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Occurrence of Secretory Glycoprotein-Specific GalNAcβ1→4GlcNAc Sequence in *N*-Glycans in MDCK Cells

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Many reports show that *N*-glycans of glycoproteins play important roles in vectorial transport in MDCK cells. To assess whether structural differences in *N*-glycans exist between secretory glycoproteins and membrane glycoproteins, we studied the *N*-glycan structures of the glycoproteins isolated from MDCK cells. Polarized MDCK cells were metabolically labeled with [^{3}H]glucosamine, and ^{3}H-labeled *N*-glycans of four glycoprotein fractions, secretory glycoproteins in apical and basolateral media, and apical and basolateral membrane glycoproteins, were released by glycopeptidase F. The structures of the free *N*-glycans were comparatively analyzed using various lectin column chromatographies and sequential glycosidase digestion. The four samples commonly contained high-mannose-type glycans and bi- and tri-antennary glycans with a bisected or non-bisected trimannosyl core. However, secretory glycoproteins in both media predominantly contained (sialyl)LacdiNAc sequences, ±Siaα2→6GalNAcβ1→ 4GlcNAcβ1→R, which linked only to a non-bisected trimannosyl core. β1→4*N*-acetylgalactosaminyltransferase (β4GalNAc-T) activity in MDCK cells preferred non-bisected glycans to bisected ones in accordance with the proposed *N*-glycan structures. This secretory glycoprotein-predominant LacdiNAc sequence was also found in the case of human embryonic kidney 293 cells. These results suggest that the secretory glycoprotein-specific (sialyl)LacdiNAc sequence and the corresponding β4GalNAc-T are involved in transport of secretory glycoproteins.

Key words: β1→4*N*-acetylgalactosaminyltransferase, LacdiNAc sequence, MDCK cells, secretory glycoprotein.

There have been many reports about intracellular sorting of (glyco)proteins in polarized Madin-Darby canine kidney (MDCK) cells. Several cytoplasmic signal peptides for basolateral sorting are known (*1–4*), but the signal peptide for apical sorting has not yet been reported. Instead, the importance of *N*-glycans (*5–10*), *O*-glycans (*11*), and glycosyl phosphatidylinositol (GPI) anchor (*12*) for apical sorting has been suggested. We recently found that vesicular integral protein of 36 kDa (VIP36) recognizes high mannose-type glycans of glycopeptides (*13*) and is involved in the intracellular transport of *N*-linked glycoproteins (*14*).

The functional roles of *N*-glycans include quality control of newly synthesized *N*-linked glycoproteins and sorting of lysosomal enzymes. Newly synthesized glycoproteins carrying monoglucosylated high-mannose-type glycans are correctly folded in the endoplasmic reticulum (ER) by binding to calnexin and calreticulin (*15, 16*), and the properly folded glycoproteins are supposed to be intracellularly transported by binding to the ER/Golgi intermediate compartment-53 (ERGIC-53) (*17*) or VIP36 (*14, 18*). Lysosomal enzymes bearing phosphorylated high-mannose-type glycans are sorted by binding to 6-phosphomannosyl receptor in the *trans*-Golgi network (TGN) and transported to lysosomes (*19, 20*). On the other hand, although most *N*-glycans of glycoproteins are processed into complex-type glycans in the Golgi apparatus, the functional roles of complex-type glycans in relation to sorting and transport are not yet clear.

In spite of many reports about the importance of *N*-glycans in the intracellular transport of glycoproteins in MDCK cells, the structures of *N*-glycans derived from MDCK cells have not been reported. If complex-type glycans are involved in the intracellular sorting and trafficking, their structures and topography should differ according

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Abbreviations: LacdiNAc, GalNAcβ1→4GlcNAcβ1→; Sia, sialic acid; GalNAc, *N*-acetyl-D-galactosamine; GlcNAc, *N*-acetyl-D-glucosamine; β4GalNAc-T, β1→4*N*-acetylgalactosaminyltransferase; MDCK, Madin-Darby canine kidney; ER, endoplasmic reticulum; VIP36, vesicular integral protein of 36 kDa; ERGIC, ER/Golgi intermediate compartment; TGN, *trans*-Golgi network; RCA, *Ricinus communis* agglutinin; DSA, *Datura stramonium* agglutinin; L_4-PHA, phytohemagglutinin-L_4; Con A, concanavalin A; WFA, *Wistaria floribunda*; AAL, *Aleuria aurantia* lectin; PVL, *Psathyrella velutina* lectin; TJA-I, *Trichosanthes japonica* agglutinin-I; MAL, *Macckia amurensis* lectin; LacNAc, *N*-acetyllactosamine; Neu5Ac, *N*-acetylneuraminic acid; Man, mannose; Fuc, fucose; αMG, methyl-α-D-glucopyranoside; αMM, methyl-α-D-mannopyranoside; Lac, lactose; XylNAc, *N*-acetylxylosamine; TBS, Tris-buffered saline; TB, 10 mM Tris-HCl buffer (pH 7.4) containing 0.02% NaN_3; D-MEM, Dulbecco's Modified Eagle Medium; HexNAc, *N*-acetylhexosamine; GnT, *N*-acetylglucosaminyltransferase; subscript $_{OH}$, alditol reduced with $NaBH_4$; subscript $_{OD}$ alditol reduced with NaB^3H_4.

to their destination. In this study, we analyzed the structures and the % molar ratios of *N*-glycans of secretory and membrane glycoproteins derived from apical and basolateral membranes of polarized MDCK cells. No difference was found in the structures of *N*-glycans between apical and basolateral membranes. However, a (sialyl)LacdiNAc se-quence, (Siaα2→6)GalNAcβ1→4GlcNAcβ1→, was specifically found in secretory glycoproteins from both sides, indicating that this carbohydrate moiety may function in the secretory mechanism. Furthermore, β1→4-*N*-acetylgalactosaminyltransferase (β4GalNAc-T), which is responsible for LacdiNAc sequence formation in MDCK cells, had a unique substrate specificity, showing preference for non-bisected glycans to bisected ones as acceptor substrates.

EXPERIMENTAL PROCEDURES

Chemicals, Lectins, and Enzymes—[^{3}H]Glucosamine hydrochloride (1.79 MBq/nmol), Expre^{35}S^{35}S (37 MBq/nmol), UDP-[^{3}H]*N*-acetylgalactosamine (GalNAc) (289 kBq/nmol) were purchased from NEN Life Science Products (Boston). Sulfo-NHS-biotin and monomeric avidin gel were obtained from Pierce Chemicals (Rockford, IL). *Ricinus communis* agglutinin-I (RCA-I)-agarose (4 mg/ml gel), *Datura stramonium* agglutinin (DSA)-agarose (3.8 mg/ml gel), phytohemagglutinin-E_4 (E_4-PHA)-agarose (4.5 mg/ml gel), and phytohemagglutinin-L_4 (L_4-PHA)-agarose (9 mg/ml gel) were purchased from Hohnen Oil (Tokyo). Concanavalin A (Con A)-Sepharose (12 mg/ml gel) was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). *Wistaria floribunda* agglutinin (WFA)-agarose (5 mg/ml gel) was from EY Laboratories (San Mateo, CA). *Aleuria aurantia* lectin (AAL)-Sepharose (7 mg/ml gel) and *Psathyrella velutina* lectin (PVL)-Sepharose (2 mg/ml gel) were kindly provided by Dr. Kochibe of Gunma University. *Trichosanthes japonica* agglutinin-I (TJA-I)-Sepharose (3 mg/ml gel) and *Macckia amurensis* lectin (MAL)-Sepharose (3 mg/ml gel) were purchased from Seikagaku Kogyo (Tokyo). L-Fucose (Fuc), lactose (Lac), GalNAc, methyl-α-D-mannopyranoside (α-MM), methyl-α-D-glucopyranoside (α-MG), *Arthrobacter ureafaciens* α-sialidase (*21*) were purchased from Nacalai Tesque (Kyoto). Bovine epididymal α-fucosidase was obtained from Sigma Chemicals (St. Louis, MO). Glycopeptidase F (*22*), *Salmonela typhimurium* LT2 α-sialidase (*23*) and *Bacillus* α-fucosidase (*24*) were obtained from Takara Biochemicals (Kyoto). Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purified from the culture fluid of *Diplococcus pneumoniae* according to the method of Glasgow *et al.* (*25*). Jack bean β-*N*-acetylhexosaminidase was prepared by the method of Li and Li (*26*). *Aspergillus saitoi* α-mannosidase (*27*) was purchased from Glyco (Novato, CA). Bio-Gel P-4 (<45 μm) was obtained from Bio-Rad Laboratory (Richmond, CA). CIM™ DEAE disk monolithic column was purchased from BIA Separations (Ljubljana, Slovenia).

Oligosaccharides—(Neu5Acα2→)$_{1\text{-}3}$[(Galβ1→4GlcNAcβ1→2)(Galβ1→4GlcNAcβ1→4)Manα1→3(Galβ1→4GlcNAcβ1→2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$] (Neu5Ac$_{1\text{-}3}$·Gal$_3$·GlcNAc$_3$·Man$_3$ GlcNAc·GlcNAc$_{OT}$) was prepared from ceruloplasmin as described in the previous paper (*28*). Man$_{5\text{-}7}$·GlcNAc·GlcNAc$_{OT}$ and Man$_{8\text{-}9}$·GlcNAc·GlcNAc$_{OT}$ were prepared from RNase B (*29*) and porcine thyroglobulin (*30*). GalNAcβ1→4GlcNAcβ1→2Manα1→3(Manα1→6)-Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GalNAc·GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$) was prepared from batroxobin (*31*). GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAc (GlcNAc$_2$·Man$_3$·GlcNAc) and GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAc (GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc) were purified from urinary oligosaccharides of a patient with Sandhoff disease (Yamashita, K., unpublished results). GlcNAcβ1→2Manα1→6(Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$) and GlcNAcβ1→2Manα1→6(GlcNAcβ1→4Manα1→3)(GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$ (GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$) were prepared from NCA-2 (*32*). GalNAcβ1→4Galβ1→4Glc$_{OT}$ and GalNAcβ1→3Galα1→4Galβ1→4Glc$_{OT}$ were prepared from asialo GM2 and globoside, respectively, by endoglycoceramidase digestion.

Metabolic Radiolabeling of Cells and Preparation of Glycoproteins and Their N-Glycans—MDCK cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) containing 4.5 g/liter of glucose plus 5% fetal bovine serum. For metabolic radiolabeling, MDCK cells were seeded at the confluent level on 24-mm tissue culture inserts (Transwell; Corstar, Cambridge, MA) and cultured for 4 days. Polarization was monitored by measuring transepithelial electric resistance. Cells were then radiolabeled in apical medium containing 1 g/liter of glucose with [^{3}H]glucosamine (3.7 MBq/ml) for 24 h at 37°C in a humidified incubator containing 5% CO_2. For ^{35}S-labeling, cells were cultured with Expre^{35}S^{35}S (925 kBq/ml) in media lacking methionine and cystein for 2 h at 37°C. After the apical and the basolateral media were pooled, the cells were washed three times with ice-cold PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. In order to domain-selectively biotinylate cell surface (glyco)proteins, 1 mg/ml membrane impermeable sulfo-NHS-biotin in PBS with the above divalent cations was added either to the apical or to the basolateral compartment of the chamber. After 45 min at 4°C, the cells were washed three times with PBS containing the divalent cations and 1 mM glycine and harvested. The cells were extracted in 10 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 1% Triton X-100, 0.1% octylglucoside, and protease inhibitor cocktail (1 mM phenylmethanesulfonylfluoride, 100 kalikrein U/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) for 30 min at 4°C. Membrane proteins were concentrated by Ultrafree-4 (cutoff: 10 kDa, Millipore), then applied to monomeric avidin columns (0.5 ml) that were sequently pre-washed with 5 mM biotin, 0.4 N acetic acid, and Tris-buffered saline (TBS) containing 0.1% Triton X-100. The biotinylated, apical and the basolateral membrane glycoproteins were eluted with 0.4 N acetic acid at 4°C and dried. The biotinylated membrane proteins were reproducibly retained on the monomeric avidin column and recovered with high-yields from the column. In contrast, the avidin column was not applicable, since the recovery of the membrane proteins from the column was too low. The glycoproteins secreted from the both sides were prepared by concentrating pooled media with Ultrafree-4 (cutoff: 10 kDa, Millipore). Secretory and membrane glycoproteins from both sides were incubated with glycopeptidase F (10–25 mU) in denatured condition (pH 8.6) at 37°C for 24 h. The released oligosaccharides

were separated by Ultrafree-4, concentrated, and reduced with $NaBH_4$. The yields of ^3H-labeled *N*-glycans in one 24-mm tissue culture are summarized in Table I. Hydrazinolysis of these glycoproteins also gave the same recovery and sugar chain characteristics.

Analytical Methods—Anion exchange HPLC was carried out on a CIM™ DEAE disk monolithic column (0.34 ml × 2) (*33*). Elution was performed at the flow rate of 4 ml/min at room temperature with a linear gradient (0–0.5 M) of pyridine-acetate buffer (pH 5.4) for 90 s. Monosaccharide analysis was performed as reported using a Shodex SP0810 column (8 mm i.d. × 30 cm long, Showa Denko, Tokyo) (*34*). Bio-Gel P-4 (<45 μm) column chromatography (2 cm i.d. × 100 cm long) was performed as reported previously (*35*).

Lectin-immobilized columns (1 ml gel) were equilibrated with 10 mM Tris-HCl buffer (pH 7.4) containing 0.02% NaN_3(TB). Tritium-labeled oligosaccharides dissolved in 100 μl of TB were applied to the column, and then elution was performed with 5–15 ml of TB at room temperature, followed by 5 ml of TB containing 5 mM Fuc (AAL-Sepharose column), 10 mM Lac (RCA-I-agarose column), 5 mM α-MG and 0.2 M α-MM (Con A-Sepharose column), 10 mM GalNAc (WFA-agarose column), 1% GlcNAc oligomer (DSA-agarose column), and 0.3 M GlcNAc (PVL-Sepharose column).

Methanolysis of sulfated oligosaccharides was performed as reported in the previous paper (*36*). Periodate oxidation was performed as previously reported (*37*). The products were analyzed by Bio-Gel P-4 column chromatography.

Glycosidase Digestion—Radioactive oligosaccharides were digested with one of the following reaction mixtures (20 μl) at 37°C for 18 h: *Arthrobacter* α-sialidase digestion, 100 mU of the enzyme in 0.1 M sodium acetate buffer, pH 5.0; *Salmonella* α2→3-sialidase digestion, 5 mU of the enzyme in 0.1 M sodium acetate buffer, pH 5.5; *Bacillus* α1→2-fucosidase digestion, 20 μg of the enzyme in 0.1 M citrate-phosphate buffer, pH 6.5; bovine epididymal α-fucosidase, 20 mU of the enzyme in 0.1 M citrate-phosphate buffer, pH 6.5; *Streptococcus* 6646k β-galactosidase digestion, 5 mU of the enzyme in 0.1 M citrate-phosphate buffer, pH 5.5; diplococcal β-galactosidase digestion, 5 mU of enzyme in 0.1 M citrate-phosphate buffer, pH 5.5; diplococcal β-*N*-acetylhexosaminidase digestion, 5 mU of the enzyme in 0.1 M citrate-phosphate buffer, pH 5.5; jack bean β-*N*-acetylhexosaminidase digestion, 0.5 or 2 U of the enzyme in 0.2 M citrate-phosphate buffer, pH 5.0; *Aspergillus* α1→2-mannosidase digestion, 50 μg of the enzyme in 0.1 M acetate buffer, pH 5.0. One drop of toluene was added to each reaction mixture to prevent bacterial growth. After incubation, each reaction mixture was heated at 100°C for 2 min to stop the reaction.

β4GalNAc-T Assay—The reaction mixtures (40 μl) containing cell extracts solubilized with 0.5% Triton X-100 in TBS (100–200 μg of protein), 10 mM $MnCl_2$, 5 mM ATP, 0.5 mM acceptor oligosaccharides, and 2 μM of UDP-[^{3}H]GalNAc in 50 mM cacodylate buffer (pH 6.5) were incubated at 37°C for 2 h, and the enzymatic reaction was stopped by heating at 100°C for 2 min. ^{3}H-labeled products passed through the mixed gel column of AG-50 (H^+ form) and AG-3 (OH^- form) were separated by Bio-Gel P-4 column chromatography.

TABLE I. **Metabolically [^{3}H]glucosamine-labeled *N*-linked glycans derived from secretory and membrane glycoproteins in polarized MDCK cells.**

Glycoproteins	^{3}H-labeled *N*-glycans in Fig 1				[^{35}S]-proteins
	Neutral (N)	Sialylated (AN)	Sulfated (AAN)	Total	
			(×10^4 dpm)		
Secretory					
Apical	366	360	60	786	53
Basolateral	196	166	18	380	53
Membrane					
Apical	70	26	5	101	90
Basolateral	46	12	1	59	96

RESULTS

Fractionation of the N-Linked Oligosaccharides of Glycoproteins Derived from MDCK Cells—MDCK cells polarized on the filter for 4 days were metabolically labeled with D-MEM containing [^{3}H]glucosamine and 1 g/liter of glucose for 24 h, then ^{3}H-labeled glycoproteins secreted from the apical and basolateral sides were pooled and plasma membrane glycoproteins on both sides were isolated by surface biotinylation and monomeric avidin gel chromatography as described in "EXPERIMENTAL PROCEDURES." *N*-Linked oligosaccharides were liberated from these glycoproteins by glycopeptidase F digestion. The oligosaccharide fractions derived from apical secretory and plasma membrane glycoproteins were both separated into one neutral and two acidic fractions by anion exchange HPLC (Fig. 1, A and F). Although acidic fractions (A) could not be hydrolyzed by *Salmonella* sialidase, which specifically hydrolyzes the Siaα2→3 linkage of both Siaα2→3Galβ1→4GlcNAcβ1→R and Siaα2→3GalNAcβ1→4GlcNAcβ1→R, large parts of A were converted to the neutral fractions (AN) by *Arthrobacter* sialidase, which can cleave both Siaα2→3 and Siaα2→6Gal(GalNAc) linkages (Fig. 1, B and G). Released ^{3}H-labeled sialic acid could not be detected, suggesting that metabolic labeling with [^{3}H]glucosamine at 37°C for 24 h did not induce the conversion of [^{3}H]glucosamine to [^{3}H]sialic acid. Furthermore, since a large parts of A bound to a TJA-I-Sepharose column, which specifically recognizes the Siaα2→6Galβ1→4GlcNAc residue (Fig. 1, D and I) (*38*), most sialic acids should be linked to the C-6 position of galactose residues. The remaining acidic fractions (AA) bound to a MAL-Sepharose column, which specifically recognizes Siaα2 (or SO_3^-)→3Galβ1→4GlcNAc (Fig. 1, E and J) (*38*), and were completely converted to neutral oligosaccharides (AAN) by methanolysis (0.05 N HCl/MeOH at 30°C for 4 h) (Fig. 1, C and H), which hydrolyses sulfate residues. These results suggested that a sulfate residue is linked to the C-3 position of Galβ1→4GlcNAc. The same results were obtained from basolateral secretory and membrane glycoproteins. Radioactivities of N, AN, and AAN derived from apical secretory and membrane glycoproteins were several times higher than those from basolateral glycoproteins, respectively, although both sides showed similar protein contents from radioactivities of [^{35}S]methionine incorporated proteins (Table I). Next, the pooled N, AN, and AAN fractions derived from apical secretory and membrane glycoproteins in Fig. 1 were chromatographed on various lectin-agarose columns having strict carbohydrate binding specificities (*39*) to search for their structural differences. No difference was observed in Con A (Fig. 2, A and F), E_4-PHA (Fig. 2, B and G), RCA-I (Fig. 2, C and H), AAL (Fig.

2, D and I), DSA, L_4-PHA, and PVL-Sepharose column chromatograms between the two fractions (data not shown). However, a striking difference was observed in the elution patterns of WFA-agarose column chromatography between apical secretory and membrane glycoproteins (Fig. 2, E and J). Most oligosaccharides derived from apical membrane glycoproteins flowed through the WFA-agarose column (Fig. 2J), while oligosaccharides derived from apical secretory glycoproteins were reproducibly separated into flow-through (WFA^-), retard (WFA^r), and bound (WFA^+) fractions (Fig. 2E). These results implied that secretory glycoproteins predominantly contain β-GalNAc-linked *N*-glycans. Furthermore, the WFA^- fractions in Fig. 2, E and J, were separated into flow-through(–), 5 mM αMG-eluted (+), and 0.2 M αMM-eluted (++) fractions by Con A column chromatography (Fig. 3, A and B). The structures of these fractions were analyzed later. The oligosaccharide fractions from basolateral secretory and membrane glycoproteins exhibited the same behaviors on these lectin column chromatographies as those from apical secretory and membrane glycoproteins, respectively (data not shown).

Structures of Oligosaccharides in WFA^r and WFA^+ Fractions—The WFA^r fraction from apical secretory glycoproteins (Fig. 2E) was separated into two components with mobilities of 15.8 and 12.2 glucose units on Bio-Gel P-4 column chromatography (Fig. 4A, components a and b). When these components were digested with jack bean β-*N*-acetylhexosaminidase, two *N*-acetylhexosamine (HexNAc) residues were released from each component, and free [^{3}H]-HexNAc residues appeared at 2 glucose units (Fig. 4, B and C). The [^{3}H]HexNAc residues were identified as GlcNAc and GalNAc in the ratio of 100:15 by use of a Shodex SP0810 column, and the components a and b flowed through a β-GlcNAc-specific PVL-Sepharose column (*39*)

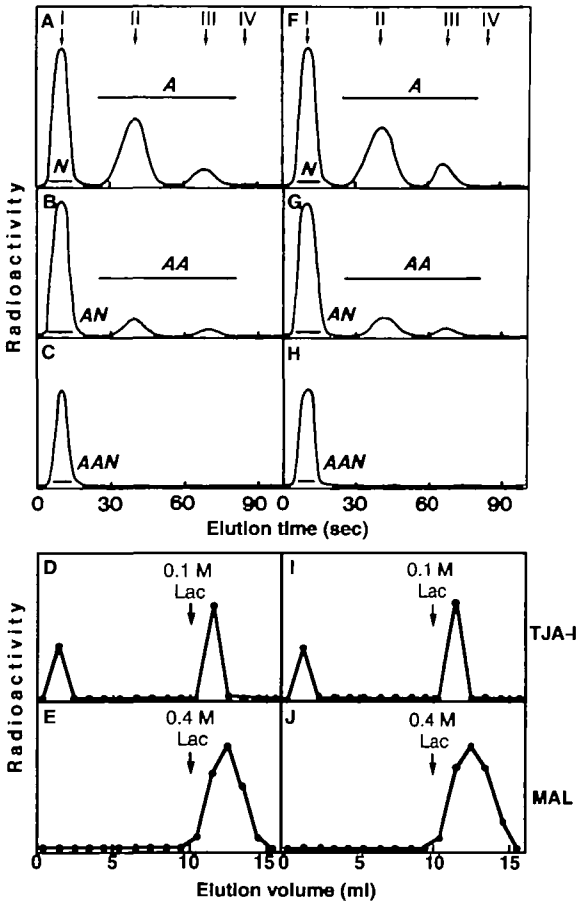


Fig. 1. **DEAE disk chromatography (A–C and F–H), TJA-I- or MAL-Sepharose column chromatography of ^{3}H-labeled *N*-glycans derived from apical secretory and membrane glycoproteins, their sialidase digests and methanolysates.** Arrows in A and F indicate the elution positions of authentic oligosaccharides: I, Gal_3·$GlcNAc_3$·Man_3 GlcNAc $GlcNAc_{OT}$; II, Neu5Ac Gal_3·$GlcNAc_3$·Man_3·GlcNAc $GlcNAc_{OT}$; III, $Neu5Ac_2$ Gal_3·$GlcNAc_3$·Man_3 GlcNAc·$GlcNAc_{OT}$; IV, $Neu5Ac_3$·Gal_3·$GlcNAc_3$·Man_3 GlcNAc·$GlcNAc_{OT}$ (A) and (F), oligosaccharides (1×10^6 dpm) derived from apical secretory and membrane glycoproteins; (B) and (G), oligosaccharides obtained from A in (A) and (F) by *Arthrobacter* sialidase digestion; (C) and (H), oligosaccharides obtained from AA in (B) and (G) by methanolysis, respectively. (D) and (I), TJA-I-Sepharose column chromatograms of fraction A (1×10^3 dpm) in (A) and (F); (E) and (J), MAL-Sepharose column chromatograms of fraction AA (1×10^3 dpm) in (B) and (G), respectively. Arrows in D, E, I, and J indicate the positions where the buffers were switched to those containing the indicated haptenic sugars. The same results were obtained with basolateral glycoproteins.

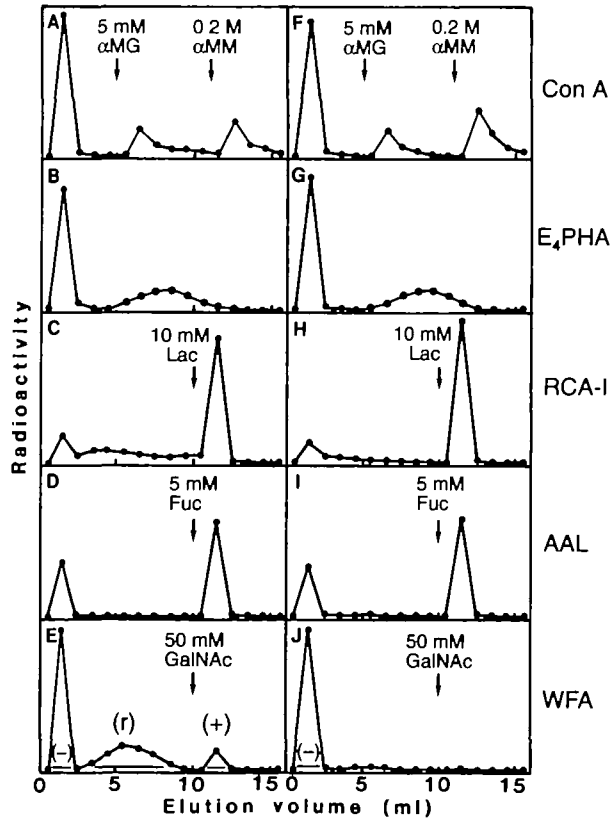
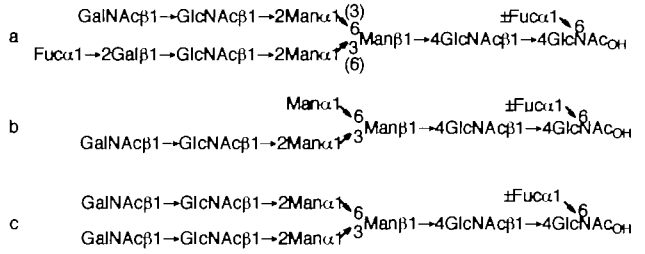


Fig. 2. **Immobilized lectin column chromatography of the pooled ^{3}H-labeled oligosaccharide fractions N+AN+AAN in Fig. 1.** Fractions N+AN+AAN (1×10^3 dpm) from apical secretory glycoproteins (A–E) and those from apical membrane glycoproteins (F–J) were chromatographed on various lectin columns. 8×10^5 dpm of the oligosaccharides were applied on WFA column. (A) and (F), Con A column; (B) and (G), E_4-PHA column; (C) and (H), RCA-I column; (D) and (I), AAL column; (E) and (J), WFA column. Arrows indicate the positions where the buffers were switched to those containing the indicated haptenic sugars. The same results were obtained with basolateral glycoproteins.

(data not shown), indicating that they contained a GalNAcβ1→GlcNAcβ1→ residue, although 4.7 and 3.6% of components a and b radioactivities are due to GalNAc residues. Moreover, by sequential digestion with *Bacillus* α1→2-fucosidase, diplococcal β1→4-galactosidase, and diplococcal β-*N*-acetylhexosaminidase, component a′ released a Fuc residue (Fig. 4D), a Gal residue (Fig. 4E), and a GlcNAc residue (Fig. 4F), respectively, and the consequent digests showed the same mobility as authentic Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$ with a small peak as Man_3 GlcNAc·$GlcNAc_{OT}$ (Fig. 4F).

The WFA⁺ fraction in Fig. 2E flowed through a PVL-Sepharose column (data not shown) and was eluted as component c with 15.5 glucose units on a Bio-Gel P-4 column (Fig. 4G). When the component c was digested with jack bean β-*N*-acetylhexosaminidase, it released 4 [³H]HexNAc residues and the digests showed the same mobility as authentic Man_3·GlcNAc·±Fuc·$GlcNAc_{OT}$ (Fig. 4H). From these results, the structures of the components a, b, and c can be summarized as follows.



Structure of the Side Chains Containing LacdiNAc Sequence—WFA-agarose column chromatograms of the fractions N, A, AN, and AAN derived from apical secretory glycoproteins (Fig. 1, A, B, and C) are shown in Fig. 5, A, B, C, and D, respectively. The radioactivity of the fraction A was recovered in the flow-through fraction (Fig. 5B), and the fraction AN was partially retarded or bound to the WFA-agarose column (Fig. 5C), suggesting that β-*N*-acetylgalactosamine residues are substituted with sialic acids. As shown in Fig. 1, none of the sialylated oligosaccharides was digested by Siaα2→3Gal-specific *Salmonella* sialidase, but most were hydrolyzed by Siaα2→3Siaα2→6Gal (GalNAc) linkage-specific *Arthrobacter* sialidase. These results indicate that most GalNAc residues were substituted at the C-6 position by sialic acids. The fraction AAN flowed through a WFA-agarose column (Fig. 5D), indicating that LacdiNAc moieties are not sulfated and that sulfated LacdiNAc moieties, as reported in sugar chains of glycohormone (*40*), are not biosynthesized in MDCK cells. To determine which hydroxyl group of GlcNAc is substituted with GalNAc, the component c in Fig. 4G was subjected to periodate oxidation-Smith degradation, and the product was analyzed by Bio-Gel P-4 column chromatography. If GlcNAc is substituted at the C-3 or C-4 position with GalNAc, 2 mol of [³H]GlcNAc should be released by periodate oxidation-Smith degradation, and if it is substituted at the C-6 position, free [³H]GlcNAc should not be detected. As a result, the products were separated into components of 5.2 and 2.0

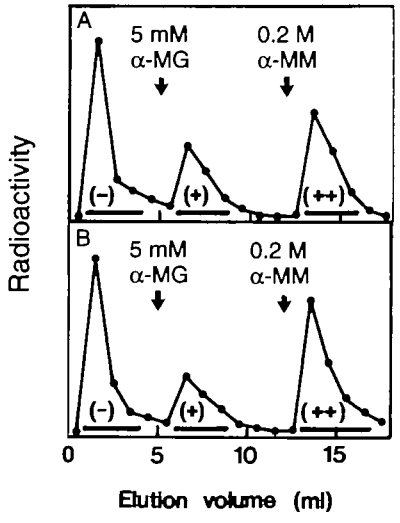


Fig. 3. **Con A-Sepharose column chromatography of the WFA– fractions in Fig. 2, E and J.** (A) WFA⁻ in Fig. 2E; (B) WFA⁻ in Fig. 2J. Arrows indicate the positions where the buffers were switched to those containing the indicated haptenic sugars.

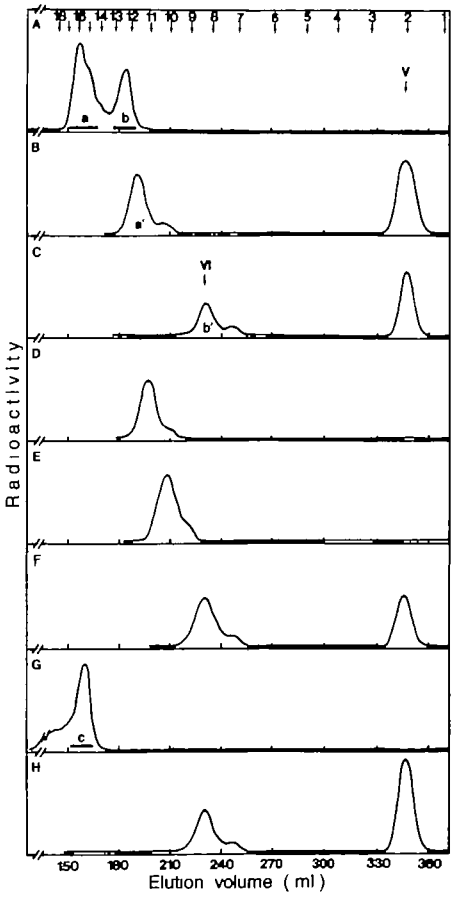
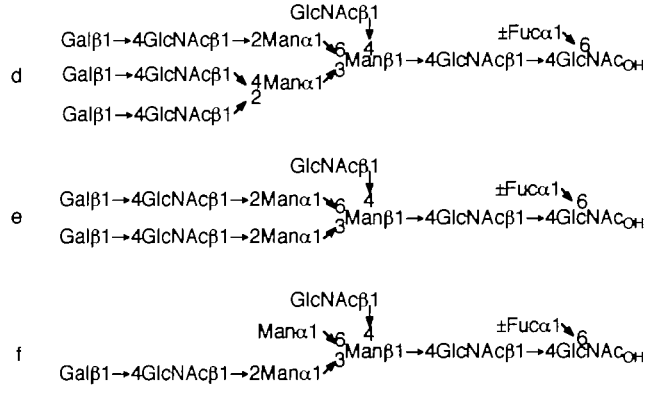


Fig. 4. **Bio-Gel P-4 column chromatography of the WFA⁻ and WFA⁺ fractions in Fig. 2E and their sequential exoglycosidase digests.** (A) and (G), WFA⁻ and WFA⁺ fractions (1×10^6 dpm each); (B), (C), and (H), jack bean β-*N*-acetylhexosaminidase digests of the components a, b, and c, respectively. (D), *Bacillus* α-fucosidase digest of the component a′; (E), diplococcal β-galactosidase digests of oligosaccharides in (D); (F), diplococcal β-*N*-acetylhexosaminidase digests of oligosaccharides in (E). V and VI indicate the elution positions of authentic [³H]GlcNAc and Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, respectively. Arrows at the top indicate the elution positions of glucose oligomers (the numbers indicate glucose units).

glucose units, corresponding to authentic Manβ1→4GlcNAcβ1→4GlcNAc$_{OH}$ (XylNAc$_{OH}$) and [^{3}H]GlcNAc as shown in Fig. 6. The component of 5.2 glucose unit was converted to monosaccharides by sequential digestion with snail β-mannosidase and jack bean β-*N*-acetylhexosaminidase, each releasing 1 mol of mannose and *N*-acetylglucosamine residue, respectively. The pooled monosaccharides were determined to be *N*-acetylglucosamine, *N*-acetylglucosaminitol and *N*-acetylxylosaminitol by using a Shodex SP0810 column (data not shown). These results indicated that the *triitols* are the mixtures of Manβ1→4GlcNAcβ1→4GlcNAc$_{OH}$ and Manβ1→4GlcNAcβ1→4XylNAc$_{OH}$. Furthermore, when we compared the elution profiles of the components a and b in Fig. 4A on a WFA-agarose column with those of authentic GalNAcβ1→3Galα1→4Galβ1→ 4Glc$_{OT}$, GalNAcβ1→4Galβ1→4Glc$_{OT}$ and GalNAcβ1→4GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$, the elution positions of components a and b were coincident with that of GalNAcβ1→4Galβ1→4Glc$_{OT}$ or GalNAcβ1→4GlcNAc·Man$_3$·GlcNAc·Fuc·GlcNAc$_{OT}$ (Fig. 5, E and F). From the results described above, the sialyl-LacdiNAc sequence synthesized in MDCK cells was estimated to be Siaα2→6GalNAcβ1→4GlcNAcβ1→R.

Structures of Oligosaccharides in WFA⁻ Fraction—The WFA⁻Con A⁻ fraction in Fig. 3A was separated into several components on Bio-Gel P-4 column chromatography (Fig. 7A). When all the radioactive oligosaccharides in Fig. 7A were incubated with *Bacillus* α1→2-specific α-fucosidase, they were separated into three components (d, e, and f in Fig. 7B). By digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase, the components d, e, and f released 3Gal+1GlcNAc, 2Gal+1GlcNAc, and 1Gal+1GlcNAc, respectively, and they were converted to components d′, e′, and f′, with the same mobilities as authentic GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)-(GlcNAcβ1→4Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$, GlcNAcβ1→2Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$, and Manα1→6(GlcNAcβ1→4)(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$, respectively (Fig. 7, C, D, and E). Furthermore, by digestion with 2 units of jack bean β-*N*-acetylhexosaminidase, these components were eluted at the same mobilities as authentic Manα1→6(Manα1→3)-Manβ1→4GlcNAcβ1→4(±Fucα1→6)GlcNAc$_{OT}$ (Fig. 7F). To elucidate the branching structure of the triantennary oligosaccharides, a part of component d was applied to a DSA column (3 ml). Since the radioactive component was slightly retarded, it was suggested that component d was C-2,4 branched triantennary oligosaccharides (*39*) (data not shown). From the results so far, the structures of components d, e, and f in the *Bacillus* α-fucosidase-treated WFA⁻ Con A⁻ fraction can be written as follows.



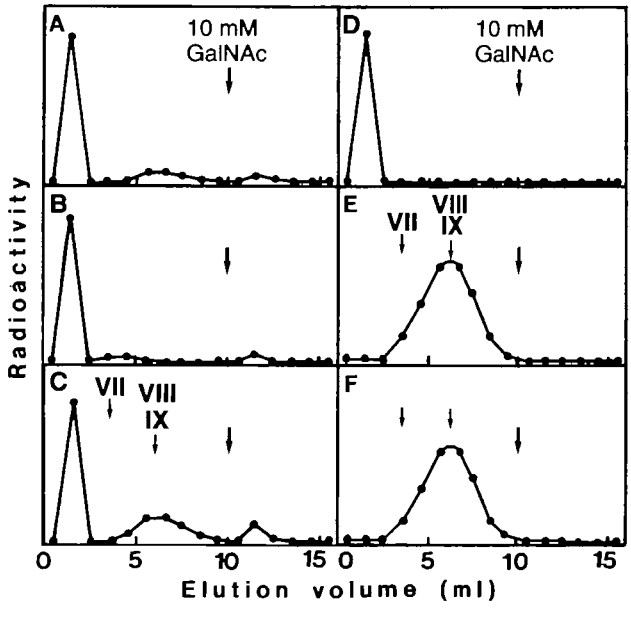


Fig. 5. **WFA-agarose column chromatography of oligosaccharides derived from apical secretory glycoproteins in Fig. 1, A, B, and C, and the components a and b in Fig. 4A.** 1 × 10^5 dpm of each oligosaccharide was applied on WFA-agarose column. (A) the fraction N in Fig. 1A; (B) the fraction A in Fig. 1A; (C) the fraction AN in Fig. 1B; (D) the fraction AAN in Fig. 1C; (E) the component a in Fig. 4A; (F) the component b in Fig. 4A. VII, VIII, and IX indicate the elution positions of authentic GalNAcβ1→3Galα1→4Galβ1→4Glc$_{OT}$, GalNAcβ1→4Galβ1→4Glc$_{OT}$, and GalNAcβ1→4GlcNAc·Man$_3$·GlcNAc Fuc·GlcNAc$_{OT}$, respectively. Arrows indicate the positions where the buffers were switched to those containing 10 mM GalNAc.

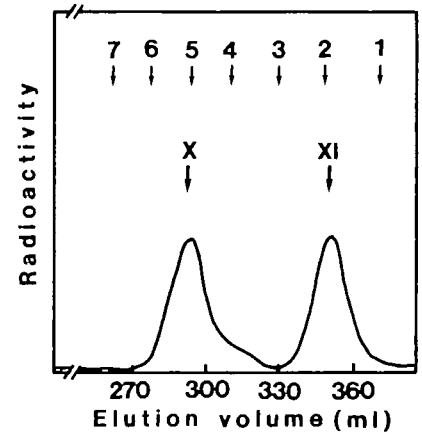


Fig. 6. **Bio-Gel P-4 column chromatography of the component c (2×10^5 dpm) in Fig. 4G after periodate oxidation-Smith degradation.** X and XI indicate the elution positions of authentic Man-GlcNAc GlcNAc$_{OT}$, and [^{3}H]GlcNAc, respectively. Arrows at the top indicate the elution positions of glucose oligomers (the numbers indicate glucose units).

The elution pattern of the WFA⁻Con A⁺ fraction in Fig. 3A on Bio-Gel P-4 chromatography is shown in Fig. 7G. When all the oligosaccharides were incubated with *Bacillus* α1→2-specific fucosidase, the oligosaccharides were separated into two components (g and h in Fig. 7H). By digestion with a mixture of diplococcal β-galactosidase and diplococcal β-*N*-acetylhexosaminidase, components g and h released 2Gal+2GlcNAc, and 1Gal+1GlcNAc, respectively, and the digests were eluted at the same mobilities as authentic Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4-(±Fucα1→6)$GlcNAc_{OT}$ (Fig. 7I). Therefore, the structures of components g and h in the *Bacillus* α-fucosidase-treated WFA⁻Con A⁺ fraction were determined as follows.

g: Galβ1→4GlcNAcβ1→2Manα1→6, Galβ1→4GlcNAcβ1→2Manα1→3 Manβ1→4GlcNAcβ1→4$GlcNAc_{OH}$; ±Fucα1→6

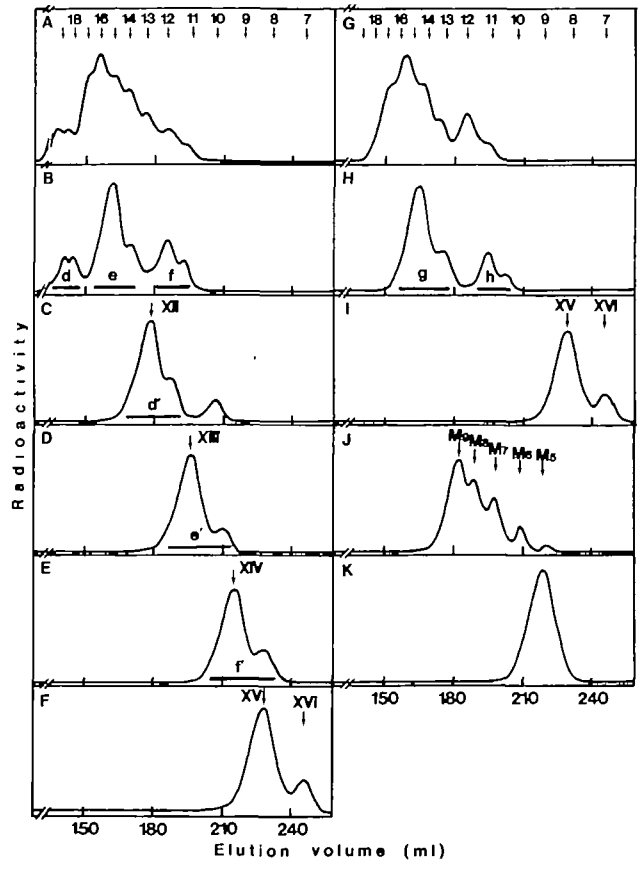


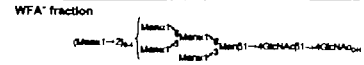
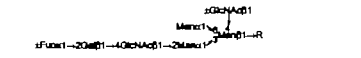
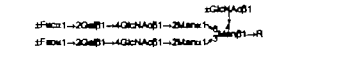
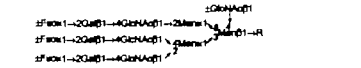
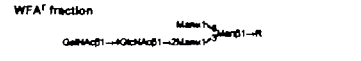
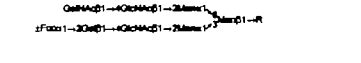
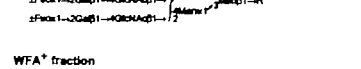
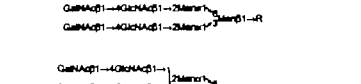
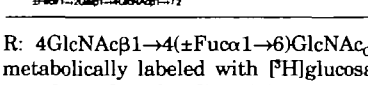
Fig. 7. **Bio-Gel P-4 column chromatography of the fractions WFA⁻ Con A⁻ (A), WFA⁻ Con A⁺ (G), WFA⁻ Con A⁺⁺ (J) in Fig. 3A and their sequential exoglycosidase digests (B–F, H, I, and K).** (B) and (H), *Bacillus* α-fucosidase digests of (A) and (G); (C), (D), and (E), diplococcal β-*N*-acetylhexosaminidase and β-galactosidase digests of the components d, e, and f, respectively. (F), jack bean β-*N*-acetylhexosaminidase digests of the pooled components d', e', or f'; (I), diplococcal β-*N*-acetylhexosaminidase and β-galactosidase digests of the pooled components g or h; (K), *Aspergillus* α1→2-specific mannosidase digest of oligosaccharides in (J). XII, XIII, XIV, XV, XVI, and M_{9-5} indicate the elution positions of authentic $GlcNAc_2$·Man_2 GlcNAc·Man·GlcNAc·Fuc $GlcNAc_{OT}$, GlcNAc Man_2·GlcNAc·Man·GlcNAc·Fuc·GlcNAc$_{OT}$, Man_2·GlcNAc·Man·GlcNAc·Fuc·$GlcNAc_{OT}$, Man_3·GlcNAc·Fuc·$GlcNAc_{OT}$, Man_3·GlcNAc·$GlcNAc_{OT}$, and Man_{9-5} GlcNAc·$GlcNAc_{OT}$, respectively. Arrows at the top indicate the elution positions of glucose oligomers (the numbers indicate glucose units).

h: Manα1→6, Manβ1→4GlcNAcβ1→4$GlcNAc_{OH}$; ±Fucα1→6; Galβ1→4GlcNAcβ1→2Manα1→3

The oligosaccharides in the WFA⁻Con A⁺⁺ fraction in Fig. 3A were separated at the same mobilities as authentic $(Manα1→)_{8-4}$Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$ on Bio-Gel P-4 column chromatography (Fig. 7J). When these five peaks were digested with *Aspergillus* α1→2-specific mannosidase, they were eluted at the same mobility as authentic $(Manα1→)_4$Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$ (Fig. 7K). This indicated that the structures of oligosaccharides in the WFA⁻Con A⁺⁺ fraction can be written as $(Manα1→)_{8-4}$-Manβ1→4GlcNAcβ1→4$GlcNAc_{OH}$. It became clear by the same analyses as described above that the oligosaccharides from basolateral secretory glycoproteins consisted of the same components as those from apical secretory ones, and that the oligosaccharides from both membrane glycoproteins consisted of the same components as those from secretory ones except for the LacdiNAc moieties, as summarized in Table II.

Distribution of LacdiNAc-Bearing Sugar Chains in Secretory Glycoproteins from MDCK Cells—To determine whether secretory glycoproteins carrying LacdiNAc-*N*-glycans are broadly distributed, *Arthrobacter* sialidase-

TABLE II. **Possible structures and % ratios of pooled desialyated-desulfated *N*-glycans derived from secretory and membrane glycoproteins in polarized MDCK cells.**

Structures	Apical secretory	Apical membrane	Basolateral secretory	Basolateral membrane
WFA⁻ fraction	(%)			
	16.1	22.3	18.3	24.2
	9.4	13.0	6.1	13.2
	44.6	49.2	43.1	48.6
	6.9	13.6	8.0	12.7
WFA⁺ fraction				
	3.7	0.4	3.8	0.2
	9.4	0.9	10.1	0.6
	1.3	0.1	1.2	0.1
WFA⁺⁺ fraction				
	7.1	0.3	7.6	0.3
	1.5	0.2	1.8	0.1

R: 4GlcNAcβ1→4(±Fucα1→6)$GlcNAc_{OH}$. % ratio: *N*-glycans were metabolically labeled with [³H]glucosamine at 37°C for 24 h, and over 98% of total radioactivities were identified as [³H]glucosamine by monosaccharide analysis using Shodex SP0810 column. Accordingly, the % ratios were calculated based on the radioactivities of the respective *N*-glycans.

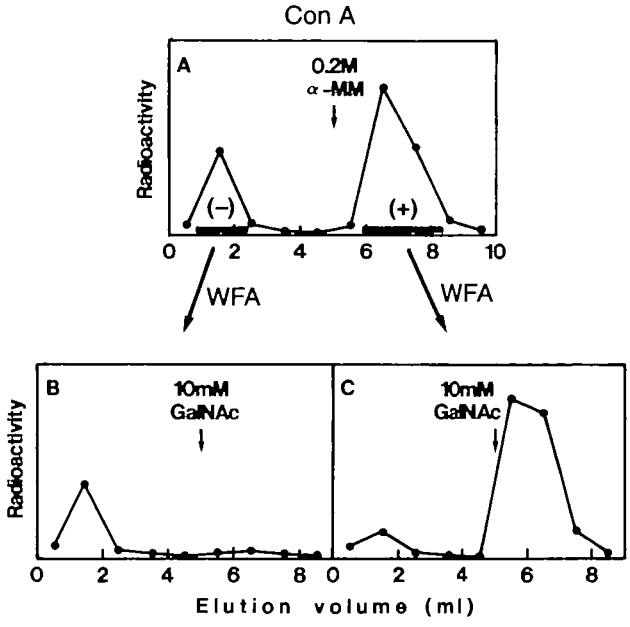


Fig. 8. **Serial lectin column chromatography of sialidase-treated, apical secretory ^{3}H-glycoproteins.** (A) Con A-Sepharose column chromatography of *Arthrobacter* sialidase-treated secretory glycoproteins (1×10^4 dpm); (B) WFA-agarose column chromatography of the Con A$^-$ fraction in (A); (C) WFA-agarose column chromatography of the Con A$^+$ fraction in (A).

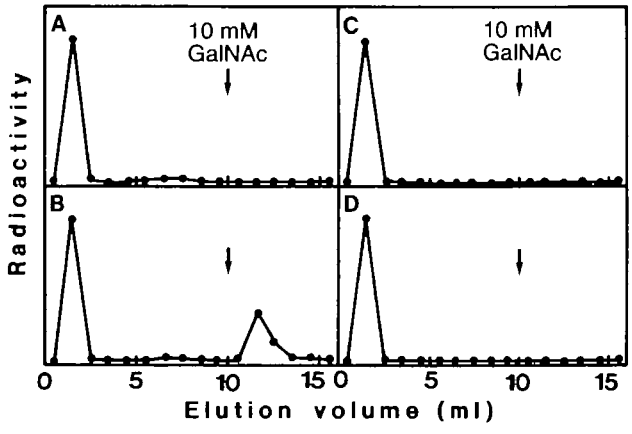


Fig. 9. **WFA-agarose column chromatography of ^{3}H-*N*-glycans (1×10^5 dpm) derived from secretory (A) and membrane (C) glycoproteins of 293 cells, and their sequential *Arthrobacter* sialidase and bovine epididymal α-fucosidase digests (B and D, respectively).** Arrows indicate the positions where the buffers were switched to those containing 10 mM GalNAc.

treated, ^{3}H-labeled secretory glycoproteins were directly analyzed by serial Con A- and WFA-column chromatographies. Over 70% of secretory glycoproteins bound to both Con A- and WFA-Sepharose columns (Fig. 8), indicating that most secretory glycoproteins carry at least 1 mol of LacdiNAc-glycan per molecule.

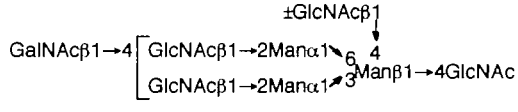
Distribution of LacdiNAc-Glycans in 293 Cells—Human embryonic kidney 293 cells produce recombinant human protein C bearing LacdiNAc residues which are modified with a Neu5Ac or Fuc residue (*41*). To assess whether LacdiNAc residues are present only in secretory glycoproteins in 293 cells, the structures of *N*-glycans were analyzed by the same methods as those in MDCK cells. Secretory and membrane glycoproteins were prepared from 293 cells, which were metabolically labeled with [^{3}H]glucosamine. *N*-linked oligosaccharides were released from these glycoproteins by glycopeptidase F. Although oligosaccharides from secretory and membrane glycoproteins scarcely bound to WFA columns (Fig. 9, A and C), only oligosaccharides from secretory glycoproteins bound to a WFA-Sepharose column by digestion with *Arthrobacter* α-sialidase and bovine epididymis α-fucosidase (Fig. 9, B and D). These results indicate that LacdiNAc-glycans occur only in secretory glycoproteins in both MDCK and 293 cells, and that LacdiNAc-glycans may participate in the secretory mechanism of both cells.

TABLE III. **Acceptor specificities for the β4GalNAc-T activities using homogenates of MDCK cells as enzyme source.**

Acceptor	Activity (pmol/h/mg protein)
GlcNAc$_2$·Man$_3$·GlcNAc	1.14
GlcNAc$_2$·Man$_2$ GlcNAc·Man GlcNAc	0.30

Enzymatic activity values were the means of three independent experiments (SDs were less than 5%).

Acceptor Specificities of β4GalNAc-T—The structures of *N*-glycans bearing LacdiNAc in MDCK cells implied that β4GalNAc-T in MDCK cells cannot transfer β-GalNAc to bisected *N*-glycans. Therefore, the enzymatic activities of β4GalNAc-T in MDCK cells were assayed by using biantennary and bisected biantennary oligosaccharides as acceptors. The radioactivities of the elution positions corresponding to authentic GalNAc·GlcNAc$_2$·Man$_3$·GlcNAc and GalNAc·GlcNAc$_2$·Man$_2$·GlcNAc·Man·GlcNAc on Bio-Gel P-4 column chromatography were calculated as the respective enzyme activities. The enzyme products were identified from the elution patterns on WFA-agarose column chromatographies. Both products showed the same elution patterns as those of components a and b in Fig. 5, E and F (data not shown), indicating that the structures of both products are as follows.



As summarized in Table III, β4GalNAc-T activity toward the bisected oligosaccharide in MDCK cells was one fourth of that toward the non-bisected oligosaccharide.

DISCUSSION

LacdiNAc sequences are found in bovine milk fat globule membrane glycoproteins (*42*), *Schistosoma* glycoproteins (*43*), human glycodelin (*44*), human urinary kallidinogenase (*45*), snake venom enzyme, batroxobin (*31*), pituitary glycohormones (*40*), and glycoproteins derived from human kidney 293 cells (*46*). Furthermore, LacdiNAc moieties of batroxobin, glycodelin, recombinant human protein C produced in 293 cells, and lutropin are substituted with a Neu5Ac, Fuc, or sulfate residue (*31, 40, 41, 44*). These results suggest that not only variously modified LacNAc sequences but also modified LacdiNAc sequences play indi-

vidual functional roles. In fact, the SO_3^-→4GalNAcβ1→4GlcNAcβ1→ sequence of lutropin functions as a clearance signal from the blood stream into hepatic endothelial cells (*47*). The GalNAcβ1→4(Fucα1→3)GlcNAcβ1→ sequence of human recombinant protein C is recognized by E-selectin (*48*), and the Siaα2→6GalNAcβ1→4GlcNAcβ1→ sequence of glycodelin interacts with CD22 in B cells (*49*), indicating that these modified LacdiNAc sequences are related to anti-inflammatory phenomena and anti-immune response.

We comparatively analyzed the structures of *N*-glycans from secretory and membrane glycoproteins which were separately prepared from apical and basolateral domains of polarized MDCK cells. The four samples commonly contained high-mannose-type glycans and bisected bi- and triantennary glycans bearing Fucα1→2Galβ1→4GlcNAc and/or Siaα2→6Galβ1→4GlcNAc. In addition, over 20% of [^{3}H]glucosamine-labeled glycans from secretory glycoproteins from both sides contained LacdiNAc sequences, which could not be detected in membrane glycoproteins. Some LacdiNAc sequences contained Sia residues, but not α1→3Fuc or SO_3^-→4′ residues. Strikingly, *N*-glycans bearing the LacdiNAc sequence did not contain bisected GlcNAc residues, which is bound to large parts of *N*-glycans bearing LacNAc sequences. These results implied that β4GalNAc-T responsible for LacdiNAc formation in MDCK cells prefers non-bisected *N*-glycans to bisected ones. Actually, we analyzed substrate specificity of β4GalNAc-T activities in MDCK cells and found that the enzyme prefers non-bisected glycans as acceptor substrates.

The (sialyl)LacdiNAc sequence was detected in over 70% of secretory glycoproteins of MDCK cells, but it was not found in the membrane glycoproteins, suggesting that it is related to a secretory mechanism of glycoproteins in MDCK cells. If this sequence functional as the intracellular transport signal to the cell surface, it should be present in both secretory and membrane glycoproteins. High-mannose-type glycans functioning as the transport signal in MDCK cells were distributed in most secretory and membrane glycoproteins (*14*). We previously reported that VIP36 is a lectin molecule involved in the intracellular transport system, and that it specifically recognizes high-mannose-type glycopeptides but not the LacdiNAc sequence (*13*). Moreover, a major secretory glycoprotein of MDCK cells, clusterin, which contains at least 1 mol of high-mannose-type glycan per molecule, is supposed to be transported *via* VIP36 from the ER to the cell surface. We investigated whether clusterin-linked complex-type glycans have LacdiNAc residues. We determined that clusterin has the same structural features as whole apical secretory glycoproteins summarized in Table II (Ohkura, T., Hara-Kuge, S., and Yamashita, K., unpublished results). All of desialylated clusterin bound to a WFA-agarose column and eluted with 10 mM GalNAc. These results prompted us to hypothesize that VIP36-bound clusterin is transported to the cell surface *via* VIP36 and the complex may be packaged/condensed into secretory granules *via* (sialyl)LacdiNAc-binding lectin. This hypothesis is also supported by the finding that a lectin, ZG16p, localized in zymogen granule of pancreatic acinar cells, functions for condensation and packaging of intracellular transported glycoproteins (*50*). We are now investigating whether such a lectin exists in MDCK cells.

We determined in this study that the (sialyl)LacdiNAc sequence specifically exists in secretory glycoproteins of MDCK cells and 293 cells. The structural difference between *N*-glycans of secretory glycoproteins and membrane glycoproteins might be tissue-specific. For example, the complex-type glycans of serum glycoproteins secreted from human liver parenchymal cells are not fucosylated (*51–53*), while the membrane glycoproteins carry the complex-type glycans with fucosylated trimannosyl core (*54–56*). These phenomena imply that tissue- or cell-specific carbohydrate moieties are present in secretory glycoproteins and are involved in their condensation, packaging, or other functions related to the secretory mechanism.

It has been reported that there exists two types of UDP-GalNAc: GlcNAcβ1→R-β4GalNAc-T. One dually recognizes the PXR/K motif of luteinizing hormone or follicle-stimulating hormone and acceptor glycans (*57*), and the other is non-specific for polypeptide backbones and expressed in lepidopteran insect cells (*58*), 293 cells and CHO cells (*46*). β4GalNAc-T in MDCK cells in this study acts on various secretory glycoproteins, suggesting its similarity to the latter type. The enzymatic character of β4GalNAc-T in MDCK cells, which prefers non-bisected glycans to bisected ones, is reflected to the structures of *N*-glycans in MDCK cells. A similar substrate specificity has been reported for α-galactosyltransferase: the expression of α-galactosyl epitope was suppressed in *N*-acetylglucosaminyltransferase (GnT)-III transfected cells which acquire the ability to synthesize bisected glycans (*59*). Of course, it remains possible that this phenomenon is due to steric hindrance of the bisecting GlcNAc residue, since β1→4 galactosyltransferase was also shown to possess much lower activity toward bisected glycans than toward non-bisected ones (*60, 61*). We are now investigating the functional roles of LacdiNAc glycans in relation to the secretory transport mechanism using GnT-III overexpressed MDCK cells. However, it is still unclear why β4GalNAc-Ts in MDCK cells and 293 cells exclusively acts on secretory glycoproteins. The hydrophobic regions of membrane glycoproteins may inhibit their accessibility to these β4GalNAc-Ts.

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