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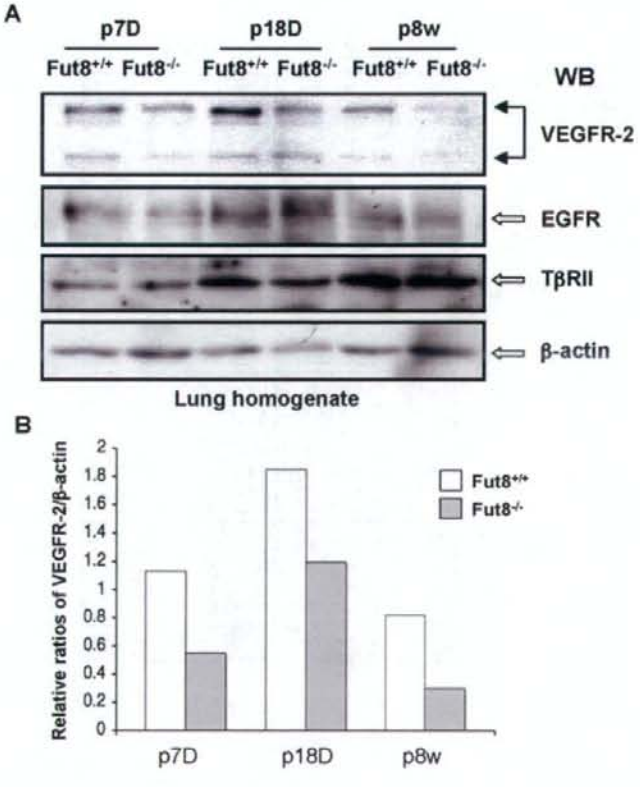

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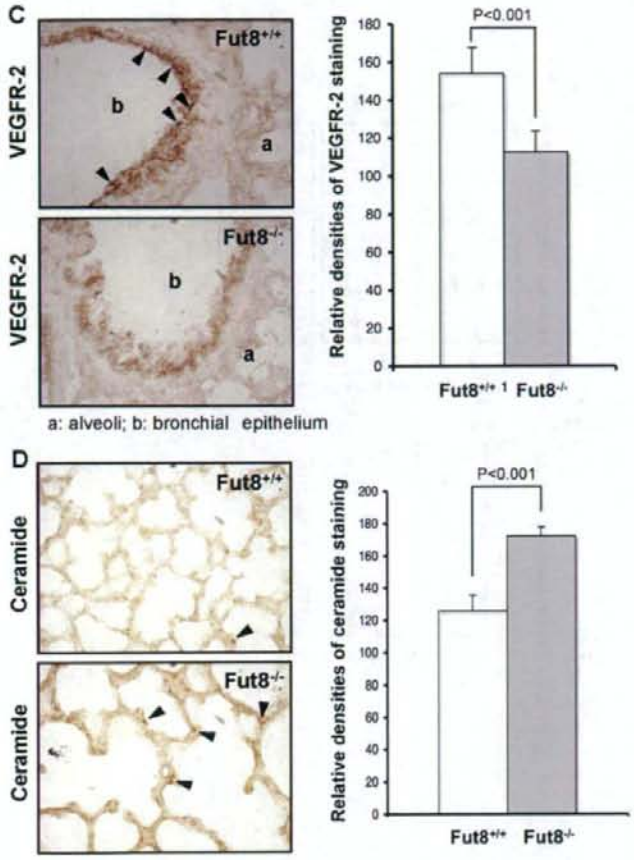

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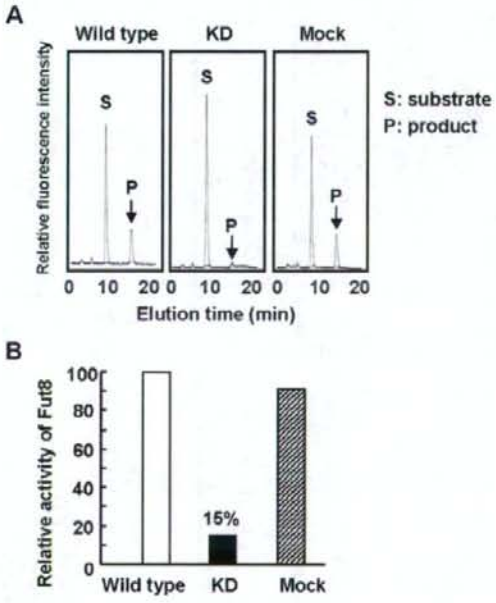

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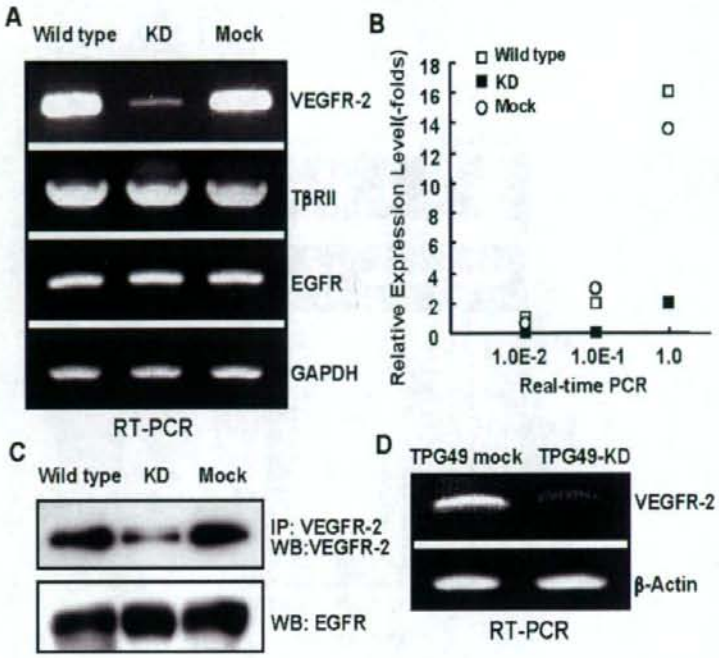

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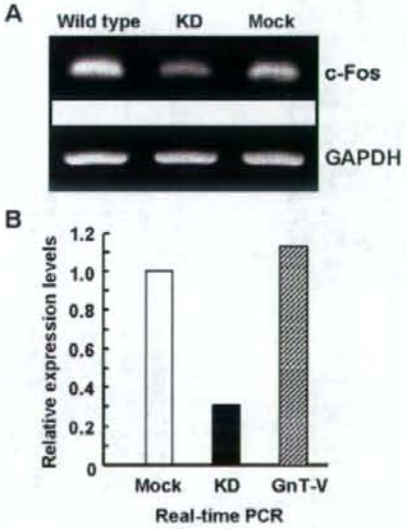

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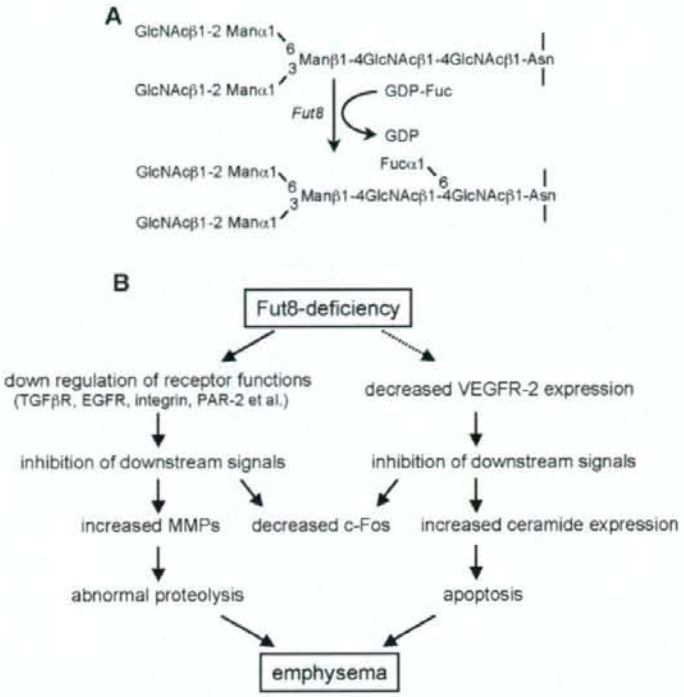

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Reviews

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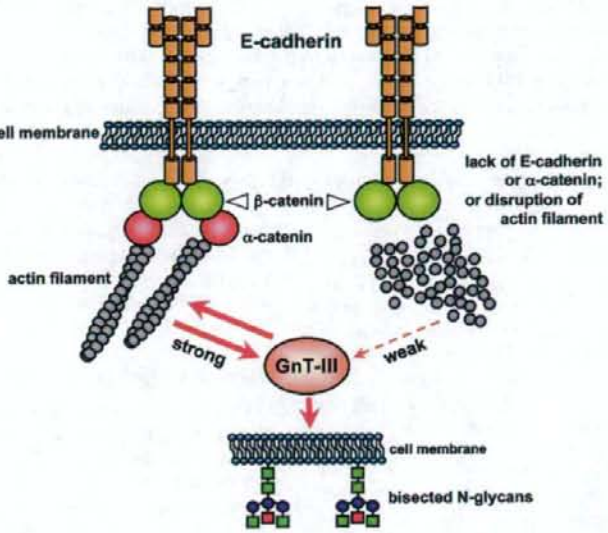


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# A Mutual Regulation between Cell−Cell Adhesion and N-Glycosylation: Implication of the Bisecting GlcNAc for Biological Functions

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Changes in oligosaccharide structures are associated with numerous physiological and pathological events. E-cadherin-mediated cell−cell adhesion is believed to be both temporally and spatially regulated during development, and represents a key step in the acquisition of the invasive phenotype for many tumors. Here, we focus mainly on a mutual regulation between E-cadherin-mediated cell−cell adhesion and *N*-acetylglucosaminyltransferase III (GnT-III) expression, and discuss its implications for biological functions.

**Keywords:** Bisecting GlcNAc • cell adhesion • E-cadherin • N-glycosylation • GnT-III • integrin

## Introduction

Oligosaccharides have various effects on the functional aspects of glycoproteins and play important roles in cell differentiation, adhesion and proliferation.[1,2] The oligosaccharides of glycoproteins are produced via catalysis by various glycosyltransferases. As a result, a specific structure is determined by the expression pattern of these enzymes and their associated molecules under physiological and pathological events, including cell growth, migration, differentiation and tumor invasion. An increasing body of evidence suggests that the structures of oligosaccharide greatly contribute to cell adhesion, cell invasion and cancer metastasis. It is known that some malignant phenotypes are highly associated with N-linked oligosaccharides (N-glycan) containing $\beta$1,6 *N*-acetylglucosamine branching, which is an association catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V). By contrast, *N*-acetylglucosaminyltransferase III (GnT-III) has been found to play an important role in the suppression of metastasis. Therefore, it is not surprising that aberrant glycosylation patterns can serve as markers for certain disease states including cancer metastasis, development and differentiation.[3] E-cadherin is the main epithelial cell−cell adhesion molecule, which is involved in $Ca^{2+}$-dependent cell−cell adhesion. It is well-known that epithelia-to-mesenchymal transition, a process associated with normal development, wound healing, cancer progression and metastasis, is associated with loss of E-cadherin expression.[4] This review reports recent advances in the effect of cell adhesion on expression of bisected N-glycans on cell surface receptors modified by GnT-III,[5,6] which, in turn, regulates the function of the cell-adhesion molecules, integrin and E-cadherin, and further addresses the important roles of N-glycans in cell biology and cancer metastasis.

## Biological Significance of GnT-III

GnT-III transfers *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc to a $\beta$1,4 mannose in N-glycans to form a "bisecting" GlcNAc linkage, as shown in Figure 1. Bisecting GlcNAc linkages are found in various hybrid and complex N-glycans on glycoproteins. GnT-III is generally regarded as a key glycosyltransferase in N-glycan biosynthetic pathways. Introduction of a bisecting GlcNAc suppresses $\beta$1,6 GlcNAc branching formation catalyzed by GnT-V, which is strongly associated with cancer metastasis, since GnT-V cannot utilize the bisected oligosaccharide as an acceptor substrate.[7−9] The $\beta$1,6 GlcNAc-branched N-glycans can be preferentially modified by $\beta$1,4 GalT and $\beta$1,3 GlcNAcT to form poly-*N*-acetyllacotosamine for elongation of N-glycans, which are further processed to form other sugar motifs such as sialyl Lewis X, which may contribute to promotion of cancer metastasis (Figure 1). It has also been reported that GnT-V activity and $\beta$1,6 GlcNAc-branched N-glycans levels are increased in highly metastatic tumor cell lines.[10,11] Consistently, cancer metastasis is greatly suppressed in GnT-V knockout mice.[12] Therefore, GnT-III has been proposed as an antagonistic of GnT-V, thereby contributing to the suppression of cancer metastasis. In fact, overexpression of GnT-III in highly metastatic melanoma cells reduced $\beta$1,6 GlcNAc branching in cell-surface N-glycans and increased bisected N-glycans,[13] which resulted in an enhancement of cell−cell adhesion due to prolonged turnover of E-cadherin on the cell surface.[14] In contrast to GnT-III, overexpression of GnT-V resulted in

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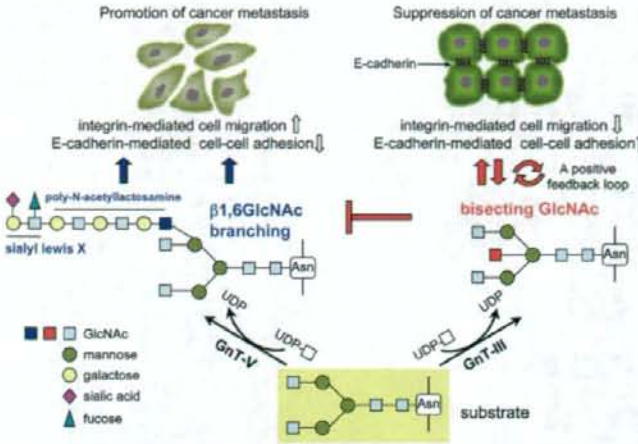


**Figure 1.** Glycosylation reactions catalyzed by the action of glycosyltransferase GnT-III and GnT-V. Enhanced expression of GnT-V in epithelial cells results in a loss of cell–cell adhesion, increasing integrin-mediated cell migration. In contrast, overexpression of GnT-III strengthens cell–cell interaction, and down-regulates integrin-mediated cell migration, which may contribute to the suppression of cancer metastasis. The $\beta$1,6GlcNAc branching is preferentially modified by polylactosamine and other sugar motifs such as sialyl Lewis X, which also contribute to promotion of cancer metastasis. It is worth mentioning that the mutual regulation of GnT-III and E-cadherin-mediated cell–cell interaction exists as a positive feedback loop.

decreased N-cadherin clustering on the cell surface and down-regulation of cadherin-associated cell–cell adhesion.[15] N-cadherin is found primarily in neural tissues and fibroblasts, where it is thought to mediate a less stable and more dynamic form of cell–cell adhesion.[16] Therefore, the molecular mechanism for the suppression of cancer metastasis by GnT-III is partly explained by an increase in E-cadherin-mediated homotypic adhesion.

GnT-III also contributes to suppression of metastasis by remodeling some important glycoproteins, such as epithelial growth factor receptor (EGFR),[17–19] and adhesion molecules such as integrin. The extracellular domain of EGFR contains 12 potential N-glycosylation sites,[20] and the remodeling of N-glycans on EGFR can modulate EGFR-mediating functions.[21–26] The binding of EGF to EGFR is reported to be significantly reduced by treatment with some N-glycosylation inhibitors.[21] In addition, EGF binding, as well as tyrosine kinase activity, is reduced in the presence of certain lectins,[22–24] and the glycosylation site on Asn-420 of EGFR is shown to suppress ligand independent spontaneous oligomerization.[27] The overexpression of GnT-III significantly reduces the ability of EGF to bind to its receptor, reduces EGFR autophosphorylation, and subsequently blocks EGFR-mediated Erk phosphorylation in U373 MG glioma cells[25] and in PC12 cells.[26] On the other hand, Partridge et al. reported that GnT-V-modified N-glycans containing poly-*N*-acetyllactosamine, the preferred ligand for galectin-3, on surface receptors oppose their constitutive endocytosis, which resulted in the promotion of intracellular signaling, and, consequently, cell migration and tumor metastasis.[28] The opposing effects of GnT-III and GnT-V have been observed for the same target protein.[29] Integrin $\alpha3\beta1$, which is believed to be highly associated with tumor metastasis, can be modified by either GnT-III or GnT-V. GnT-V stimulates $\alpha3\beta1$ integrin-mediated cell migration, while overexpression of GnT-III inhibits GnT-V-induced cell migration. The modification of the $\alpha3$ subunit by GnT-III supersedes modification by GnT-V, and as a result, GnT-III inhibits GnT-V-induced cell migration. These results strongly suggest that N-glycan plays important roles in biological functions.

In addition, GnT-III also inhibits the formation of the $\alpha$-Gal epitope, which is a major xenotransplantation antigen that is problematic in swine-to-human organ transplantation.[30] Moreover, GnT-III affects antibody-dependent cellular cytotoxicity (ADCC) activity,[31] although the effect of GnT-III on ADCC activity appears to be less than that of core fucose structures.[32,33] Transgenic mice, in which GnT-III was expressed specifically in the liver by use of a serum amyloid P component gene promoter, exhibited fatty liver. It has been proposed that ectopic expression of GnT-III disrupts the function of apolipoprotein B, resulting in abnormal lipid accumulation.[34] To explore the physiological roles of GnT-III, GnT-III-deficient mice have been established using gene targeting. These mice are viable and reproduce normally, suggesting that GnT-III and the bisected N-glycans apparently are not essential for normal development.[35,36] Because no physical abnormalities were apparent, the physiological roles of GnT-III are yet to be identified.

## E-Cadherin-Mediated Cell Adhesion

The Adherens junction (AJ) provides important adhesive contacts between neighboring epithelial cells, and forms intracellular links to the actin cytoskeleton and signaling pathways including the regulation of gene transcription.[37] E-cadherin is a single-pass, transmembrane glycoprotein (Figure 2) that belongs to the classical cadherin family of $Ca^{2+}$-dependent adhesion proteins; other members of this family include N-, P-, and R-cadherin.[38] E-cadherin is the core transmembrane protein of the adherens junction and is required for binding and localization of a number of important cytoplasmic proteins, termed catenins, that connect the cadherin complex to the actin cytoskeleton (Figure 2) and several signaling pathways. The catenin family comprises $\alpha$-catenin, $\beta$-catenin and $\gamma$-catenin (plakoglobin). It is believed that E-cadherin directly binds

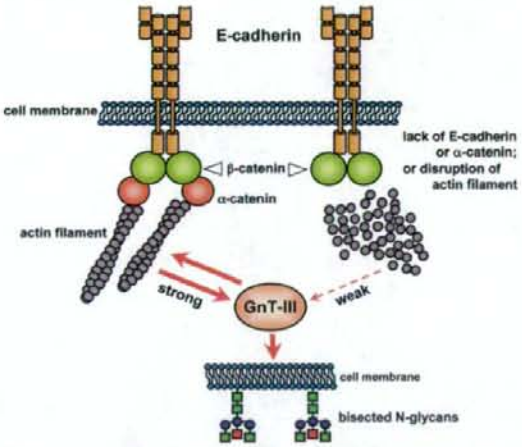


**Figure 2.** A working model for cell–cell adhesion and regulation of GnT-III expression. The GnT-III expression and its products, the bisected N-glycans, were up-regulated by cell–cell interaction via the E-cadherin–catenin–actin complex. Conversely, overexpression of GnT-III enhanced E-cadherin-mediated cell adhesion. The weak signal could be considered that GnT-III expression is regulated by an E-cadherin–catenin-independent mediated cell–cell adhesion.

to either $\beta$- or $\gamma$-catenin, resulting in a recruitment of α-catenin, which links the E-cadherin complex to the actin cytoskeleton. Indeed, α-catenin contains multiple interaction sites: actin-binding sites, $\beta$-catenin-binding sites and binding sites for other actin-binding proteins, such as α-actinin[39] and vinculin.[40] However, recent evidence has shown that this simultaneous interaction could not be reconstituted *in vitro*.[41] Alpha-catenin exists in either a monomeric or homodimeric state. *In vitro* binding assays demonstrated that monomeric α-catenin binds $\beta$-catenin, but not actin. Conversely, homodimeric α-catenin binds actin filaments but not $\beta$-catenin.[41] A dynamic crosstalk between the α-catenin plasma membrane pool (monomeric, $\beta$-catenin bound) and cytoskeleton pool (dimeric, actin-bound) has been proposed where α-catenin switches between the adherens complex and binding the actin cytoskeleton.[42] In fact, without α-catenin, tight cell–cell associations do not form despite cadherin expression.[43]

Cadherin-mediated cell–cell adhesion is highly dynamic and enables the reorganization and dispersal of cells, for example, during epithelial-to-mesenchymal transition in normal development and carcinogenesis.[4] In epithelial derived tumors, loss of cell–cell adhesion is correlated with down-regulation of E-cadherin as well as increased proliferation and tumor invasiveness. For example, E-cadherin regulates normal cell–cell adhesion in the mammary gland and expression of E-cadherin in breast cancer has been studied in relation to prognosis, diagnosis, and potential therapy.[44] Ductal carcinoma, accounting for ~80% of breast cancer, is associated with a reduction in both E-cadherin and α-catenin;[44] moreover, the loss of α-catenin was associated with advanced stages and poor patient survival.[45] Taken together, these observations provide strong evidence that regulation of E-cadherin and associated protein expression and localization are factors involved in carcinogenesis.

## A Mutual Regulation between GnT-III Expression and E-Cadherin-Mediated Cell Adhesion

As described above, regulation of cadherin-mediated adhesion and associated adherence junctions is thought to underlie the dynamics of intercellular adhesive interactions, which are regulated during tissue development and homeostasis, as well as during tumor cell progression. During normal development, E-cadherin-mediated cell adhesion is vital to gastrulation, which recognizes embryonic germ layers, as well as to the development of other migratory cell types, such as the neural crest.[46] E-cadherin engagement at cell–cell contacts is known to suppress proliferation; this effect has been best described in the context of tumorigenesis.[47] Conversely, the disruption of E-cadherin-mediated cell adhesion appears to be a central event in the transition from noninvasive to invasive carcinomas. Therefore, most studies have focused on the identification and characterization of transcriptional repressors of E-cadherin expression in epithelial tumor cells. The most prominent factors identified in these studies included the related factors, Slug, Snail, SIP1 and Twist, which are best known for their roles in early embryogenesis and tumor progression.[48]

However, E-cadherin can be post-translationally modified by phosphorylation, O-glycosylation and N-glycosylation. Casein kinase II, a serine-threonine kinase, phosphorylates the cytosolic tail of E-cadherin and enhances binding to $\beta$-catenin.[49] Cytoplasmic O-glycosylation of the E-cadherin cytosolic tail has been shown to occur in response to endoplasmic reticulum (ER) stress and inactivate E-cadherin-mediated intercellular adhesion by preventing its transport to the cell membrane.[50] In addition, E-cadherin can be N-glycosylated and the N-glycosylation at Asn-633 is essential for E-cadherin expression, folding and trafficking.[51,52] In fact, our earlier study showed that E-cadherin-mediated cell–cell adhesion is regulated by post-transcriptional modification of N-glycans. Overexpression of GnT-III increased the retention of E-cadherin at the cell border, which resulted in an enhancement of E-cadherin-mediated homotypic adhesion. And the increased GnT-III product on E-cadherin reduces phosphorylation of $\beta$-catenin either by EGFR or by Src signaling.[14,53] As a result, $\beta$-catenin remains tightly complexed with E-cadherin and is not translocated into the nuclei. Otherwise, $\beta$-catenin enhances expression of genes that promote cell growth or oncogenesis. Thus, expression of E-cadherin may be regulated, not only by transcriptional factors, but also by post-transcriptional processing, maturation and modifications.

Conversely, GnT-III is regulated by E-cadherin-mediated cell–cell adhesion.[54] GnT-III activity was increased under dense culture conditions compared with sparse culture conditions. A significant up-regulation of GnT-III expression was observed only in epithelial cells that express basal levels of E-cadherin and GnT-III, but not in the following: the MDA-MB231 cell, an E-cadherin-deficient cell line; the MDCK cell, in which GnT-III expression is undetectable; and fibroblasts, which lack E-cadherin. The expression levels of GnT-III and its products, the bisected N-glycans, were up-regulated by cell–cell interaction via the E-cadherin–catenin–actin complex. Disruption of actin polymerization by treatment with cytochalasin D or lack of α-catenin expression interfered with regulation of GnT-III (Figure 2). Interestingly, reintroduction of α-catenin into α-catenin-deficient cells rescued GnT-III expression enhanced under dense culture conditions.[55] Given the previously described important biological functions of GnT-III,[36,56] the present study provides new insight into the molecular mech-

anism of relationships among cell−cell interaction, normal development and cancer metastasis. The E-cadherin−catenin−actin complex formation is very important for regulation of GnT-III expression, but not exclusive, since the modest increase in GnT-III expression under dense culture conditions could be observed in α-catenin-deficient DLD-1 cells as well as in E-cadherin-deficient MDA-MB231 cells,[54] which may be modulated by an E-cadherin−catenin-independent pathway (weak signal), as shown in Figure 2. Therefore, the mutual regulation of GnT-III expression and E-cadherin-mediated cell−cell interaction exists as a positive-feedback loop.

It is worth mentioning that sialylation is also associated with cell density. Wieser et al. reported that cell-contact mediated hyposialylation of contactinhibin,[57] which is involved in contact-dependent inhibition of growth. It could be explained that introduction of a bisecting GlcNAc by GnT-III expression induced by a dense culture suppressed GlcNAc branching formations catalyzed by GnT-IV or/and GnT-V, which resulted in a decrease in sialylation on nonreducing terminal of N-glycans. The detailed molecular mechanism remains further study.

## Future Perspectives

To a certain extent, cells cultured under sparse and dense culture conditions can be viewed as cells in the proliferative and differentiative maintenance states, respectively. GnT-III expression was significantly up-regulated in cells cultured under dense conditions. This would reasonably maintain cell differentiation rather than cell proliferation, since growth factor-mediated activation can be suppressed by the up-regulation of GnT-III. In fact, the results of several studies suggest that E-cadherin can induce ligand-independent activation of the EGFR and subsequent activation of Rac1 and MAP kinase, which appears to be involved in cell migration and proliferation.[58,59] Thus, it is possible that up-regulation of GnT-III by cell−cell interaction might neutralize the signals responsible for maintenance of the cell differentiation phenotype. In conclusion, it would be quite interesting and important to further examine whether the phenomena *in vitro* can also be observed *in vivo*, using animal models such as GnT-III knockout mice and epithelia tumor models, to elucidate the relationship between bisected N-glycosylation by GnT-III and cell adhesion.

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# Dual-gradient high-performance liquid chromatography for identification of cytosolic high-mannose-type free glycans

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ABSTRACT

It has been shown that free oligosaccharides derived from N-linked glycans accumulate in the cytosol of animal cells. Most of the glycans have only a single GlcNAc at their reducing termini (Gn1 glycans), whereas the original *N*-glycans retain *N,N'*-diacetylchitobiose at their reducing termini (Gn2 glycans). Under the conditions of high-performance liquid chromatography (HPLC) mapping established for pyridylamine (PA)-labeled Gn2 *N*-glycans, Gn1 glycans are not well retained on reversed-phase HPLC, making simultaneous analysis of Gn1 and Gn2 glycans problematic. We introduced a dual gradient (i.e., pH and butanol gradient) for the separation of Gn1 and Gn2 glycans in a single reversed-phase HPLC. Determination of elution time for various standard Gn2 high-mannose-type glycans, as well as Gn1 glycans found in the cytosol of animal cells, showed that elution of Gn1 and Gn2 glycans could be separated. Sufficient separation for most of the structural isomers could be achieved for Gn1 and Gn2 glycans. This HPLC, therefore, is a powerful method for identification of the structures of PA-labeled glycans, especially Gn1-type glycans, isolated from the cytosol of animal cells.



Pyridylamination of the reducing end of oligosaccharides is widely used for highly sensitive labeling of oligosaccharides [1,2]. Two- and three-dimensional mapping is a powerful characterization method to identify oligosaccharide structure [2–5]. Using this method, various structures can be deduced based on the elution position of given *N*-glycans by two- or three-dimensional high-performance liquid chromatography (HPLC)[3] methods (anion exchange, reversed phase, and size fractionation). This method has proven to be sufficiently powerful to analyze the structure of Gn2 *N*-glycans retaining *N,N'*-diacetylchitobiose at their reducing termini. The same strategy cannot be applied for Gn1-type glycans bearing only a single GlcNAc at their reducing termini because the glycans are not retained well in the octadecylsilica (ODS) column under the conditions usually employed. The separation method for Gn1-type glycans has been reported [6], but there is no established method to obtain sufficient separation for structural isomers of Gn1 and Gn2 glycans using a single analytical method.

Besides butanol concentration, pH is a key factor for the partition of pyridylamine (PA)-labeled oligosaccharides with the ODS column [7]. Using this principle, we established an HPLC condition in which a gradient of butanol concentration and pH was generated. Under this condition, we analyzed various Gn1- and Gn2-type, high-mannose-type oligosaccharides, which are the major free oligosaccharides found in animal cells [6,8–14]. Our results clearly showed that Gn1 and Gn2 glycans give reproducible retention times, expressed as glucose units (GUs), with sufficient separation of structural isomers. Although the empirical additive rule with regard to the reversed-phase HPLC had been used successfully [15–18], ratio comparison was found to be more consistent than the additive scale when compared with Gn1 and Gn2 glycans with the same isomeric oligosaccharide structures. Through ratio analysis of Gn1/Gn2 glycans, the value for G3M9A was found to be considerably higher than that for the other glycans examined. The

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[3] *Abbreviations used:* HPLC, high-performance liquid chromatography; Gn2 glycan, *N*-glycan-derived free glycan with *N,N'*-diacetylchitobiose at its reducing terminus; ODS, octadecylsilica; Gn1 glycan, *N*-glycan-derived free glycan with a single GlcNAc at its reducing terminus; PA, pyridylamine; GU, glucose unit; MALDI–TOF, matrix-assisted laser desorption/ionization time-of-flight; RNase B, ribonuclease B; PNGase, peptide:*N*-glycanase; MS, mass spectrometry; ENGase, *endo*-β-*N*-acetylglucosaminidase; ESI–MS, electrospray ionization mass spectrometry.

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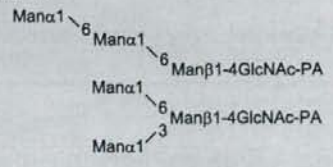
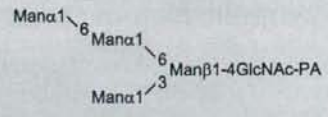
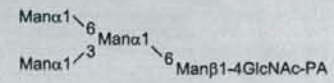
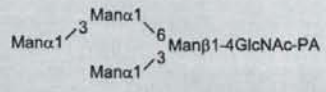
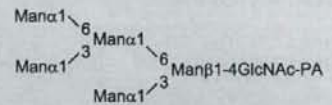
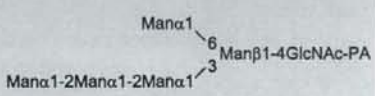
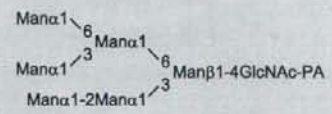
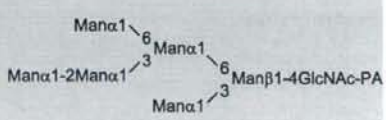
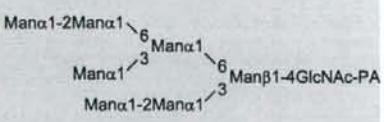
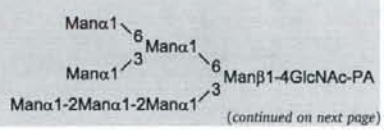
**Table 1**
Structures of Gn1 PA-oligosaccharides used and their abbreviations

| Abbreviation | Structure |
|---|---|
| GN | GlcNAc-PA |
| M3A′<br>M3B′ |  |
| M4A′ |  |
| M4B′ |  |
| M4C′ |  |
| M5A′ |  |
| M5B′ |  |
| M6B′ |  |
| M6C′ |  |
| M7A′ |  |
| M7B′ |  |

(continued on next page)

**Table 1** (*continued*)

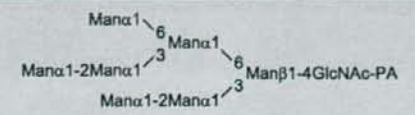
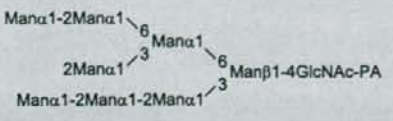
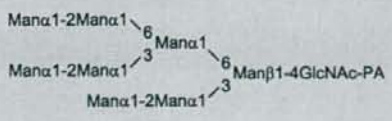
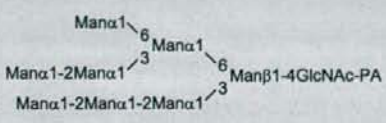
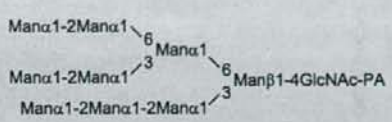
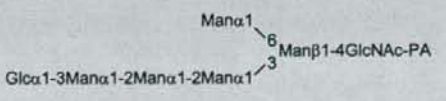
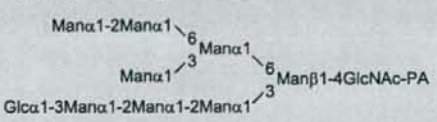
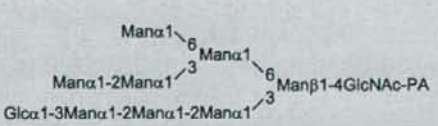
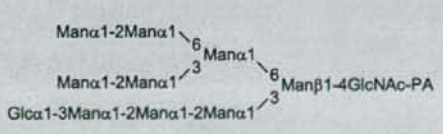
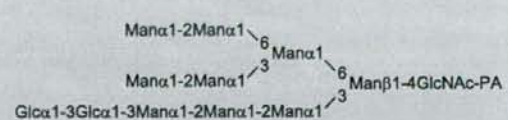
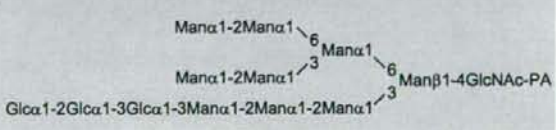
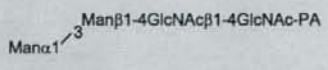
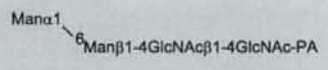
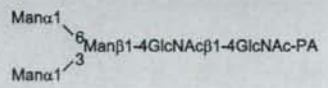
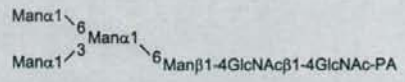
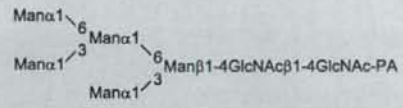
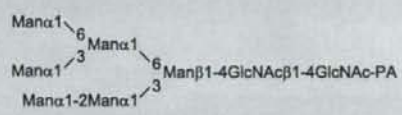
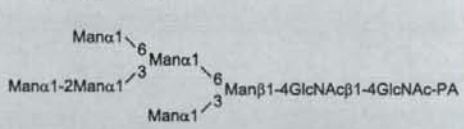
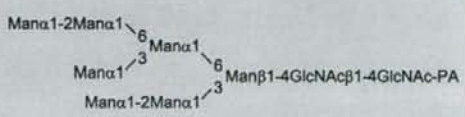
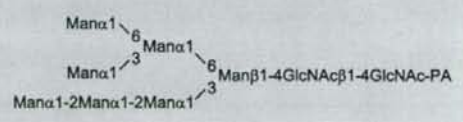
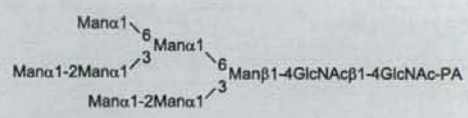
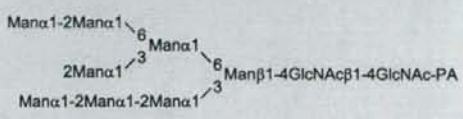
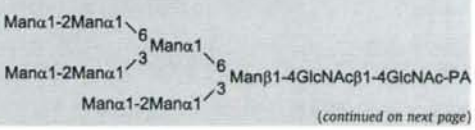
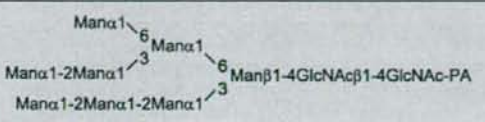
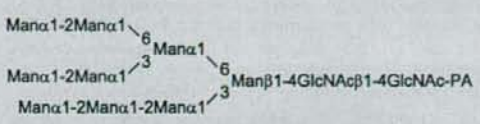
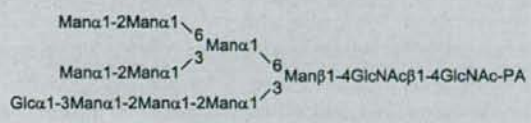
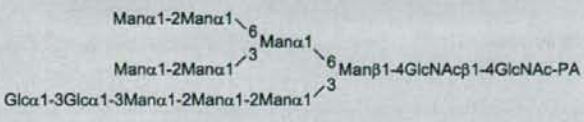
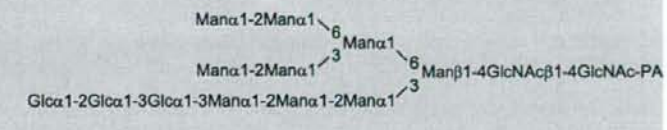
| Abbreviation | Structure |
|---|---|
| M7D′ |  |
| M8A′ |  |
| M8B′ |  |
| M8C′ |  |
| M9A′ |  |
| G1M5B′ |  |
| G1M8A′ |  |
| G1M8C′ |  |
| G1M9A′ |  |
| G2M9A′ |  |
| G3M9A′ |  |

**Table 2**
Structures of Gn2 PA-oligosaccharides used and their abbreviations

| Abbreviation | Structure |
|---|---|
| GN2 | GlcNAcβ1-4GlcNAc-PA |
| M2A |   |
| M2B |   |
| M3B |   |
| M4B |   |
| M5A |   |
| M6B |   |
| M6C |   |
| M7A |   |
| M7B |   |
| M7D |   |
| M8A |   |
| M8B |   |

(continued on next page)

**Table 2** (*continued*)

| Abbreviation | Structure |
|---|---|
| M8C |  |
| M9A |  |
| G1M9A |  |
| G2M9A G |  |
| G3M9A |  |

exceptionally small GU for G3M9A–PA may reflect its unusual conformation in solution. This method can, after size fractionation of glycans by HPLC, serve as a powerful secondary step of two-dimensional HPLC for the preparation of a mixture of Gn1 and Gn2 such as free *N*-glycans isolated from the cytosol of animal cells.

## Materials and methods

### *Preparation of standard PA-labeled oligosaccharides*

Standard Gn1 and Gn2 oligosaccharides are listed in Tables 1 and 2, respectively. Abbreviations of oligosaccharides are given according to previous studies [6,10,11]. The following compounds were purchased from Takara Bio (Shiga, Japan): PA–Glc, PA–GlcNAc, PA–ManNAc, PA–M2A, PA–M3B, PA–M4B, PA–M5A, PA–M6B, PA–M6C, PA–M7A, PA–M7B, PA–M7D, PA–M8A, PA–M8B, PA–M8C, PA–M9A, and PA–Glc oligomers. PA–M2B was purchased from Masuda Chemical Industry (Kagawa, Japan). Isomaltose (Glcα1-6Glc), isomaltotriose (Glcα1-6Glcα1-6Glc), and *N,N'*-diacetylchitobiose (GlcNAcβ1-4GlcNAc) were purchased from Sigma Chemical (St. Louis, MO, USA). These compounds (20 μg each) were labeled with PA using Palstation (Takara Bio) according to manufacturer's instructions. The following Gn1 compounds were isolated from the cytosol of animal cells and prepared as described previously [6,10,11]: PA–M3A′, PA–M4A′, PA–M4B′, PA–M4C′, PA–M5A′, PA–M5B′, PA–M7B′, PA–M8A′, PA–M8B′, PA–M8C′, PA–GM5B′, PA–G1M8A′, and PA–G1M8C′. PA–M3B′ was prepared by α1,2-mannosidase digestion of PA–M5B′ as described previously [19]. PA–M6B′, PA–M6C′, PA–7A′, PA–7D′, and PA–M9A′ were prepared by digestion of 500 pmol of the equivalent Gn2 PA–oligosaccharides with 10 mU of Endo H (Roche Diagnostics, Mannheim, Germany) at 37 °C overnight, followed by pyridylamination of released oligosaccharides using Palstation.

Glucosylated oligosaccharides (G1M9A, G2M9A, and G3M9A) were chemically synthesized as described previously [20]. Each oligosaccharide (20 μg) was directly labeled with PA by Palstation. For the Gn1 series of PA-labeled oligosaccharides (PA–G1M9A′, PA–G2M9A′, and PA–G3M9A′), 20 μg of each oligosaccharide was digested with 5 mU of Endo H before PA labeling.

PA-labeled oligosaccharides obtained by Palstation were purified using a Shodex NH2P-50 4E column (4.6 mm i.d. × 250 mm, Showa Denko, Tokyo) as described previously [19]. For several newly prepared PA-labeled oligosaccharides (PA–G1M9A, PA–G2M9A, PA–G3M9A, PA–GM9A′, PA–G2M9A′, and PA–G3M9A′), matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) analysis was carried out using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector (Bruker Daltonics, Bremen, Germany). The analytical condition of the MALDI–TOF mass spectrometer was described previously [19].

### *Preparation and identification of PA-labeled Gn1 and Gn2 oligosaccharides from ribonuclease B*

Preparation of Gn1 oligosaccharides ($Man_{5-9}GlcNAc$–PA) was carried out using Endo H as described previously [19]. For preparation of Gn2 oligosaccharides ($Man_{5-9}GlcNAc_2$–PA), 1 mg of ribonuclease B (RNase B) was digested with 5 U of peptide:*N*-glycanase F (PNGase F, Roche Diagnostics) at 37 °C overnight. Released oligosaccharides were labeled using Palstation. The PA-labeled sample was applied to a column of Sephadex LH-20 (1.0 × 30 cm, GE Healthcare, Buckinghamshire, UK) equilibrated with 50% aqueous methanol. Fractions eluted earlier than PA were collected and

evaporated to dryness. Samples were further purified using a Shodex NH2P-50 4E column as described previously [19].

For identification of peaks obtained from Gn1/Gn2 oligosaccharides from RNase B, elution positions were compared with the standard PA-labeled Gn1 and Gn2 oligosaccharides. Peaks that did not match any of the available standard PA-labeled oligosaccharides were collected manually and were evaporated to dryness. Fractions were desalted using C18 + Carbon NuTip (Hypercarb, Glygen, Columbia. MD, USA) as described previously [19]. Eluate (0.5 μl) was mixed with an aqueous solution (0.5 μl) of 2,5-diydroxybenzoic acid (5 mg/ml). Mass spectra of the samples were obtained by AXIMA-CFR (Shimadzu, Kyoto, Japan).

For identification of reducing sugar, PA-labeled Gn2 glycans from RNase B (10 pmol) were digested with 50 mU of Endo H, and the digests were analyzed with TSKgel Sugar AXI (4.6 mm i.d. × 150 mm, Tosoh, Tokyo, Japan). Isocratic elution was performed in 0.7 M $H_3BO_3$–KOH buffer (pH 9.0) at 65 °C for 1 h with a flow rate of 0.3 ml/min. The elution position, as well as the amount of PA–ManNAc and PA–GlcNAc, in samples was determined by analyzing the 1 pmol of authentic PA-labeled sugars (PA–ManNAc and PA–GlcNAc) with the same column as standards.

*Reversed-phase HPLC*

For the analysis of reversed-phase HPLC, an Inertsil ODS-3 column (2.1 mm i.d. × 150 mm, GL Sciences, Tokyo, Japan) was used in conjunction with a GL Sciences HPLC system (PU611 double pumps/CO630 column oven) with a fluorescence detector (LaChrom, Hitachi High Technologies, Tokyo, Japan). Elution was carried out at a flow rate of 200 μl/min at 25 °C. Standard sample (10 μl, 0.1–1 pmol) was injected for a single run. Elution was done using two solvent gradients: solvent A (0.1 M ammonium acetate buffer [pH 6.4]) and solvent B (0.1 M ammonium acetate buffer [pH 4.0] and 0.5% 1-butanol). Elution was carried out at a flow rate of 0.2 ml/min. The gradient program (expressed as percentages of solvent A) was as follows: 0–10 min, isocratic 99%; 10–110 min, 99% to 30%; and 110–120 min, 0% isocratic. Eluted samples were detected by fluorescence with $\lambda_{ex}$ = 320 nm and $\lambda_{em}$ = 400 nm. Samples were automatically injected using MidasASBio2 (GL Sciences), and the column was washed with 99% solvent A for 20 min between samples. GU of each PA-labeled oligosaccharide was determined by comparing its elution position with the elution positions of standard glucose oligomers. Values were calculated by the following formula:

$$GU = n + [E_{sample} - E_{Glc(n)}]/[E_{Glc(n+1)} - E_{Glc(n)}], \quad (1)$$

where $E_{sample}$ is elution time of the sample, $E_{Glc(n)}$ is elution time of the closest standard PA-labeled glucose oligomer eluted before the sample, and $E_{Glc(n+1)}$ is elution time of the closest standard PA-labeled glucose oligomer eluted after the sample.

## Results and discussion

*Establishment of conditions for HPLC analysis*

We aimed to establish conditions in which (i) Gn1 and Gn2 glycans could be clearly separated and (ii) Gn1 and Gn2 glycans had considerable separation for various oligosaccharides, especially for isomer separation. We used a dual-gradient system of pH and 1-butanol concentration. We initially used selected standard Gn1 and Gn2 glycans, and several HPLC conditions were tested for the elution time of these samples. Inclusion of a steep gradient during analysis (conditions **1** and **2**) caused elution of most of Gn2 glycans around the same time, making the identification of each peak quite difficult (Table 3). Therefore, we chose the gradient condition with the least steep gradient conditions tested (condition **4**) in which satisfactory separations not only between Gn1 and Gn2 glycans but also between structural isomers were achieved. These results

**Table 3**
Elution time for various glycans under different gradient conditions

| | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| Gradient Conditions | 0–5 A99<br>5–8 A99-75<br>8–110 A75-30<br>110.1–120 A0 | 0–5 A99<br>5–8 A99-75<br>8–110 A75-45<br>110–120 A0 | 0–10 A99<br>10–110 A99-0<br>110–120 A0 | 0–10 A99<br>10–110 A99-30<br>110–120 A0 |
| Abbreviations | Elution time (min) | | | |
| GN2 | n.d.[1] | n.d. | 59.56 | 49.72 |
| M5A | 42.90 | 43.50 | 62.43 | 63.52 |
| M6B | 42.90 | 43.43 | 59.56 | 59.20 |
| M7A | 42.87 | 43.39 | 56.60 | 54.83 |
| M7B | 42.85 | 43.18 | 58.21 | 57.90 |
| M7D | 42.82 | 43.23 | 61.20 | 63.44 |
| M8A | 42.82 | 43.45 | 57.67 | 54.09 |
| M8B | 42.81 | 43.52 | 58.08 | 57.70 |
| M8C | 42.82 | 43.53 | 60.47 | 61.23 |
| M9A | 42.79 | 43.51 | 57.78 | 55.93 |
| M4A′ | n.d. | n.d. | 36.94 | 31.97 |
| M4B′ | n.d. | n.d. | 37.00 | 31.94 |
| M4C | n.d. | n.d. | 43.80 | 37.38 |
| M5A′ | n.d. | n.d. | 40.16 | 34.81 |
| M5B′ | n.d. | n.d. | 38.10 | 32.84 |
| M6B′ | n.d. | n.d. | 35.96 | 32.19 |
| M7A′ | n.d. | n.d. | 33.84 | 27.83 |
| M7B′ | n.d. | n.d. | 38.63 | 30.83 |
| M7D′ | n.d. | n.d. | 47.25 | 33.23 |
| M8A′ | n.d. | n.d. | 31.96 | 28.85 |
| M8B′ | n.d. | n.d. | 33.12 | 29.88 |
| M8C | n.d. | n.d. | 39.51 | 35.03 |
| M9A′ | n.d. | n.d. | 32.66 | 28.48 |
| G3M9A′ | n.d. | n.d. | 54.07 | 40.47 |

[1] n.d.; not done.

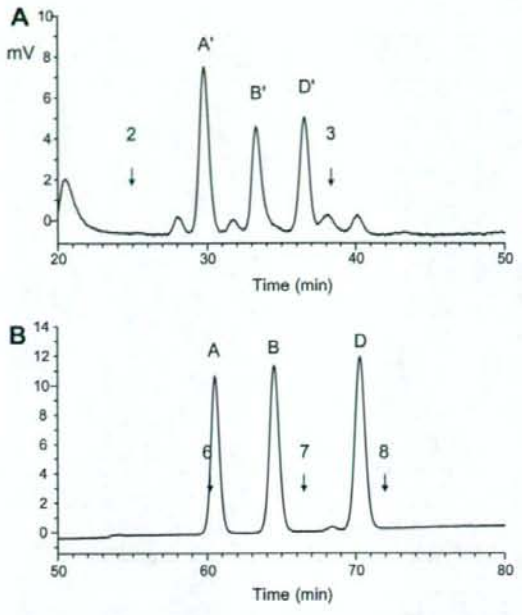



**Fig. 1.** HPLC analysis for Man7 isomers of Gn1 (A) and Gn2 (B) glycans. A': M7A'; B': M7B; D': M7D'; A: M7A; B: M7B; D: M7D. Arrows and numbers indicate elution positions for standard PA-labeled Glc oligomers.

indicate that, after size fractionation of PA-labeled glycans by an amine- or amide-based column, isomeric structures could be readily separated and quantified by this method. Separation of Man7 isomers (M7A', M7B', and M7D' for Gn1 glycans (Fig. 1A) and M7A, M7B, M7D, for Gn2 glycans (Fig. 1B) clearly showed that sufficient separation was achieved under the analytical conditions. During the repeated analysis under the HPLC conditions used, we observed variable retention times for the standard samples (e.g., cf. Figs. 1 and 2 for elution time of standard PA-labeled Glc oligomers). We observed quite consistent GU values for given standard oligosaccharides, indicating that the elution time relative to the standard PA-labeled Glc oligomer remained constant (see below).

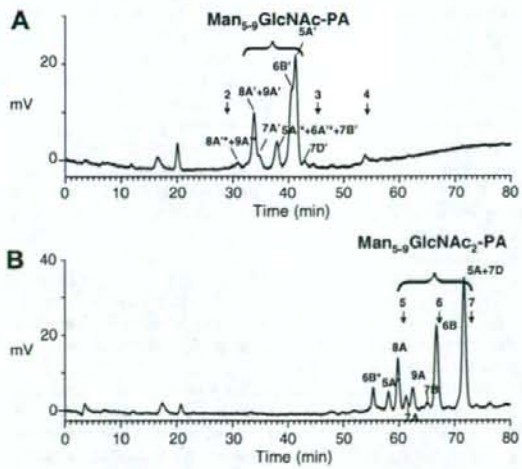



**Fig. 2.** Reversed-phase HPLC chromatogram for RNase B-derived Gn1 (A) and Gn2 (B) glycans. Shown are elution positions for $Man_{5-9}GlcNAc$–PA (A) and $Man_{5-9}GlcNAc_2$–PA (B). Assigned peaks are labeled. Asterisks (•) indicate the peaks where reducing sugar was converted from GlcNAc to ManNAc due to epimerization of the *N*-acetyl group.

To confirm separation of Gn1 and Gn2 glycans, reference high-mannose-type glycans were obtained from bovine RNase B; this protein contains typical high-mannose-type glycan ($Man_{5-9}GlcNAc_2$) [21]. PA-labeled Gn1 glycans ($Man_{5-9}GlcNAc$–PA) were prepared by Endo H digestion, whereas Gn2 glycans were prepared by PNGase F digestion. This condition enabled separation between Gn1 and Gn2 glycans (Fig. 2A and B). During the identification of peaks in Fig. 2, we observed peaks that did not comigrate with the standard PA-labeled glycans. We hypothesized that these peaks were formed by epimerization of the *N*-acetyl group of the innermost GlcNAc (to form ManNAc) during reductive amination. Consistent with this hypothesis, mass spectrometry (MS) analysis of these peaks showed the same MS numbers as the control high-mannose glycans, and PA–ManNAc as the reducing end for glycans was confirmed by monomeric sugar analysis of the reducing end of the PA-labeled sugars. After Endo H digestion of Gn2 glycans, it was found that 12% of glycans underwent epimerization to form glycans in which innermost PA–GlcNAc were converted to PA–ManNAc (data not shown).

In summary, this method can serve as rapid separation/quantification of total Gn1 and Gn2 glycans formed in the cytosol of animal cells.

*Determination of GUs for standard PA-labeled oligosaccharides*

We used various reference PA-labeled high-mannose glycans to examine their elution positions. Most of the reference Gn1 glycans used in this study were those previously isolated from the cytosol of animal cells [6,10,11]. PA-labeled Glc oligomers (PA-labeled isomalto-oligosaccharides, $PA–Glc_{1-15}$) were used as the standard for determination of GUs for all reference PA-labeled oligosaccharides. Analysis was carried out at least three times, and the elution position for each glycan was expressed as a GU. Quite consistent data were obtained with respect to GU, showing the high reproducibility of this HPLC analysis (Table 4). When GU values were compared for structural isomers, most of the glycans were sufficiently separated from each other except M4A' (2.52 ± 0.03) and M4B' (2.54 ± 0.02) (Table 4). This result was consistent with the previous findings that these two glycans were not separable by reversed-phase HPLC and that the separation of M4A' and M4B' was possible only by anion exchange HPLC [10]. The biggest GU for Gn1 glycans (3.49 for G3M9'A–PA) was considerably smaller than that of $GlcNAc_2$–PA (4.23), the smallest Gn2 glycan tested.

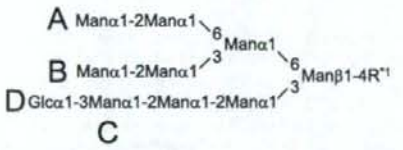
*Comparison of GUs for Gn1 and Gn2 glycans with identical isomeric outer structures*

We compared the GU values of Gn1 and Gn2 glycans with the same outer structures. The difference between Gn1 and Gn2 could be more consistent when compared by ratio (0.44 ± 0.03) than by subtraction (3.54 ± 0.54) (Table 4). Based on the "additive rule" principle, the addition of a monosaccharide residue to a PA-labeled sugar chain causes an increase or decrease in elution time, with the difference being defined as the partial elution time of the residue [15–18]. It is unknown whether this rule can also be applied to Gn1 glycans. Partial elution time for the outermost Man residues (A, B, and C in Table 5), as well as for the innermost Glc (GlcD) for Gn1 glycans, was calculated based on the current analysis. Partial elution time was not very consistent as a whole, but the

**Table 4**
Elution time of PA-oligosaccharides tested

| Gn2 glycans Abbreviations | GU1 | Gn1 glycans Abbreviations | GU | $GU_{GN2}$–$GU_{GN1}$ | $GU_{GN1}$/$GU_{GN2}$ |
|---|---|---|---|---|---|
| GN2 | 4.23 ± 0.04 | GN | 2.85 ± 0.03 | | |
| M2A | 5.29 ± 0.08 | | | | |
| M2B | 6.74 ± 0.03 | | | | |
| | | M3A′ | 2.39 ± 0.03 | | |
| M3B | 6.56 ± 0.03 | M3B′ | 2.70 ± 0.01 | 3.86 | 0.412 |
| | | M4A′ | 2.52 ± 0.03 | | |
| M4B | 6.63 ± 0.03 | M4B′ | 2.54 ± 0.02 | 4.09 | 0.383 |
| | | M4C′ | 2.96 ± 0.00 | | |
| M5A | 6.69 ± 0.06 | M5A′ | 2.75 ± 0.01 | 3.94 | 0.411 |
| | | M5B′ | 2.62 ± 0.02 | | |
| M6B | 5.95 ± 0.07 | M6B′ | 2.71 ± 0.02 | 3.24 | 0.456 |
| M6C | 7.51 ± 0.03 | M6C′ | 2.96 ± 0.01 | 4.55 | 0.394 |
| M7A | 5.10 ± 0.07 | M7A′ | 2.38 ± 0.04 | 2.72 | 0.467 |
| M7B | 5.75 ± 0.01 | M7B′ | 2.65 ± 0.01 | 3.10 | 0.461 |
| M7D | 6.71 ± 0.01 | M7D′ | 2.85 ± 0.03 | 3.86 | 0.425 |
| M8A | 4.99 ± 0.03 | M8A′ | 2.32 ± 0.01 | 2.67 | 0.465 |
| M8B | 5.62 ± 0.06 | M8B′ | 2.39 ± 0.02 | 3.23 | 0.425 |
| M8C | 6.35 ± 0.01 | M8C′ | 2.79 ± 0.01 | 3.56 | 0.439 |
| M9A | 5.31 ± 0.06 | M9A′ | 2.28 ± 0.01 | 3.03 | 0.429 |
| | | G1M5B′ | 3.23 ± 0.03 | | |
| | | G1M8A′ | 2.76 ± 0.00 | | |
| | | G1M8C′ | 3.41 ± 0.01 | | |
| G1M9A | 6.57 ± 0.07 | G1M9A′ | 2.74 ± 0.02 | 3.83 | 0.417 |
| G2M9A | 7.34 ± 0.07 | G2M9A′ | 3.28 ± 0.03 | 4.06 | 0.447 |
| G3M9A | 6.91 ± 0.03 | G3M9A′ | 3.49 ± 0.07 | 3.42 | 0.505 |
| | | | Average | 3.54 ± 0.54 | 0.44 ± 0.03 |

**Table 5**
Calculated partial elution time of the outermost Man residues




| | | Calculated Partial Elution Time (GU) |
|---|---|---|
| ManA | Gn1 | −0.46 ± 0.14 |
| | Gn2 | −0.94 ± 0.14 |
| ManB | Gn1 | 0.07 ± 0.10 |
| | Gn2 | 0.60 ± 0.20 |
| ManC | Gn1 | −0.07 ± 0.02 |
| | Gn2 | −0.25 ± 0.11 |
| GlcD | Gn1 | 0.53 ± 0.10 |
| | Gn2 | 1.26*2 |

*1 R = GlcNAc-PA (Gn1) or $GlcNAc_2$-PA (Gn2).
*2 Data based on the GU of PA-G1M9A and PA-M9A.

additive rule may be applicable only within the same group of glycans (i.e., Gn1 or Gn2) (Table 5).

Although the values of ratio comparison were fairly consistent, the ratio value for G3M9A′/G3M9A was found to be much larger than the ratio values for other isomeric structures. This result was evident from the fact that G3M9A–PA (GU: 6.91 ± 0.03) had a much smaller GU than did G2M9A–PA (GU: 7.34 ± 0.07), whereas this was not the case with Gn1 glycans (G3M9A′–PA: 3.49 ± 0.07; G2M9A′–PA: 3.28 ± 0.03). This may suggest an unusual structural conformation that is specific for G3M9A–PA. There may be a specific intramolecular interaction in G3M9A–PA (e.g., PA and the outermost glucose), but not in G3M9A′–PA, that makes the elution position of the former abnormal in our HPLC analysis. Further analyses are needed to validate this hypothesis.

*General discussion*

It is well known that during N-glycosylation of proteins, significant amounts of free *N*-glycans, which are structurally related to *N*-glycans but not attached to proteins, are observed [22–24]. Free glycans are known to be formed in the cytosol by the action of cytosolic PNGase [25–27] or in the endoplasmic reticulum by an unclarified mechanism. In both cases, the glycans released retain the *N,N′*-diacetylchitobiose structure (GlcNAcβ1–4GlcNAc) at their reducing termini and are often called Gn2 species. Most free glycans recovered in the cytosol have only one GlcNAc at their reducing termini (Gn1 glycans). Conversion of Gn2 to Gn1 was predicted to be due to the cytosolic *endo*-β-*N*-acetylglucosaminidase (ENGase) [28,29] or chitobiase [30] and were further processed, in the case of mammalian cells, by the cytosolic α-mannosidase Man2C1 [19,31,32]. These successive reactions constitute well-ordered, nonlysosomal processing of *N*-glycans in the cytosol [22–24,33].

In this study, we established a reversed-phase HPLC condition for separation of various high-mannose-type glycans, especially Gn1-type glycans, isolated from the cytosol of animal cells. Our HPLC method successfully separated Gn1 and Gn2 glycans. Extensive analyses for reference glycans indicated that Gn1 and Gn2 glycans showed sufficient resolution for structural isomers, further supporting the validity for this analysis. The difference in GU between Gn1 and Gn2 glycans with the same outer structures was analyzed, and the ratio value was less variable than the subtraction value (Table 4). It may be preferable to use the ratio value to predict the tentative GU of the equivalent Gn1 glycan if only the GU for a Gn2 glycan is known.

Whether the additive rule can be applied for structural assignment of Gn1-type glycan chains has not been studied. We showed in this study that it could be the case within the same reducing end group (Table 5). More thorough analysis is required to validate this hypothesis, and for this purpose systematic synthesis of high-mannose-derived *N*-glycans by a combinatorial approach may be a useful way to prepare an entire set of diverse structures [20].

This HPLC method can serve as a second step for the separation of PA-labeled oligosaccharides isolated from the cytosol of animal