Koch Light Laboratories Ltd., England), respectively.

Preparation of L-929 PNGase

The C3H mouse loose connective tissue-derived cell line L-929 was kindly donated by Dr Hiroshi Sakagami, Showa University School of Medicine. All preparation procedures were done at 4 °C. Cell culture of L-929 and preparation of the purified L-929 PNGase have been described elsewhere [19]. In some experiments, we used the partially purified PNGase fraction, which was prepared from L-929 cell lysate

as follows (cf. [19]). The soluble fraction obtained by ultracentrifugation of the lysate was chromatographed first on a TSK Butyl-Toyopearl 650 M column [Tosoh Co., Japan; 2.5 × 4.5 cm; equilibrated with 10 mM MES buffer (pH 7.5) containing 1.2 M ammonium sulfate, 0.25 M sucrose, and 2 mM DTT; eluted with a linear gradient (1.2–0 M) of ammonium sulfate in the same buffer] and second on a Sephacryl S-300 column [0.9 × 93 cm; eluted with 100 mM MES buffer (pH 6.8) containing 0.25 M sucrose and 2 mM DTT]. The PNGase activity-positive fraction thus obtained was subjected to 50% saturated ammonium sulfate precipitation. The precipitate was dissolved in 100 mM MES buffer (pH 7.0) containing 0.25 M sucrose and 10 mM DTT, and used as the partially purified PNGase. Protein was quantitated by the modified Lowry method (BCA, Pierce) with bovine serum albumin (BSA, Pierce) as the standard.

Effects of various compounds on PNGase activities

The following compounds were added to examine their effects on the PNGase activities: 1 mM GlcNAc-Asn (Sigma, USA), 10 mM D-mannose, Man (Wako Pure Chemicals, Japan), 10 mM α-methyl-D-mannoside (Seikagaku Kogyo Co., Japan), 10 mM triomannose, Manα1 → 3(Manα1 → 6)-Man (Dextra Laboratories Ltd., England) and 1.8 mg ml^{-1} of yeast mannan (Nacalai Tesque Co.). The concentration of triomannose as well as yeast mannan required for 50% inhibition of the partially purified L-929 PNGase activity was determined by adding various concentrations (1–20 mM for triomannose and 0.1–1.8 mg ml^{-1} for yeast mannan) in 10 μl of the reaction mixture containing 210 pmol of fetuin [^{14}C]asialoglycopeptide I.

Binding of L-929 PNGase to yeast mannan-coupled Sepharose 4B

Yeast mannan was immobilized using cyanogen bromide-activated Sepharose 4B [23] and the affinity matrix was washed as described by the manufacturer (Pharmacia, LKB). One ml of gel was coupled with 2.0 mg mannan. All the experiments were carried out at 4 °C, unless otherwise stated. Immediately before use, the gels were packed in a column (0.5 × 1.0 cm) and washed with at least five column volumes of Buffer A, consisting of 100 mM MES buffer (pH 7.0) containing 0.25 M sucrose, 10 mM DTT, and 1.6 mg ml^{-1} of BSA (Nacalai Tesque Co.).

Three hundred μl of the partially purified L-929 PNGase fraction was added to the mannan-conjugated Sepharose 4B column. The column was eluted first with 15 column volumes of Buffer A, secondly with 15 column volumes of 10 mM triomannose in Buffer A, and finally with 2 M NaCl in Buffer A. Fractions (0.5 ml) were collected and an aliquot of each fraction was assayed for PNGase activity after dialysis against 100 mM MES buffer containing 0.25 M sucrose and 10 mM DTT. As a control experiment, mannan-Sepharose 4B gel was incubated at 37°C in 5 ml 50 mM sodium acetate buffer (pH 4.5) with 0.5 U of Jack bean α-mannosidase (Seikagaku Kogyo Co.). Twelve h after incubation, 0.5 U α-mannosidase was added and incubated at 37 °C for an additional 16 h. The amount of mannose released was 1.7 mg ml^{-1} of gel, as estimated by the phenol-sulfuric acid method [24] using mannose as a standard.

The same experiments were carried out for PNGase A (77 μU) and PNGase F (7.5 μU) except that Buffer A was replaced by 50 mM sodium citrate-phosphate buffer, pH 5.0, (Buffer B) for PNGase A and by 50 mM sodium phosphate buffer, pH 8.5 (Buffer C) for PNGase F, both of which contained 1.6 mg ml^{-1} of BSA. PNGase activity of an aliquot of each fraction was determined after dialysis against Buffer B (for PNGase A) and Buffer C (for PNGase F) as described under 'Assay for PNGase activities' except that incubation time was 40 min for PNGase A and 90 min for PNGase F.

Enzyme kinetics for PNGase A- and F-catalysed deglycosylation of fetuin glycopeptide I

The Michaelis constants (K_m) for PNGase A and PNGase F were determined using fetuin glycopeptide I as the substrate which was previously used in estimation of K_m for L-929 PNGase [19]. Enzyme reaction was carried out as described except that concentration of the substrate was varied from 0.1 to 4.0 mM and incubation time was 3 h. The value of K_m was determined by Lineweaver-Burk plot as previously described [19].

Preparation of ^{3}H-labelled asialofetuin derived oligosaccharide alditol and determination of the dissociation constant (K_d) of its binding with L-929 PNGase

Asialofetuin was prepared by digestion of fetuin with 0.5 U of *Arthrobacter ureafaciens* sialidase (Nacalai Tesque Co., Japan) in 5.2 ml of 0.1 M sodium acetate buffer, pH 5.5, at 37 °C for 16 h. After desalting by passage through a Sephadex G-25 column (1.2 × 100 cm; eluted with water), asialofetuin was digested with 19 μU of PNGase F in 4 ml of 50 mM sodium phosphate buffer, pH 8.5, at 37 °C for 18 h. The reaction mixture was applied on a Sephadex G-50 column (1.2 × 96 cm; eluted with 0.1 M NaCl), 100 ml fractions were collected, and monitored by the phenol-sulfuric acid method [24]. Free oligosaccharide-containing fractions 56–65 was pooled and further purified by Bio-Gel P-4 chromatography (1.2 × 99 cm; eluted with 50 mM acetic acid). Carbohydrate composition of the free oligosaccharide thus prepared was determined by GLC analysis [25] to be Man:Gal:GlcNAc = 3:3:5. Tritium-labelling of asialofetuin-derived free oligosaccharide was carried out as previously described [26] at the Radioisotope Centre, University of Tokyo. Specific radioactivity for the asialofetuin-derived [^{3}H]oligosaccharide alditol was 3.4×10^4 cpm $nmol^{-1}$.

The dissociation constant (K_d) of L-929 PNGase-[^{3}H]oligosaccharide alditol was determined at 4 °C. L-929

Table 1. Comparison of enzymatic properties of L-929 PNGase with those of PNGase A from almond and PNGase F from *F. meningosepticum* (quoted in part from [19]).

	L-929 PNGase	*PNGase A*	*PNGase F*
Molecular weight (K)	212	66.8	34.8
pH optimum	7.0	4.5	8.5
K_m (mM)[a]	0.114	1.46	0.525
Subunit	Dimeric	Monomeric	Monomeric
Requirement of -SH group(s)	yes	no	no
Inhibition by free oligosaccharides	yes	no	no

[a] Values for fetuin glycopeptide I.

Table 2. Effects of various compounds on activities of the known PNGases.

		Relative activity[a] (%)		
Compound	*Concentration*	*L-929 PNGase*	*PNGase A*	*PNGase F*
Control	—	*100*	*100*	*100*
GlcNAc-Asn	1 mM	107	99	101
Man	10 mM	103	113	109
α-methyl-D-mannoside	10 mM	99	103	102
Manα1 → 3(Manα1 → 6)Man	10 mM	24	101	113
Yeast mannan	1.8 mg ml^{-1}	28	126	131

[a] Relative activity was expressed as the control activity set to 100. Each value represents the mean of duplicate assays.

PNGase (224 nM) was incubated in 100 µl of 100 mM MES buffer (pH 7.0) containing 0.25 M sucrose and 10 mM DTT with 0.15–2.9 nmol of [^{3}H]oligosaccharide alditol for 1 h, and an equal volume of saturated ammonium sulfate (pH 7.1) was slowly added to the reaction mixture. After 24 h, the solution was centrifuged at 17 000 × **g** for 30 min. Under these conditions, L-929 PNGase was recovered in the precipitate. The precipitate was resuspended in 100 µl of saturated ammonium sulfate (pH 7.1), centrifuged at 17 000 × **g** for 30 min, and radioactivity in the precipitate was determined on an Aloka liquid scintillation system LSC-700 with ACS-II (Amersham) as a scintillant. Non-specific binding of each incubation was estimated by measuring radioactivity in the precipitate in the presence of 20-fold excess amount of the unlabelled oligosaccharide alditol, and accounted for less than 1% of the input counts. The dissociation constant was estimated by Scatchard plot analysis as previously described [27].

Results

K_m *of the three PNGases, PNGase A, PNGase F, and L-929 PNGase, for fetuin glycopeptide I*

We determined the K_m value for L-929 PNGase to be 114 µM for fetuin glycopeptide I in a previous paper [19]. To compare this value with the corresponding values for the known PNGases A and F, we carried out a kinetic analysis for these enzymes using fetuin glycopeptide I as the substrate. The apparent K_ms are 1.46 mM and 525 µM for PNGase A and PNGase F, respectively (Table 1).

Effects of various compounds on PNGase activities

Highly purified L-929 PNGase, which was prepared as previously described [19], was used in the inhibition studies, and the enzymatic properties are compared with those of the known PNGases (Table 1). The specific activity was 107 mU per mg protein. GlcNAc-Asn, α-methyl mannoside, triomannose, and yeast mannan were examined for their ability to inhibit the de-*N*-glycosylation reaction of fetuin glycopentapeptide catalysed by three different PNGases, i.e. L-929 PNGase, PNGase A, and PNGase F. The results are summarized in Table 2. The only compounds showing significant inhibitory properties on L-929 PNGase activity were yeast mannan and triomannose. Note that the L-929 PNGase was not inhibited by the addition of 1 mM GlcNAc-Asn, 10 mM Man or 10 mM α-methyl mannoside. Yeast mannan and triomannose were also tested for their ability to inhibit the PNGase A and PNGase F activities. In contrast to L-929 PNGase, both yeast mannan and triomannose had no inhibitory effects on the activities of PNGase A and PNGase F.

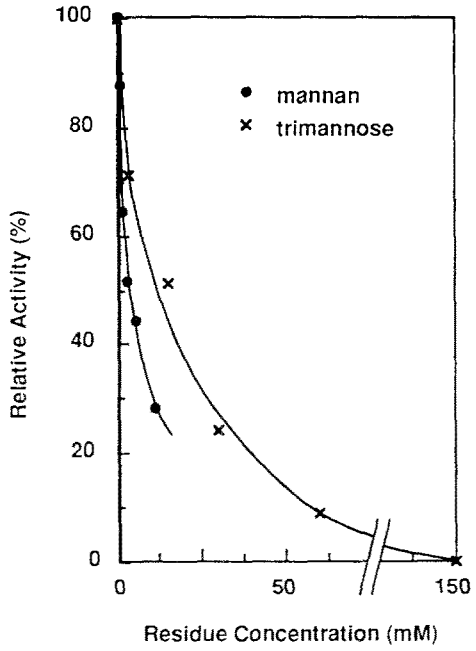

Figure 1. Inhibitory effects of triomannose (×) and yeast mannan (●) on L-929 PNGase activity. A partially purified enzyme was used in these experiments. Triomannose and yeast mannan were separately added to the reaction mixture at varying concentrations as described under 'Materials and methods'. Residue concentration in this Figure represents that of mannose in each compound added.

Inhibitory effects of triomannose and yeast mannan on L-929 PNGase activity

Inhibitory effects of triomannose and yeast mannan on L-929 PNGase reaction were examined at varying concentrations of these inhibitors (Fig. 1). The concentrations required for 50% inhibition of this PNGase activity under the conditions used were 3.5 mM for triomannose and 0.56 mg ml^{-1} for yeast mannan.

Binding of L-929 PNGase to mannan-coupled Sepharose 4B and asialofetuin-derived oligosaccharide alditol

Because a solution of the purified L-929 PNGase lost significant proportions of its original activity, the enzyme fraction was partially purified to 62 mU per mg protein with 33.6% yield and 250-fold purification from the crude L-929 cell lysate. This fraction was found to be devoid of endo-β-*N*-acetylglucosaminidase, β-galactosidase, and α-mannosidase activities, while a negligible amount of β-*N*-acetylglucosaminidase activity was detected, i.e. 2.7% of GlcNAcβ1 → PNP was cleaved on incubation, at 25 °C for 24 h, of 1 mM of the substrate with the enzyme fraction containing 0.51 mU of L-929 PNGase activity.

Due to the observed lability and shortage of the highly purified form of L-929 PNGase, partially purified enzyme fraction (300 µl or 3.1 mU) was loaded on the mannan-conjugated Sepharose 4B column, and eluted first with Buffer A, second with 10 mM triomannose in Buffer A, and finally with 2 M NaCl in Buffer A. As shown in Fig. 2A, 0.85 mU (28% recovery) of PNGase activity was eluted in 10 mM triomannose-pulsed fractions, while unbound activity (1.1 mU, 34% recovery) was detected in the pass-through fraction and negligible activity was observed in 2 M NaCl fractions. In the latter experiment the total recovery of activity was 69% and the corresponding value was 74% when chromatographed on the α-mannosidase-treated mannan-Sepharose 4B column, showing that approximately 30% of the activity was lost during passage through the column. The binding capacity of the affinity gel to L-929 PNGase was calculated to be 4.3 mU ml^{-1} (duplicate experiments). The activity found in the pass-through fractions was found to be adsorbed to the same mannan-Sepharose 4B column (not shown). Bound L-929 PNGase was not eluted with 5 mM EDTA (data not shown). To eliminate the possibility that the L-929 PNGase enzyme would interact with the protein moiety of mannan or the resin itself, α-mannosidase-treated mannan-Sepharose 4B column was used for the same experiment, which showed that almost all activity was recovered in the pass-through fractions from the α-mannosidase-treated column (Fig. 2B). These results clearly showed that L-929 PNGase bound yeast mannan conjugated Sepharose 4B in a carbohydrate dependent manner, and that binding of L-929 PNGase to the mannan-column required the mannose-containing glycan moiety of yeast mannan. Contrary to L-929 PNGase, neither PNGase A nor PNGase F bound to the mannan column (Fig. 2C and D).

The K_d value for the dissociation of L-929 PNGase-[^{3}H]oligosaccharide alditol was determined as 1.1×10^{-5} M (triplicate experiments) as described under Materials and methods, indicating that L-929 PNGase can bind quite strongly with the free oligosaccharide.

Discussion

In a recent study, we found the presence and developmental expression of the de-*N*-glycosylating enzyme (PNGase) in Medaka embryos as the first example of animal-originated PNGase [16, 28]. In subsequent studies we have also demonstrated the ability of mammalian cultured cell extracts and the homogenates of mammalian organs and tissues to convert an *N*-glycan linked glycopeptide into the free glycan having the GlcNAcβ1 → 4GlcNAc sequence at its reducing end and a de-*N*-glycosylated peptide where the glycan-linked Asn residue was deglycosylated to the Asp residue [17, 18]. Based on these findings, we proposed possible biological roles of PNGase as a de-*N*-glycosylating enzyme, which could be involved in regulation of physiological and physicochemical properties of glycoproteins by converting site-specifically the glycosylasparagine residue(s) to the aspartic acid residue(s), thereby introducing negative charge(s) to the core protein [16–19].

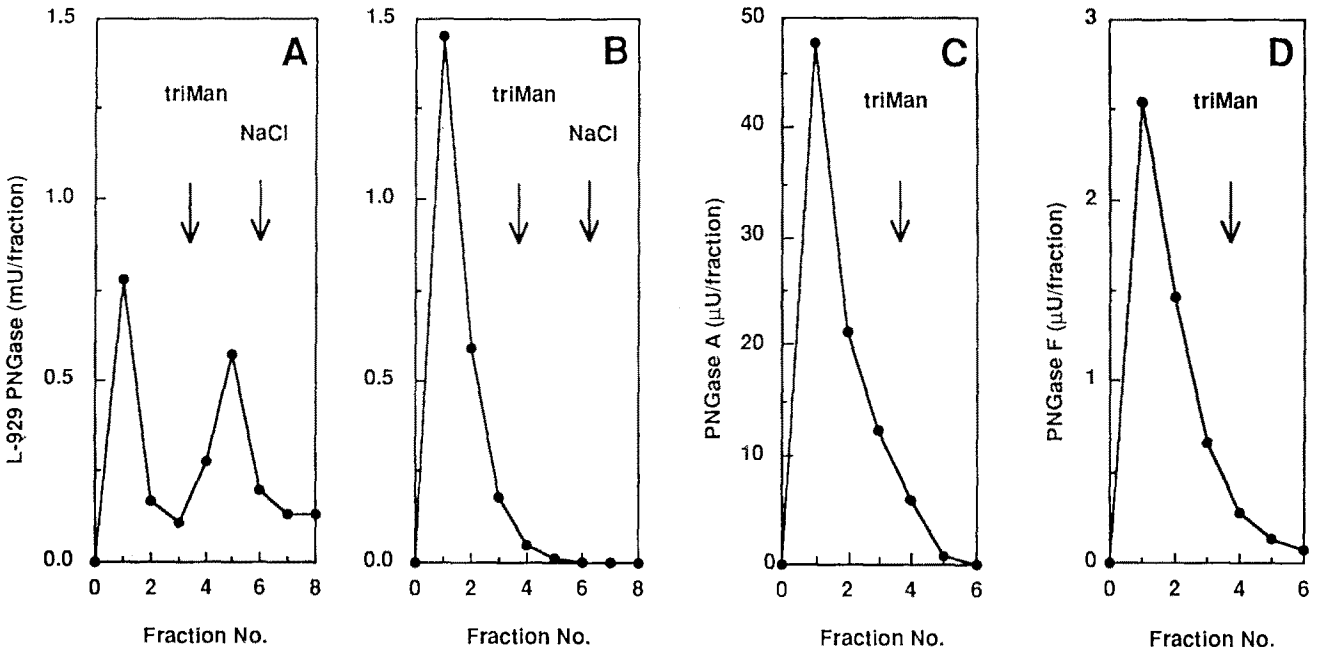

Figure 2. Elution profiles of (A) L-929 PNGase from a mannan-Sepharose 4B column, (B) L-929 PNGase from an α-mannosidase-treated mannan-Sepharose 4B column, (C) PNGase A and (D) PNGase F from a mannan-Sepharose 4B column. To the column were separately applied: 3.1 mU of L-929 PNGase in (A) and (B), 77 μU of PNGase A in (C), and 7.5 μU of PNGase F in (D), and the column was eluted first with 15 column volumes of the assay buffer used for each enzyme. Subsequently each column was eluted with the same volume of the assay buffer containing 10 mM triomannose (denoted as **triMan**) followed by elution with the buffer containing 2 M NaCl (denoted as **NaCl**) in (A) and (B). Fractions (0.5 ml) were collected and an aliquot of each fraction was assayed for PNGase activities after dialysis against the assay buffer.

Most recently, we have reported the isolation and purification of PNGase to homogeneity from mouse fibroblast L-929 cells [19]. The enzyme was designated as L-929 PNGase, and the results of Sephacryl S-300 chromatography and SDS polyacrylamide gel electrophoresis indicated that the native enzyme (M_r = 212 000) is a dimer of identical (or nearly identical) subunits (M_r = 105 000). L-929 PNGase is characterized by: (a) having relatively high molecular weight; (b) being a sulfhydryl protein whose enzymatic activity is destroyed by a sulfhydryl reagent; and (c) requiring a neutral pH for maximum activity. These properties distinguish the L-929 PNGase from other known PNGases such as PNGase A from almond seeds and PNGase F from *F. meningosepticum* (Table 1). The most intriguing and unexpected findings are: (i) L-929 PNGase has a relatively low K_m value (114 μM) with fetuin glycopeptide I as a substrate; and (ii) L-929 PNGase catalysis is strongly inhibited by fetuin-derived free *N*-glycan chains. These results suggest that L-929 PNGase may have a glycan-binding activity and may possibly function by a dual mechanism as a lectin-like receptor and a de-*N*-glycosylating enzyme, and this prompted us to examine a carbohydrate-binding activity of L-929 PNGase.

In this study, we were able to establish that L-929 PNGase possesses a strong carbohydrate-binding (lectin-like) activity by showing that L-929 PNGase was inhibited by yeast mannan and had a mannan glycan-binding activity. Yeast mannan is a carbohydrate-rich (approximately 97% by weight [23]) glycoprotein and contains both *O*- and *N*-linked glycan chains [29] on its polypeptide backbone. The *O*-glycans having a linear oligomannosyl saccharide chain are linked to Ser/Thr residues, and *N*-glycans have highly branched polymannose structures on a triomannosyl *N,N'*-diacetylchitobiose core. L-929 PNGase activity was also inhibited by triomannose but not by mannose and α-methyl mannoside. These results suggest that L-929 PNGase recognizes oligomannosyl structures and the triomannone structure appears to be a minimal sequence which is required for binding. How such an oligomannosyl structure could result in the inhibition of the enzyme activity of L-929 PNGase remains to be determined, although experiments to test whether these free glycans act as competitive substrate analogues or as negative allosteric effectors are underway. In this regard it should be noted that L-929 PNGase has a dimeric structure with a molecular weight (212 K) much larger than those (66.8 K and 34.8 K) of the known single polypeptidic PNGase A and PNGase F, respectively (Table 1).

Binding experiments showed that: (a) L-929 PNGase bound to mannan-conjugated Sepharose 4B and was eluted with 10 mM triomannose; and (b) it did not bind to α-mannosidase-treated mannan-Sepharose 4B, indicating

that glycan moieties of yeast mannan, but not glycan-peptide linkage regions, are involved in binding. Binding of L-929 PNGase to mannan-Sepharose 4B is independent of the presence of 5 mM EDTA, indicating that it does not require a calcium ion and thus the mechanism of carbohydrate binding is different from C-type lectins, virtually all of which are calcium ion-requiring [23, 27, 30]. It should also be noted that as evidenced from the data presented herein, i.e. the association constant for the binding of L-929 PNGase with the fetuin-derived oligosaccharide alditol ($K_a = 8.8 \times 10^4\ M^{-1}$), L-929 PNGase possesses a strong carbohyrate-binding (lectin) activity.

Many studies have illustrated that lectins and enzymes are present as carbohydrate-recognition proteins [1–3, 6–12, 20, 23, 27, 30–37]. Following the finding by Ashwell of the specific asialoglycoprotein-binding receptor protein of liver membranes, a number of animal lectins were identified suggesting that such carbohydrate binding may be involved in intercellular interactions on the cell surface [1–3, 32, 33] besides having a role in glycoprotein catabolism and transport [34–36], although a specific cellular function cannot yet be unequivocally assigned to any lectin. The relevance of the findings of animal cell-surface lectins is also obvious in the context of the proposed role of surface-bound glycosyltransferases in the binding and adhesive properties of membranes [20]. Mono- and polyvalent carbohydrate-binding surface enzymes such as glycosyltransferases and glycosidases have been suggested to be lectin-like receptors that have the ability to bind and catalytically release their respective potential substrates on apposing cell surfaces [20, 37]. Certain glycosyltransferases and glycosidases are considered to be specific cell-surface receptors for respective glycan chains on some gamete cells at fertilization and embryogenesis [6–12]. C-type rattlesnake venom lectin has recently been shown to possess anti-freeze properties [38]. It should also be noted that lectin and lectin-like activities have been shown to be covalently associated with other functional proteins including mosaic proteins such as selectin families [32, 33], mannose-binding protein [39], neural cell adhesion molecule [40], and tumour necrosis factor [41]. A growing number of studies indicate that many proteins, including enzymes [42], have lectin consensus sequences [1–3] although lectin activities were not tested [42–46].

The present study extends these results by demonstrating that PNGase functions as a specific carbohydrate-binding protein. Presently, we favour the hypothesis that PNGase may be an enzyme active in certain specialized cell types or during certain stages of development, such as during embryogenesis [16, 28, 47, 48]. What remains to be identified is the physiological ligand(s) for L-929 PNGase *in vivo*. Also it is not known if L-929 PNGase would belong to mosaic proteins, but its carbohydrate-binding activity and de-*N*-glycosylating activity together with the widespread occurrence of PNGases among animal phyla suggest that PNGases may function as mediators or receptors in glycoprotein transport and as regulators in the biosynthesis and catabolism of glycoproteins.

Finally, it should be noted that L-929 PNGase was concluded to be a cytosolic enzyme as evidenced from the centrifugation studies [17, 19]. Most intracellular glycosyltransferases are known to be bound with varying degrees of tenacity to the membrane system of the cell. However, soluble forms of the transferases have previously been described [49–51], and the occurrence of cytosolic glycosidases has recently been reviewed [52], although the exact location and function of these soluble enzymes are still not clear. It does of course remain possible that some functions of soluble forms of enzymes, including L-929 PNGase, may be mediated by properties that have not yet been identified. While further studies are required to elucidate the exact role of this enzyme in cellular processes, one important conclusion can now be drawn from our experiments: PNGase can no longer be considered as only occurring in the plant kingdom and at least some, if not all, of the animal PNGases may function as specific carbohydrate-binding molecules.

Acknowledgements

This research was supported by Grant from Mizutani Foundation for Glycoscience (to Y.I.) and by Grant-in-Aid 05808053 for General Scientific Research from the Ministry of Education, Science, and Culture of Japan (to K.K.).

References

1. Drickamer K (1988) *J Biol Chem* **263**:9557–60.
2. Drickamer K, Taylor ME (1993) *Annu Rev Cell Biol* **9**:237–64.
3. Hirabayashi J (1993) *Trends Glycosci Glycotechnol* **5**:251–70.
4. Goldstein IJ, Hughes RC, Monsigny M, Osawa T, Sharon N (1980) *Nature* **285**:66.
5. Sharon N, Lis H (1972) *Science* **177**:949–59.
6. Hoshi M, De Santis R, Pinto MR, Cotelli F, Rosati F (1985) *Zool Sci* **2**:65–69.
7. Hoshi M (1986) *Adv Exp Med Biol* **207**:251–60.
8. Shur BD (1989) *Biochim Biophys Acta* **988**:389–409.
9. Shur BD (1991) *Glycobiology* **1**:563–75.
10. Skedlarek MD, Tulsiani DRP, Nagdas SK, Orgebin-Crist M-C (1993) *Biol Reprod* **49**:204–13.
11. Miller DJ, Gong X, Decker G, Shur BD (1993) *J Cell Biol* **123**:1431–40.
12. Litscher ES, Wassarman PM (1993) *Trends Glycosci Glycotechnol* **5**:369–88.
13. Takahashi N (1977) *Biochem Biophys Res Commun* **76**:1194–1201.
14. Plummer TH Jr, Phelan AW, Tarentino AL (1987) *Eur J Biochem* **163**:167–73.
15. Plummer TH Jr, Elder JH, Alexander S, Phelan AW, Tarentino AL (1984) *J Biol Chem* **259**:10700–4.
16. Seko A, Kitajima K, Inoue Y, Inoue S (1991) *J Biol Chem* **266**:22110–14.

17. Suzuki T, Seko A, Kitajima K, Inoue Y, Inoue S (1993) *Biochem Biophys Res Commun* **194**:1124–30.
18. Suzuki T, Seko A, Kitajima K, Inoue Y, Inoue S (1993) *Glycoconjugate J* **10**:223.
19. Suzuki T, Seko A, Kitajima K, Inoue Y, Inoue S (1994) *J Biol Chem* **269**:17611–18.
20. Roseman S (1970) *Chem Phys Lipids* **5**:270–97.
21. Nomoto H, Inoue Y(1983) *Eur J Biochem* **135**:243–50.
22. Li Y-T, Li S-C (1972) *Methods Enzymol* **28**:702–13.
23. Kawasaki T, Etoh R, Yamashina I (1978) *Biochem Biophys Res Commun* **81**:1018–24.
24. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Chem* **28**:350–56.
25. Nomoto H, Iwasaki M, Endo T, Inoue S, Inoue Y, Matsumura G (1982) *Arch Biochem Biophys* **218**:335–41.
26. Kitazume S, Kitajima K, Inoue S, Inoue Y (1992) *Anal Biochem* **202**:25–34.
27. Maynard Y, Baenziger JU (1982) *J Biol Chem* **257**: 3788–94.
28. Inoue S, Iwasaki M, Seko A, Kitajima K, Inoue Y (1993) *Glycoconjugate J* **10**:223.
29. Ballou CE, Lewis MS (1991) *J Biol Chem* **266**:8255–61.
30. Colley KJ, Beranek MC, Baenziger JU (1988) *Biochem J* **256**:61–68.
31. Ofek I, Sharon N (1990) *Curr Topics Microbiol Immunol* **151**:91–113.
32. Lasky LA (1992) *Science* **258**:964–69.
33. Aruffo A (1992) *Trends Glycosci Glycotechnol* **4**:146–51.
34. Ashwell G, Harfold J (1982) *Annu Rev Biochem* **51**:531–54.
35. Hoyle GW, Hill RL (1991) *J Biol Chem* **266**:1850–57.
36. Kornfeld S (1992) *Annu Rev Biochem* **61**:307–30.
37. Roth S (1973) *Quart Rev Biol* **48**:541–63.
38. Rubinsky B, Coger R, Ewart KV, Fletcher GL (1992) *Nature* **360**:113–14.
39. Schweinle JE, Nishiyasu M, Ding TQ, Sastry K, Gilles SD, Ezekowitz AB (1993) *J Biol Chem* **268**:364–70.
40. Horstkorte R, Schachner M, Magyar JP, Vorherr T, Schmitz B (1993) *J Cell Biol* **121**:1409–21.
41. Lucas R, Magez S, De Leys R, Fransen L, Scheerlinck J-P, Rampelberg M, Sablon E, De Baestselier P (1994) *Science* **263**:814–17.
42. Ackerman SJ, Corrette SE, Rosenberg HF, Bennett JC, Mastrianni DM, Nicholson-Weller A, Weller F, Chin DT, Tenen DG (1993) *J Immunol* **150**:456–68.
43. Wen D, Dittman WA, Ye RD, Deaven LL, Majerus PW, Sadler JE (1987) *Biochemistry* **26**:4350–57.
44. Nakayama E, Von Hogen I, Parnes JR (1989) *Proc. Natl Acad Sci USA* **86**:1352–56.
45. Rouquier S, Verdier J-M, Iovanna J, Dagorn J-C, Giorgi D (1991) *J Biol Chem* **266**:786–91.
46. Takeya H, Nishida S, Miyata T, Kawada S, Saisaka Y, Morita T, Iwanaga S (1992) *J Biol Chem* **267**:14109–17.
47. Seko A, Kitajima K, Iwasaki M, Inoue S, Inoue Y (1989) *J Biol Chem* **264**:15922–29.
48. Seko A, Kitajima K, Inoue S, Inoue Y (1991) *Biochem Biophys Res Commun* **180**:1165–71.
49. Richard M, Martin A, Louisot P (1975) *Biochem Biophys Res Commun* **64**:109–14.
50. Shaper NL, Hollis GF, Douglas JG, Kirsch IR, Shaper JH (1988) *J Biol Chem* **263**:10420–28.
51. Gonzalez-Yanes B, Cicero JM, Brown RD Jr, West CM (1992) *J Biol Chem* **267**:9595–605.
52. Haeuw J-F, Michalski J-C, Strecker G, Spik G, Montreuil J (1991) *Glycobiology* **1**:487–92.