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Note

Glycosylation and ligand-binding activities of rat plasma fibronectin during liver regeneration after partial hepatectomy

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Abstract—Fibronectin (FN) is a multifunctional glycoprotein present in the extracellular matrix (ECM) and plasma. We previously reported that the glycosylation and ligand-binding of vitronectin (VN) change markedly after partial hepatectomy (PH). Here we show the changes of FN during liver regeneration. The yields of purified sham-operated (SH-) and PH-FN were higher than that of non-operated (NO)-FN, while binding activities of FNs to ECM ligands were changed only slightly by hepatectomy. The carbohydrate concentration of PH-FN decreased to 66% of that of NO- and SH-FN. By using LC/MS^n, eight kinds of complex-type *N*-glycan structures were found to be present in all FNs, and bi- and trisialobiantennary glycans were the major structures. Fucosylation was markedly increased, while O-acetylation of sialic acid was found to be decreased in PH-FN. The alterations in glycosylation and biological activities of FN after PH are different from those of VN, suggesting that these glycoproteins play different biological functions in tissue remodeling.

Keywords: Fibronectin; Glycosylation; Liver regeneration; Vitronectin; Partial hepatectomy

Numerous biological phenomena are mediated by the recognition of specific oligosaccharide signals. Among the functions of protein glycosylation are the stabilizing active conformations, protecting against proteolysis, and affording solubility to proteins.[1] Clarification of the molecular mechanisms by which glycosylation plays these roles would enable the use of glycosylation in molecular engineering for therapeutic purposes.

Partial (70%) hepatectomy is often used to study liver regeneration mechanisms. The remaining liver recovers its former weight within about two weeks in humans or 10 days in the rat.[2] Matrix degradation occurs in the early stage of this process, followed by biosynthesis of the matrix, cell proliferation, and cell differentiation. Previously, collagen-binding activities of VN were found to be significantly affected by alterations in N-glycosylation.[3] A marked increase in VN was found to be controlled by the increased multimerization induced by glycosylation changes during liver regeneration after PH of rats.[4]

FN is a typical ECM glycoprotein synthesized by various cells, including hepatocytes, that plays critical roles in many biological and pathological processes including embryogenesis, wound healing, metastasis, fibrosis, and thrombosis.[5] It exists primarily as a soluble dimer in body fluids such as plasma at high concentrations (0.3 mg/mL in humans)[6] that is assembled into insoluble fibrils with a fibrillar polymeric matrix during matrix assembly.[7] In addition to regulation of the biological activities of FN by alternative splicing of the III-type modules or V segment,[8,9] N- and O-glycosylation of human FN affects the interaction with biological ligands including cell receptors,[10] suggesting that glycosylation

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modulates the physiological functions of FN. The concentration of FN has been reported to triple at the protein and mRNA levels during liver regeneration,[11] but a change in glycosylation and biological activity has not been demonstrated yet. In this study, we focused on the glycan structure and ligand-binding activities of rat FN during liver regeneration and found differential changes in ECM glycoproteins to regulate biological activities. This research is the first to elucidate the details of the glycan structure of a particular glycoprotein synthesized in the early stage of liver regeneration.

The protein concentration in SH-plasma increased slightly 6–24 h after operation, while in PH-plasma it had decreased to about 60% of the level in NO-plasma at 24 h after PH; then it gradually returned to a normal level (data not shown).[3] In contrast, the FN concentration in the plasma increased by 10% between 24 and 120 h after PH compared with NO, while after SH it was increased by 25% at 6 h and had returned to a normal level in 7 days (data not shown). The relative increase of FN in plasma proteins after PH and SH indicates that FN is an acute phase reactant.

FN from rat plasma at 24 h after PH, SH, and NO was purified by affinity chromatography on a gelatin-Sepharose column. FN was scarcely eluted at pH 5.2, but was completely eluted with 6 M urea.[12,13] Because urea-eluted FN recovered biological activities after removal of the urea,[12] it was used for the following experiments. As shown in Figure 1A, each FN produced major double bands at the migration position around 230 kDa on SDS-PAGE. Compared with NO-FN, the amounts of purified FN were increased 1.6–2 times after SH and 1.2–1.4 times after PH (Fig. 1B). The increased yields of urea-purified PH- and SH-FN coincided with the elevated FN concentrations in plasma. The ratio of purified FN to total FN in plasma was also increased about 1.7 and 1.3 times after SH and PH, respectively (data not shown), suggesting that the gelatin-binding activity of FN was enhanced after surgery.

Ligand-binding activities of FN: FN binding to gelatin was slightly increased in SH-FN in plasma (Fig. 2A) in accordance with the high yield of SH-FN in gelatin affinity chromatography (Fig. 1B). The binding to other ligands, strongly to collagen types I–III and heparin-BSA but weakly to collagen types IV and V, hardly changed after surgery (data not shown for collagen types II, III, and V, and heparin-BSA), although both the concentrations in plasma and the yields of purified FN were increased in SH and PH. This is probably because other factors in plasma interfered with FN binding to the ligands.

As shown in Figure 2B, slightly more purified PH-FN than SH- and NO-FN bound to collagen type I, and more PH- and SH-FN than NO-FN bound to collagen type IV, indicating that surgery only increased collagen binding activities slightly. The very small alterations in collagen-binding activities of FN by PH compared to those of VN[3] indicate that FN and VN contribute differently to matrix remodeling during liver regeneration. In contrast, the binding activities of purified FNs to gelatin and collagen types IV and V were significantly higher than those of plasma FN before purification (Fig. 2A), indicating that urea-denaturation of FN activates the binding activities to some ligands. Consistent with our results, urea-denaturation of FN has been reported to induce the sequential unfolding and exposure of the type III domain to regulate the interaction with the ECM proteins.[14]

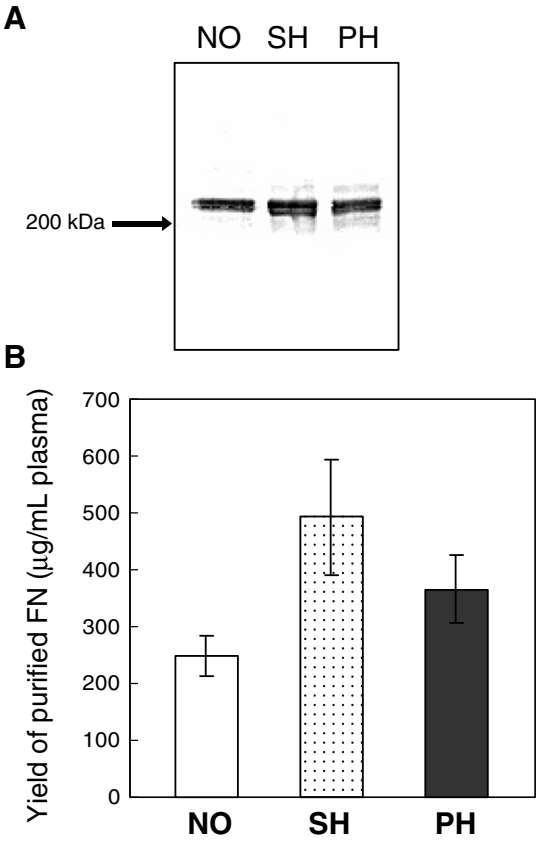


Figure 1. Purification of FN from NO, SH, and PH plasma at 24 h after operations. (A) SDS-PAGE of purified FNs using 6% polyacrylamide gels under reducing condition. (B) Yield of purified FN from each plasma. Error bars were obtained from three experiments. Open bar, NO; dotted bar, SH; solid bar, PH.

As shown in Figure 3, the ligand-binding activities of de-N-glycosylated FN were significantly increased, in contrast to the only slight increase in the binding activities after surgery, showing that glycosylation affects the ligand-binding of FN (Fig. 2). The 2.9-fold increase in binding of VN to type I collagen after de-N-glycosylation[3,4] is similar.

Changes in glycosylation of FN during liver regeneration: As summarized in Table 1, the most conspicuous change after PH was the increase in FN reactivity to *Aleuria aurantia* lectin (AAL), suggesting an increase of fucosylation in PH-FN. The reactivity to peanut

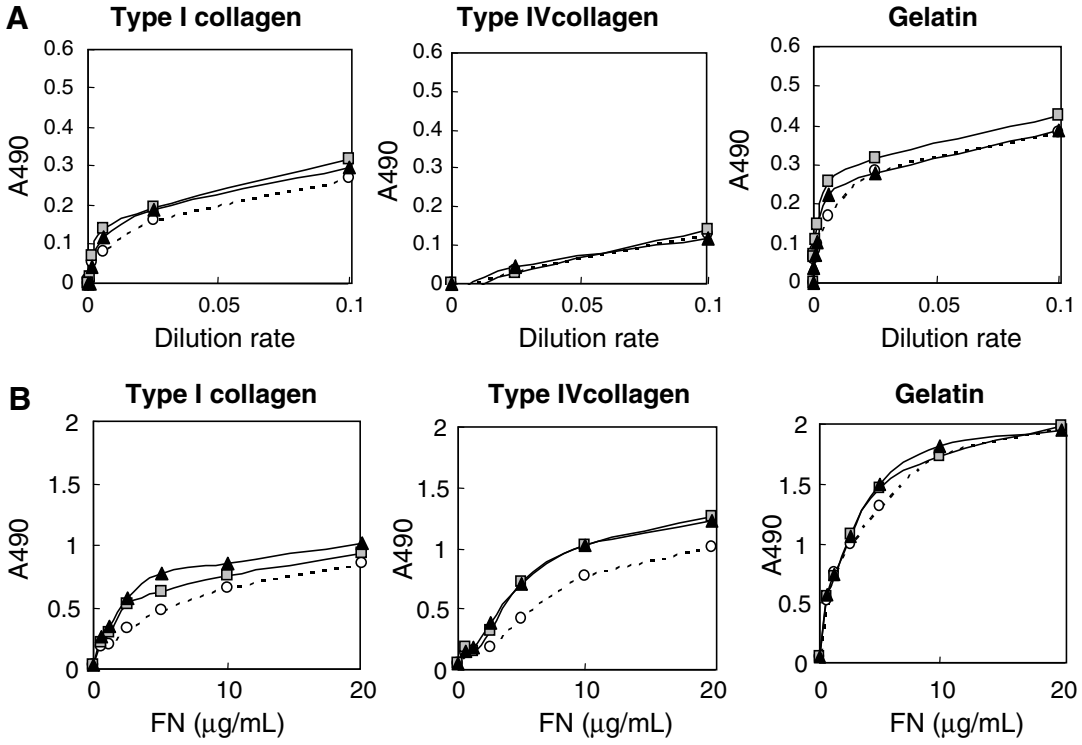


Figure 2. Binding activity of FNs in NO, SH, and PH plasma (A) and purified FNs (B) to collagen types I, and IV and gelatin by ELISA. (A) Binding of FNs in plasma. (B) Binding of purified FNs. Symbols used are circle, NO-FN; square, SH-FN; solid triangle, PH-FN.

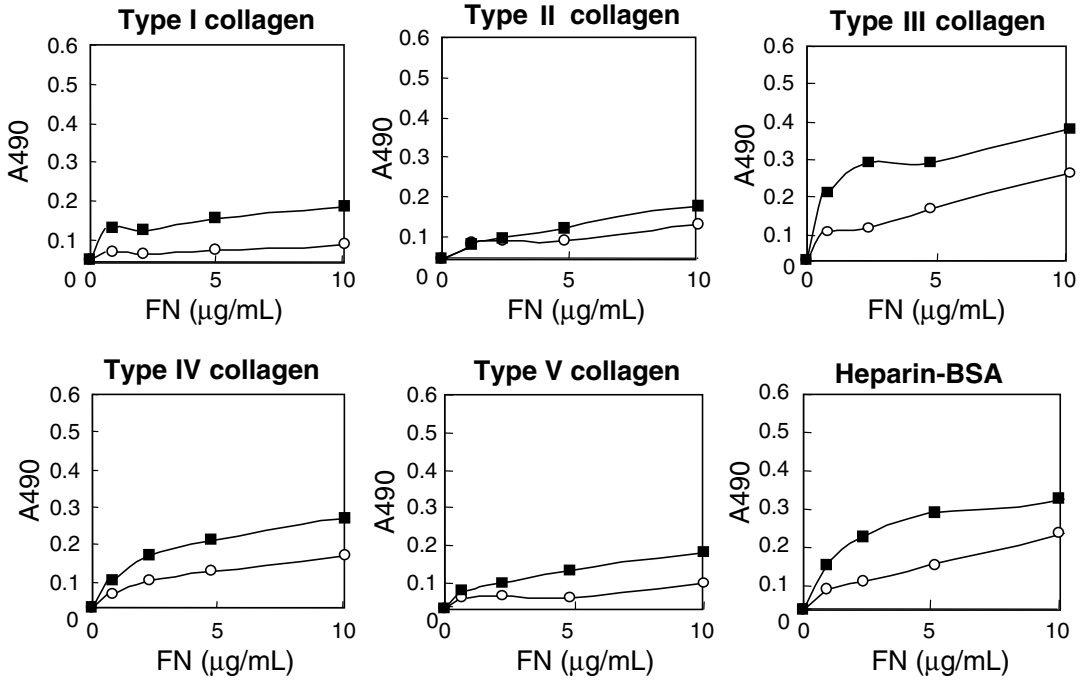


Figure 3. Effect of de-N-glycosylation on the binding activity of FN to collagen types I–V and heparin-BSA by ELISA. Symbols used are circle, control human FN treated without PNGase F; solid square, de-N-glycosylated human FN by PNGase F treatment.

lectin (PNA) after mild acid desialylation of FNs clearly indicated the presence of O-type glycans. The reactivity to *Psathyrella velutina* lectin (PVL) was increased in PH-FN but disappeared in all FNs after desialylation, indicating that sialylated glycans having more than trisialyl residues[15] increased after PH.

Table 1. Reactivity of various lectins with FNs

	NO-FN	SH-FN	PH-FN	Specificity
Con A	++	++	++	N-Linked biantennary or high Man-type
SNA	++	++	++	Sialyl α2-6 Gal
MAM	+	+	++	Sialyl α2-3 Gal
L-PHA	+++	+++	+++	N-Linked tri- or tetraantennary
E-PHA	++	++	+++	N-Linked bi- or triantennary
	(+++)	(++)	(+)	
AAL	++	+	+++	Core or outer fucosylated
LCA	++	++	++	N-Linked, core-fucosylated
PNA	−	−	−	O-Linked, unsialylated Galβ1-3GalNAc
	[++]	[+++]	[+++]	
PVL	++	++	+++	Non-reducing terminal GlcNAc or tri- or tetra sialyl
	[−]	[−]	[−]	
RCA	+++	+++	+++	Non-reducing terminal Galβ
WGA	++	++	++	GlcNAc

Reactivity of FNs with biotinyl lectins by dot blotting is expressed as staining intensity. +, indicates positive staining; −, negative staining; Reactivity after digestion with hexosaminidase is indicated in parentheses, and reactivity after mild acid desialylation is indicated in brackets. Abbreviations used are: Con A, Concanavalin A; SNA, *Sambucus nigra* agglutinin; MAM, *Maackia amurensis* mitogen; L-PHA, *Phaseolus vulgaris* leukoagglutinin; E-PHA, *Phaseolus vulgaris* erythroagglutinin; AAL, *Aleuria aurantia* lectin; LCA, *Lens culinaris* agglutinin; PNA, peanut agglutinin; PVL, *Psathyrella velutina* lectin; RCA, *Ricinus communis* agglutinin; WGA, wheat germ agglutinin.

As shown in Table 2, the total carbohydrate concentration of PH-FN was markedly decreased compared with those of NO- and SH-FN. The decrease in the ratio of the concentrations of GalNAc to total carbohydrates was notable in PH-FN, indicating that FN is highly O-glycosylated, but that the ratio is lowered in PH-FN. The Fuc concentration increased to about twice that in NO- and SH-FN, in contrast to plasma VN, in which the relative molar ratio of fucose was not changed by PH, although both of these glycoproteins are synthesized in the liver. The isoelectric points of NO-, SH-, and PH-FN, as determined by 2D-PAGE, showed no difference ($pI = 5.7$), suggesting that these FNs are sialylated to the same degree (data not shown).

The *N*-glycan structures of FN were determined by LC/MS to be the sugar alcohols. Rat plasma FN has seven potential N-glycosylation sites. As shown in Table 3 and Figure 4A, the most frequent *N*-glycan structures of rat plasma FN were biantennary complex type disialoglycans (BiNA(2)), which are very similar to human plasma FN,[16] and then biantennary trisialoglycans (BiNA(3)). As summarized in Table 3, eight kinds of *N*-glycan backbones were found. If variations of the number of O-acetylations of sialic acid residues were included, the varieties of structures would total more than 15 (Fig. 4). The increased ratios of fucosylation in PH-FN were in accordance with the carbohydrate composition results and reactivity with AAL. Because fucosylated glycans are known to participate in embryonic growth, differentiation, cell recognition, cancer formation, and inflammation,[17] the glycans of FN synthesized during liver regeneration may regulate tissue remodeling by increasing fucosylation through a change in binding to other ligands.

Table 2. Carbohydrate composition of rat FNs

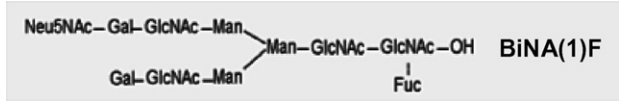
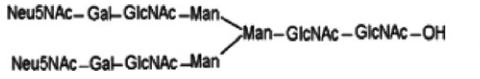
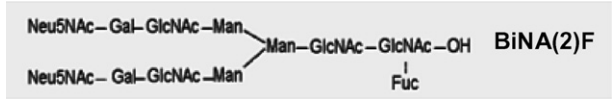
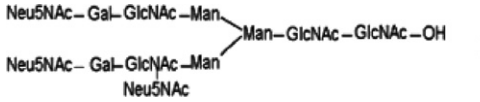
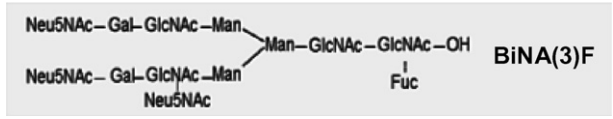
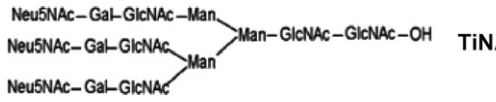
Carbohydrate	NO-FN	SH-FN	PH-FN
GalNAc	92 (9.3)	61 (5.3)	33 (4.1)
GlcNAc	33 (3.4)	37 (3.2)	21 (2.6)
Man	30 (3.0)	35 (3.0)	24 (3.0)
Fuc	1.9 (0.2)	2.5 (0.2)	4.5 (0.6)
Gal	37 (3.7)	57 (5.0)	44 (5.5)
Total	194	193	127

Values are expressed as mol/mol FN. Relative molar ratios of each carbohydrate are expressed in parentheses by taking the value of Man as 3.0.

The glycans contained various concentrations of O-acetylated Neu5NAc (Fig. 4B). The ratio of non-O-acetylated glycans in each backbone structure was higher in PH-FN than that in NO- and SH-FN. For example, Ac(0) in BiNA(2), a major glycan, was 70% in PH-FN, much higher than those of NO- (52%) and SH-FN (57%). This indicates that the O-acetylation of neuraminic acid is lowered after PH. Sialic acids from total membranes of rat liver have surprisingly high levels (approximately 20%) of O-acetylation at the 7- or 9-position.[18] These modifications modulate many biological interactions,[19–21] such as that the binding of Siglec-1 (sialoadhesin) and Siglec-2 (CD22) to glycoconjugates, which are hindered by 9-O-acetylation.[22,23] In the early stage of liver regeneration, the immune system may be regulated by the interaction of FN glycans with Siglecs via the change in O-acetylation.

These results indicate that the alterations in ligand-binding and glycosylation of FN and VN were significantly different in the early stage of liver regeneration and demonstrate that these glycoproteins play different biological roles in the promotion of tissue remodeling processes. This study provides insight into the regulation of individual ECM glycoproteins by glycosylation.

Table 3. Oligosaccharide structures of rat FN

Glycan structures	Abbreviation
	BiNA(1)
	BiNA(1)F
	BiNA(2)
	BiNA(2)F
	BiNA(3)
	BiNA(3)F
	TiNA(3)
	TriNA(3)F

Abbreviations used for glycan backbone structures are: BiNA(1), biantennary glycan with one *N*-acetylneuraminic acid; BiNA(1)F, biantennary glycan with one *N*-acetylneuraminic acid and a fucose linked to a penultimate GlcNAc; BiNA(2), biantennary glycan with two *N*-acetylneuraminic acids; BiNA(2)F, biantennary glycan with two *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc; BiNA(3), biantennary glycan with three *N*-acetylneuraminic acids; BiNA(3)F, biantennary glycan with three *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc; TiNA(3), triantennary glycan with three *N*-acetylneuraminic acids; and TiNA(3)F, triantennary glycan with three *N*-acetylneuraminic acids and a fucose linked to a penultimate GlcNAc.

1. Experimental

1.1. Animals and purification of rat FN from plasma

Male Wistar rats aged 5 weeks (weighing about 110 g; Nihon Clea, Tokyo, Japan) were subjected to two-thirds PH or SH as described previously.[3,24] Plasma was collected from NO rats, or 6, 24, 48, 120, and 168 h after SH and PH and stored at −80 °C until use. FN was purified from PH-, SH-, or NO-plasma using gelatin-Sepharose as described previously.[25] The purity of FNs was checked by SDS-PAGE.[10]

1.2. Determination of FN concentration in plasma (sandwich ELISA)

Wells of microtiter plates (Immulon 1, Dynatech Laboratories Inc., Chantilly, VA) were coated with rabbit anti-human FN IgGs (50 μL in 0.5% BSA/PBS) at 4 °C overnight. The wells were washed with TBS, and various concentrations of rat plasma or purified FN (50 μL) were added and incubated for 2 h at room temperature. After washing, the wells were blocked with 0.5% BSA/PBS for 1 h, then bound FN was detected with horseradish peroxidase (HRP)-anti-FN IgGs and visualized with *o*-phenylenediamine/0.007% H_2O_2. After stopping the reaction with 4 M H_2SO_4, absorbance was measured at 490 nm using a microplate reader.

1.3. Assays of FN binding to immobilized ligands (ELISA)

Wells of microtiter plates were coated with gelatin, heparin-BSA, or collagen types I–V (10 μg/mL, 50 μL) in 0.1 M carbonate buffer (pH 9.0), and reacted with various concentrations of plasma or FN. The bound FN was detected by ELISA.[26,27]

1.4. Carbohydrate analyses of FNs

For lectin reactivity, FNs (0.5 μg) were dot-blotted onto a PVDF membrane and reacted with HRP-lectins as previously described.[28] The staining intensities were measured by the software program Scion Image. To determine carbohydrate composition, FNs (4.5 μg) were hydrolyzed, and the carbohydrates were analyzed according to the previously described method.[3]

1.5. Analysis of *N*-glycan structures by mass spectrometry (LC/MS^n)

FN (30 μg/about 200 μL) was reduced by boiling with 2% of 2-mercaptoethanol for 1 min. N-Linked oligosaccharides were released from the FN with *N*-glycosidase F (4.8 U) and reduced with 0.25 M $NaBH_4$. The oligosaccharides were desalted with ENVI-Carb C (Supelco Co., Ltd, Bellefonte, PA, USA) and subjected to LC/MS^n. The eluents were 2% acetonitrile/5 mM ammonium acetate (pH 9.6) (pump A), and 80% acetonitrile/5 mM ammonium acetate (pH 9.6) (pump B). The oligosaccharides were separated on a Hypercarb column (0.2 × 150 mm, Thermo Fisher Scientific, Waltham, MA, USA) with a 5–30% linear gradient of the pump B eluent over 60 min at a flow rate of 2 μL/ min. The MS spectra were acquired by using an LTQ-FT system (Thermo Fisher Scientific) with a single mass scan (*m*/*z* 7000–2000) on a Fourier transformation ion cyclotron resonance mass spectrometer, and data-dependent

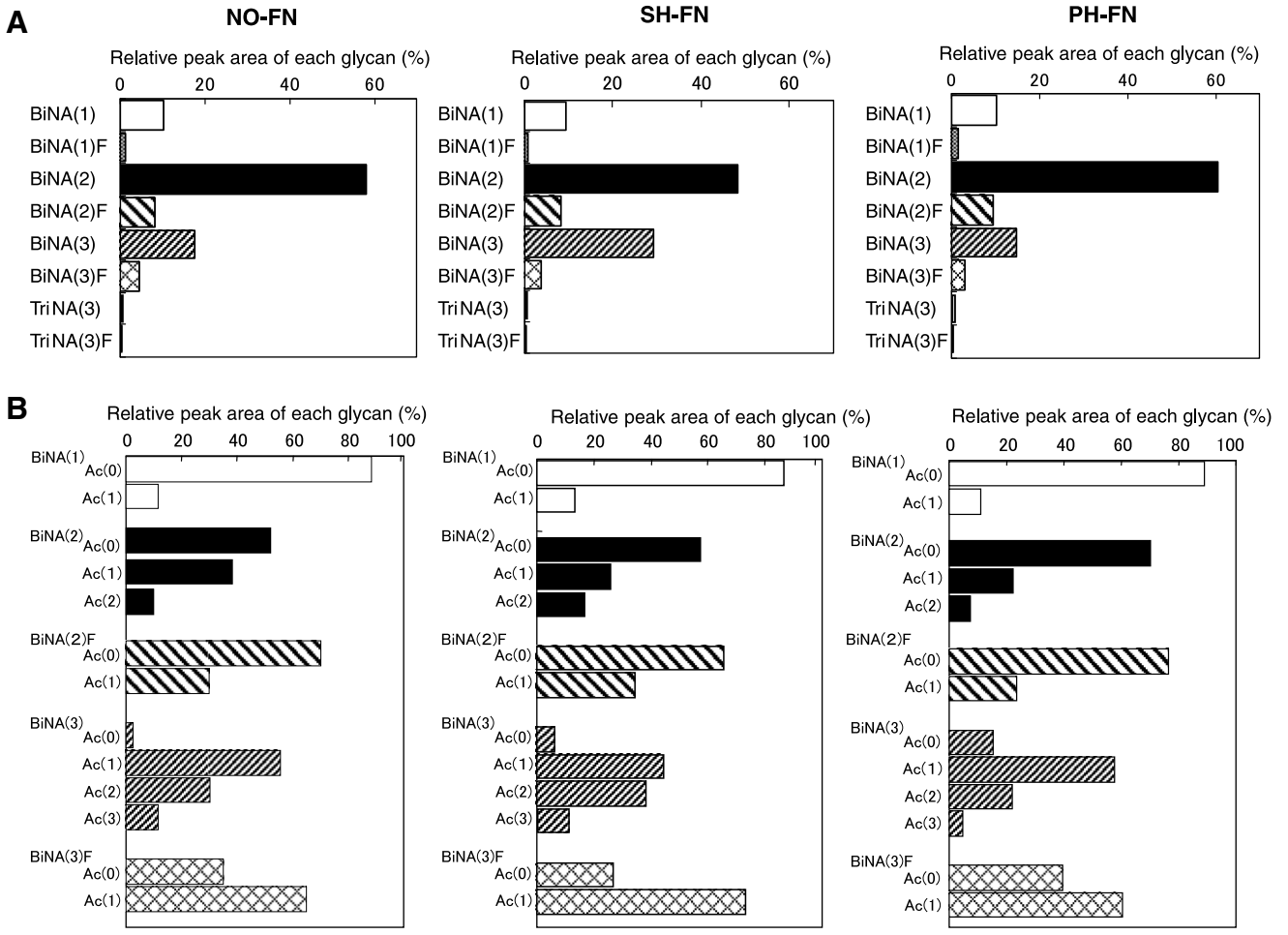


Figure 4. Ratios of each FN *N*-glycan. (A) The ratio of each glycan structure. (B) Ratios of glycans possessing various numbers of *O*-acetyl groups at the *N*-acetylneuraminic acid residues. The ratio of each glycan (the abbreviations are summarized in Table 3) was calculated from the relative peak area of mass chromatography signals in HPLC by taking the total peak area of each FN (A) or of each glycan (B) as 100%. Of the structures not expressed in (B), BiNA(1)F, TriNA(3), and TriNA(3)F, all without *O*-acetyl group (Ac(0)) were detected.

MS/MS and MS/MS/MS scans in both positive and negative ion modes.

Acknowledgments

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