Identification and Structural Determination of the KDN-Containing N-Linked Glycan Chains Consisting of Bi- and Triantennary Complex-Type Units of KDN-Glycoprotein Previously Isolated from Rainbow Trout Vitelline Envelopes

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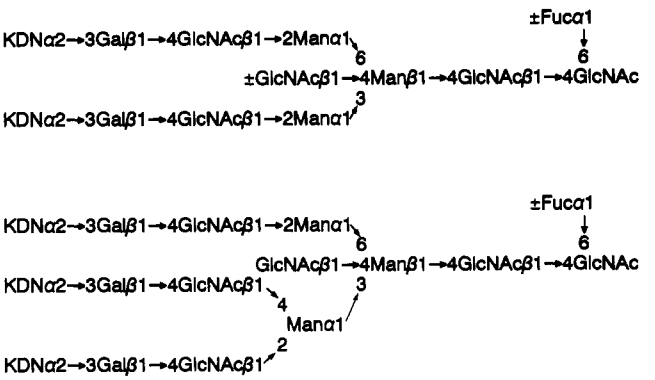
ABSTRACT: KDN-gp, which is the unique glycoprotein of the rainbow trout egg envelope, was shown to have a small amount of N-linked oligosaccharide units in addition to a large number of O-linked glycan units. Structural analysis based on chemical analysis in combination with 400 MHz ^{1}H NMR spectroscopy revealed the presence of fully KDNosylated bi- and triantennary complex-type oligosaccharide chains, mostly fucosylated at the innermost GlcNAc residue and bisected by the GlcNAc residue linked β1→4 to the β-Man residue. The structures thus determined represent the first demonstration of N-linked glycan unit containing the KDN residues in the KDN-containing glycoproteins (see Chart 1). The KDN-gp of the rainbow trout egg envelope is a molecule that is present in the second layer of the vitelline envelope but is exposed to the outer surface around the micropyle through which sperm can get in at fertilization. Like human hematopoietic cell surface glycoproteins such as glycophorin A and leukosialin, KDN-gp, which is now characterized to contain N-linked complex-type glycan chains as minor components, is heavily O-glycosylated with α2→8-linked oligo/polyKDN-containing glycan units attached O-glycosidically to Ser/Thr residues. Although little is known about the functional roles of these glycan chains, KDN-gp appears to form a model for further study on the function of cell surface receptor for sperm in fertilization.

Deaminated neuraminic acid (2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid; KDN[1]) is a unique nonulosonic acid found first in polysialoglycoprotein isolated from unfertilized eggs of rainbow trout (*Oncorhynchus mykiss*) (Nadano et al., 1986). Subsequently, a number of KDN-containing glycoproteins and glycosphingolipids have been found in living organisms (Iwasaki et al., 1987, 1990; Inoue et al., 1988; Kanamori et al., 1989, 1990; Yu et al., 1991, 1993; Knirel et al., 1989; Strecker et al., 1990, 1992a,b). In KDN-containing glycoproteins so far known, all the KDN-glycan chains were the O-linked type. A KDN-rich glycoprotein (KDN-gp) was found in and isolated from the vitelline envelope of rainbow trout egg coats (Inoue et al., 1988) and demonstrated to be composed of an average of a large number (about 1500) of oligosaccharide chains, most of which were linked to serine or threonine residues. All of serine- or threonine-linked sugar chains released by alkaline borohydride treatment were oligoKDN-containing glycans with the structure (Kanamori et al., 1990) KDNα2→(8KDNα2→)$_n$→6[KDNα2→3Galβ1→3GalNAcα1→3]GalNAcol. However, our previous finding of Man and GlcNAc as minor components of KDN-gp in addition to Gal, GalNAc, and KDN (Inoue et al., 1988) indicated the possible presence of an asparagine-linked oligosaccharide unit in KDN-gp and prompted us to identify the KDN-containing N-linked glycan unit(s).

Chart 1



Our search for N-linked KDN-containing glycan units indeed led to the finding of bi- and triantennary complex-type glycan chains in KDN-gp. Although KDN-gp is polydisperse in molecular mass (1000–4000 kDa; on average 2900 kDa) and the carbohydrate units accounted for 80% of the mass of KDN-gp, the present study showed that 1 N-glycan unit was attached to an Asn residue via an N-glycosidic bond and that about 17 of the glycan units were linked to Ser/Thr residues via O-glycosidic linkages. Thus, about 60 N-linked glycan chains and 1000 O-glycan chains are present in a 2900-kDa KDN-gp molecule. It is interesting to note that this feature is essentially similar to those found on major glycoproteins of all human hematopoietic cells in having high contents of sialic acid, containing a large number of O-linked glycan and a small proportion of N-linked glycan units as demonstrated in the major red cell sialoglycoprotein, glycophorin A, and the major leukocyte sialoglycoprotein with an apparent molecular

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[1] Abbreviations: KDN, 2-keto-3-deoxy-D-*glycero*-D-*galacto*-nononic acid or naturally occurring deaminoneuraminic acid; KDN-gp, KDN-rich glycoprotein from rainbow trout vitelline envelope; PSGP, polysialoglycoprotein; NeuAc, *N*-acetylneuraminic acid; TBA, thiobarbituric acid; TFA, trifluoroacetic acid; GLC, gas–liquid chromatography; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; 2D HOHAHA, two-dimensional homonuclear Hartmann–Hahn spectroscopy.

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weight between 100 000 and 170 000, which recently has been named leukosialin (Carlsson & Fukuda, 1986) or sialophorin (Mentzer et al., 1987). Other receptor glycoproteins at the cell surface which may be included in this family are, for example, the low-density lipoprotein receptor (Cummings et al., 1983), the human leukocyte common antigen CD45 (Aruffo et al., 1992), and the interleukin-2 receptor (Leonard et al., 1984; Nikaido et al., 1984). It should be interesting to note that there are a group of mucin-like macromolecules present in cell surfaces directly associated with the plasma membranes, which include episialin (Hilkens et al., 1992), ascites sialoglycoprotein 1 (Carraways & Hull, 1991), and cancer-associated mucins and mucin-type glycoproteins (Bhavanandan, 1991). Although much is known about the structures of these proteins, the role of such glycan chains in cell surface protein functions will still continue to be an important issue of study. However, it is suggested that changes in the glycosylation of the some of these proteins during cellular differentiation result in altered reactivities with antibodies, microbes, and other ligands.

The primary purpose of this study was to demonstrate the existence of KDN in N-linked glycans, and a new type of KDN-containing glycan units was discovered in KDN-gp although the analogous glycan chains containing NeuAc instead of KDN had been found in human glycophorin A (Irimura et al., 1981), human term amniotic fluid fibronectin (Takamoto et al., 1989), *Meconium* nonspecific cross-reacting antigen-2 (Yamashita et al., 1989), and human chorionic gonadotropin β subunit from the choriocarcinoma cell line BeWo (Hård et al., 1992).

MATERIALS AND METHODS

Preparation of KDN-Rich Glycoprotein (KDN-gp) Fraction from the Vitelline Envelope of Rainbow Trout Eggs. Unfertilized mature eggs of rainbow trout were supplied by courtesy of the Gunma Prefectural Fisheries Station at Kawaba and the Okutama Fish Farm, Department of Fishery, Tokyo Metropolitan Government. Eggs (9.45 kg) were crushed and squeezed through Tetoron gauze, and vitelline envelopes (577 g) on gauze were washed three times with 0.8% NaCl to remove yolk and cytoplasmic materials extensively.

KDN-gp was prepared as previously described (Inoue et al., 1988) or alternatively by the following modified method. Vitelline envelope (536 g) was suspended in 3000 mL of 0.8% NaCl, stirred at 4 °C for 4 days under toluene until the vitelline envelope became amorphous, and then filtered through Tetoron gauze. The filtrate was centrifuged (10000*g*, 4 °C, 1 h), and both supernatant and precipitate were used for the preparation of KDN-gp. From the supernatant, KDN-gp was purified as previously described (Inoue et al., 1988). The precipitate (60 mL) was blended with 180 mL of Tris-HCl buffer (pH 8). Methanol (240 mL) and chloroform (480 mL) were added to this precipitate, stirring at room temperature for 30 min, and centrifuged (14000*g*, 4 °C, 15 min). After the supernatant was removed, the precipitate was suspended with chloroform/methanol/10 mM Tris-HCl, pH 8.0 (=4:8:3, v/v), stirred, and centrifuged. The precipitate was air-dried.

The delipidated powder (15.5 g) was divided into three parts. Each (~5 g) was suspended with 75 mL of 0.1 M Tris-HCl (pH 8.0) containing 1 mM $CaCl_2$ and 0.75 g of Actinase E (750 000 Tyr units, Kaken Corp.) and incubated at 37 °C for 70 h with shaking. At 24 and 48 h, 0.75 g of Actinase E was added to the reaction mixture. The Actinase E digests were centrifuged (10000*g*, 4 °C, 1 h), and the supernatant was divided into six parts. Each part was applied on a Sephacryl S-500 column (2.5 × 160 cm; equilibrated and eluted with 10 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl). The elution profile was monitored by the absorbance at 230 nm and the thiobarbituric acid (TBA) method (Aminoff, 1961; Uchida et al., 1977; Kitajima et al., 1992). The high molecular weight KDN-rich fractions (designated as KDN-gp-I and KDN-gp-II, see Figure 1) obtained by six chromatographies were combined, concentrated through ultrafiltration (YM-10 membrane, Amicon), extensively dialyzed against water, and lyophilized. These fractions were subjected to hydrazinolysis to prepare N-glycan chains (see below).

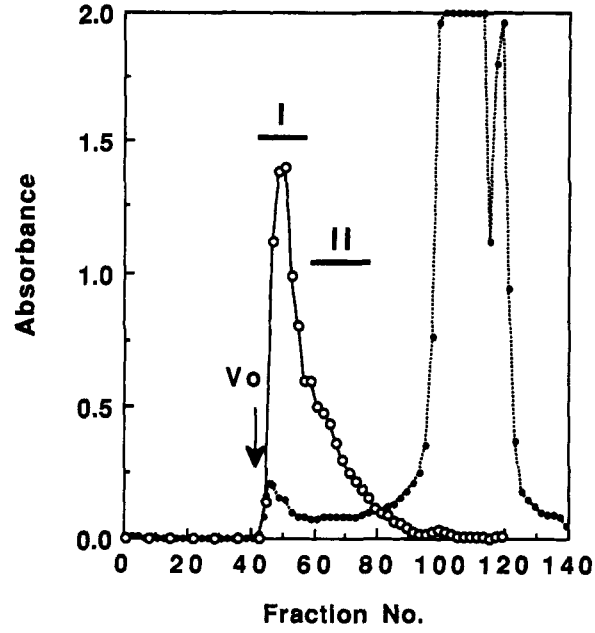


FIGURE 1: Gel filtration chromatography on Sephacryl S-500 of KDN-gp prepared from the Actinase E digests of vitelline envelope crude extract. The column (2.5 × 160 cm) was equilibrated and eluted with 0.1 M NaCl in 10 mM Tris-HCl, pH 8.0, and fractions of 5.5 mL were collected. Elution profile was monitored by the absorbance at 230 nm (●) and by the TBA method (at 549 nm; ○) for KDN as described in Materials and Methods. Vo, void volume. Fractions under the bars were pooled and designated as KDN-gp-I and KDN-gp-II, respectively.

The KDN-gp-I fraction (30 mg as KDN) was subjected to DEAE-Toyopearl 650S chromatography: column, 1.8 × 50 cm; equilibrated with 10 mM Tris-HCl, pH 8.0; eluted with a 0–0.5 M NaCl gradient in the same buffer; fraction volume, 5.5 mL.

Preparation of Oligosaccharide Fractions from KDN-gp. (a) Hydrazinolysis. The hydrazinolysis/re-N-acetylation procedure was used to release oligosaccharide chains from KDN-gp-I. The lyophilized KDN-gp-I fraction (52 mg) was heated at 110 °C for 1 h with 2 mL of anhydrous hydrazine in a commercial hydrazinolysis apparatus, Hydraclub S-204, equipped with a glass reaction chamber (Honen Corp.). After removal of hydrazine under reduced pressure over concentrated H_2SO_4, re-N-acetylation was performed at room temperature basically according to the procedure by Takasaki et al. (1982). In brief, the hydrazinolysate was dissolved in 52 mL of saturated $NaHCO_3$ solution, and then 520 μL of distilled acetic anhydride was added 5 times at a 5-min interval. After that 2.6 mL of distilled acetic anhydride was added and stirred for 15 min. The reaction mixture was, after adjusting the pH to 7 with NaOH, divided into four parts. Each was applied on a Sephacryl S-200 column (1.3 × 133 cm; eluted with 0.1 M NaCl/10 mM Tris-HCl, pH 8.0). Fractions of 2.8 mL were collected. The lower molecular weight fractions con-

taining glycans and glycopeptides were combined and desalted by Sephadex G-25 (fine) column (1.3 × 105 cm; eluted with 5% ethanol).

(b) Isolation of KDN-Containing N-Linked Glycans. The glycan-containing fraction thus obtained was chromatographed on a DEAE-Toyopearl 650S column (1.8 × 50 cm; Cl^- form; preequilibrated with 10 mM Tris-HCl, pH 8.0) eluted with a linear gradient of 0–0.25 M NaCl in 10 mM Tris-HCl, pH 8.0. The elution profile was monitored by the absorbance at 230 nm and by the TBA method. Each TBA reaction positive fraction was desalted by a Sephadex G-25 column (1.3 × 88 cm; equilibrated and eluted with water) and then analyzed for carbohydrate composition by GLC. The fractions containing Man, GlcNAc, and KDN were subjected to gel chromatography on a Bio-Gel P-4 (extrafine) column (1.3 × 105 cm; eluted with 0.1 M NaCl) and purified by rechromatography on the same column.

Chemical Analyses. The KDN residue was quantitated or monitored by the TBA method as previously described (Aminoff, 1961; Uchida et al., 1977; Kitajima et al., 1992). The hexose content was estimated by the phenol–sulfuric acid method (Dubois et al., 1956). The molar ratios of component monosaccharides were determined by GLC as trimethylsilyl derivatives (Nomoto et al., 1982). Amino acids were determined quantitatively by the methods described previously (Heinrikson et al., 1984; Taguchi et al., 1993).

Mild Acid Hydrolysis of KDN-Containing N-Linked Glycans. To remove KDN residues from KDN-containing N-linked glycans, the samples were treated with 0.1 N trifluoroacetic acid at 80 °C for 20 min. De-KDNosylated glycans were purified by Bio-Gel P-4 chromatography (see above).

Methylation Analysis. Permethylation of oligosaccharides and glycopeptides was carried out according to either Hakomori (1964) or Ciucanu and Kerek (1984) with slight modification (Iwasaki & Inoue, 1985). For KDN-containing oligosaccharides, Hakomori's procedure was used, and for the de-KDNosylated sample, the simple procedure of Ciucanu and Kerek was employed. Permethylated glycans were hydrolyzed, reduced, and O-acetylated according to Levery and Hakomori (1987). Mixtures of partially methylated alditol and hexosaminitol acetates thus obtained were analyzed by GLC using a Shimadzu GC-14A gas chromatograph equipped with a capillary column CBJ5 (0.32 mm × 30 m, Shimadzu) at 180–260 °C at 2 °C per min (Taguchi et al., 1993).

400-MHz ^1H NMR Spectroscopy. For NMR measurements, the samples were exchanged twice with 99.8% ^2H_2O, lyophilized, and finally dissolved in high-purity ^2H_2O (99.95%) (Kanamori et al., 1990). The 1D spectra were measured at 25 and 50 °C with a Bruker WM-400 spectrometer. For 2D HOHAHA (Bax & Davis, 1985) spectrum, a MLEV-17 mixing sequence of 100 ms was used. The 90° pulse width was set to 27 μs, and the spectral width was 2500 Hz. The chemical shifts were expressed in parts per million as referenced to the sodium 2,2-dimethyl-2-silapentane-5-sulfonate methyl signal (set to 0 ppm).

RESULTS

Preparation of the KDN-Rich Glycoprotein (KDN-gp) Fraction from Rainbow Trout Vitelline Envelope. KDN-gp was a deaminated neuraminic acid (KDN)-rich (50% by weight) mucin-like glycoprotein and had no *N*-acylneuraminic acid residue (Inoue et al., 1988; Kanamori et al., 1989). Most of the KDN residues occurred as oligo/polyKDN groups attached to a number of O-linked glycan chains. Our previous results suggested the occurrence of a small amount of N-linked glycan chains(s) in KDN-gp as judged from the analytical data showing the presence of the Man and GlcNAc residues as the minor components (2~3 mol %) of sugar residues in KDN-gp (Inoue et al., 1988).

Although KDN-gp is a saline-solubilizable component of the water-insoluble vitelline envelope, it is difficult to extract KDN-gp in high yield unless detergents such as SDS were used, presumably because of its adhesive nature and low solubility (less than 5 mg/mL). Vitelline coats (536 g) were stirred in salt solution until they became amorphous and separated into the supernatant and precipitate fractions by centrifugation. From the supernatant we obtained KDN-gp (168 mg as KDN) as previously described (Inoue et al., 1988). We found that the controlled proteolysis of the precipitate fraction of vitelline envelope crude extract facilitated solubilization of KDN-gp and, consequently, raised the yield of KDN-gp by 2.4-fold (245 mg as KDN, see below) without significant reduction of molecular mass in comparison with the previous results (Inoue et al., 1988). KDN-gp was resistant to Actinase E (Pronase) digestion in the presence of 1 mM $CaCl_2$ while a larger proportion (~50%) of KDN-gp (~2900 K) was proteolyzed into relatively smaller (100–1000 K) glycopeptides in 10 mM $CaCl_2$ (data not shown).

Figure 1 shows the Sephacryl S-500 chromatography of Actinase E digests of the crude extract of vitelline envelope. The fraction denoted as KDN-gp-I (170 mg) eluted at the same position of KDN-gp, and the smaller molecular mass fraction KDN-gp-II (75 mg as KDN) was obtained. KDN-gp-I was further chromatographed on a DEAE-Toyopearl 650S column, and more than 98% recovery was attained in the fractions eluted at the NaCl concentration of 0.3 M where KDN-gp were eluted. KDN-gp-I and KDN-gp had the identical carbohydrate (Man/Gal/GalNAc/GlcNAc/KDN = 2.3/14/25/2.9/56 in mol/mol) and amino acid (data not shown; see Kanamori et al., 1989) compositions except that Lys and Tyr residues disappeared in KDN-gp-I (data not shown). The shoulder on the KDN-gp-I peak was designated as KDN-gp-II, which contained KDN only two-fifths of that in KDN-gp-I (Figure 1). KDN-gp-I and KDN-gp-II were also found to have the same carbohydrate and amino acid compositions besides KDN, indicating that KDN-gp-II was a smaller molecular form of KDN-gp-I. In this papers we used the KDN-gp-I fraction as the source for preparation of N-linked glycan chains.

Preparation of Oligosaccharide Fractions from the KDN-gp-I Fraction. The KDN-gp-I (52 mg as KDN) was subjected to hydrazinolysis to obtain N-linked glycans. This procedure also results in the degradation of the polypeptide chain, yielding O-linked glycopeptides. Sephacryl S-200 chromatography of the products (29 mg as KDN) obtained from KDN-gp-I by treatment with hydrazine, followed by acetylation, gave a rather broad TBA-positive peak (data not shown). The TBA-positive fractions (fractions 55–95) were collected and then subjected to anion-exchange chromatography on DEAE-Toyopearl 650S. Figure 2 shows an elution profile, when monitored by the TBA method, from which 11 fractions, labeled a–k were pooled in a 76% yield based on KDN. Separated fractions were subjected to carbohydrate composition analysis (not shown). Man and GlcNAc were shown to be enriched only in a and d, of which yields were 0.7 and 6.6 mol %, respectively, of all KDN-positive fractions. For further purification, a and d were separately applied on a Bio-Gel P-4 column. As shown in Figure 3a, a was separated into four

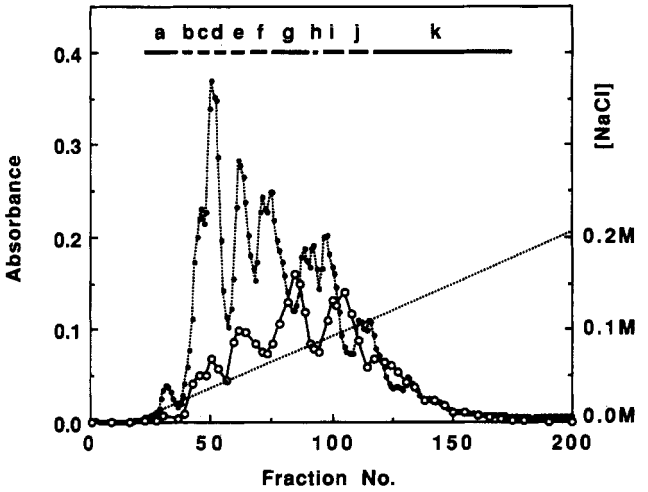


FIGURE 2: Anion-exchange chromatography on DEAE-Toyopearl 650S of the products obtained by hydrazinolysis/re-N-acetylation of KDN-gp-I. The column (1.8 × 50 cm) was equilibrated with 10 mM Tris-HCl, pH 8.0, and eluted with a linear gradient of 0–0.25 M NaCl in the same buffer. Fractions of 5.5 mL were collected and monitored by the absorbance at 230 nm (●) and at 549 nm (○) for KDN by the TBA method as described in Materials and Methods. Fractions a–k were pooled as indicated.

Table 1: Chemical Composition of a-1, a-2, and d-1

	a-2	a-1	d-1
Fuc	0.57	0.80	0.41
Man	3.0	3.0	3.0
Gal	2.0	1.8	2.8
GlcNAc	4.8	4.7	5.6
KDN	2.1	1.9	3.3
Asx		1.0	1.0
Ala		1.4	1.4

fractions, a-1 through a-4. No Man and GlcNAc were detected in fractions a-3 and a-4. a-1 (150 μg as Gal) and a-2 (150 μg as Gal) had virtually identical carbohydrate compositions (Table 1). Fraction a-1 was found to contain a small peptide covalently linked to the glycan chain, i.e., glycopeptide, whereas a-2 was devoid of amino acid residues. Fractions a-1 and a-2 were demonstrated to be of diKDNosylated biantennary complex type, the former being a glycopeptide and the latter being a free glycan. These fractions were subjected to structural analysis as described below.

The trianionic fraction d was also subjected to Bio-Gel P-4 chromatography, and fractions 48–54 were collected, pooled, and designated d-1 in a yield of 150 μg as Gal (Figure 3b), which appeared to be of the triKDNosylated triantenna type of N-linked glycopeptide (Table 1). As a-1, a-2, and d-1 turned out to contain the searched material, attention will only be paid to these fractions in this paper.

Structural Elucidation of KDN-Containing N-Linked Glycan Chains in KDN-gp. (i) a-1 and a-2. The methylation analysis of the KDN-depleted a-2 is shown in Table 2, and in comparison with that of the intact a-2 indicates the absence of 3-O-substituted Gal and the appearance of a new terminal Gal, indicating the substitution of this residue in the 3-position by KDN in a-2. Considering the data obtained by carbohydrate composition analysis and methylation analysis, the structures of a-2 and therefore the KDN-depleted a-2 were biantennary oligosaccharides. Methylation analysis of a-2 showed Man derivatives (3,4,6-O-substituted Man:3,6-di-O-substituted Man:3-O-substituted Gal = 0.9 mol:0.2 mol:2.0 mol) and GlcNAc (unsubstituted GlcNAc:3-O-substituted Gal = 0.5 mol:2.0 mol) in proportions suggesting a mixture of structures (Table 2). The major species (80–90% of a-2) corresponds to the fully KDNosylated biantennary complex-type oligosaccharide chain bisected by the GlcNAc residue linked β1→4 to the β-Man residue. Furthermore, the presence of 0.5 mol each of the unsubstituted Fuc and 4,6-di-O-substituted GlcNAc residues indicated that about 50% of a-2 were fucosylated at the innermost GlcNAc residue.

The fraction a-2 was further studied by 400-MHz ^{1}H NMR, and the spectrum obtained is shown in Figure 4. The spectrum was basically similar to that for N2.2, obtained from BeWo human chorionic gonadotropin, which was a fucosylated and bisecting GlcNAc-substituted biantennary oligosaccharide (Hård et al., 1992), except that the biantennary structure in a-2 was fully substituted by KDN instead of Neu5Ac in a form of KDN2→3Gal. Comparison of the various resonances in the anomeric region of the spectrum with the literature values published previously (Hård et al., 1992; Vliegenthart et al., 1983) allowed us to determine the anomeric configuration of the glycosidic linkages of the Fuc, Man, Gal, and GlcNAc residues in a-2 shown in Figure 5. The assignments of most of the resonances in our spectrum of a-2 to specific hydrogen atoms as summarized in Table 3 were based on 2D HOHAHA experiments (data not shown) as well as previous literature assignments (Hård et al., 1992; Vliegenthart et al., 1983) for glycans having the basic structures similar to a-2. The presence of the free di-*N*-acetylchitobiosyl structure at the reducing end in a-2 was evident from the chemical shift of GlcNAc[1](α) H-1 (δ = 5.19 ppm) and its $J_{1,2}$ value (~1 Hz). The NMR features of a-2 (Man[3] H-1, δ 4.70 ppm; Man[4] H–1, 5.07 ppm; Man[4′] H-1, δ 5.01 ppm), presented in Table 3, resembled those of the reference biantennary structure terminated with α2→3-linked Neu5Ac in both the Manα1→6 and Manα1→3 branches and having a β1→4-linked bisecting GlcNAc residue at the β-Man (Hård et al., 1992). Concommitant with these resonances, minor signals were also observed at 4.77, 5.12, and 4.92 ppm, which agreed with the literature values published for H-1 protons of Man-[3], Man[4], and Man[4′] of a biantennary glycan having no bisecting GlcNAc residue (Vliegenthart et al., 1983), indicating that the major molecular species (80–90%) in a-2 corresponded to the bisecting GlcNAc-substituted biantennary oligosaccharide. Further evidence for this was provided by the H-1, H-4, H-5, and *N*-acetyl CH_3 signals for the bisecting GlcNAc residue at 4.47, 3.29, 3.42, and 2.06 ppm, respectively (Table 3). The chemicals shifts of the Fuc structural-reporter groups, H-1, H-5, and CH_3 H-6 as well as that of GlcNAc[2] H-1 (Table 3), were characteristic of a typical biantennary structure having an α1→6-linked Fuc residue at the reducing terminal GlcNAc unit (Hård et al., 1992; Vliegenthart et al., 1983). The ^{1}H NMR spectroscopy of a-2 also showed that it was partly fucosylated since integration data indicated less than Fuc H-1/molecule resonating near 4.9 ppm. Termination of both antennae with KDN, linked α2→3 but not α2→6 to Gal, was suggested by the H-3ax/H-3eq signals for the KDN residues at 1.77/2.72 ppm [cf. Hård et al. (1992) and Vliegenthart et al. (1983)]. This was verified by the 2D HOHAHA spectral measurement (100-ms mixing time) of a-2 by showing the chemical shift values for H-3 of the Gal residues at 4.11 ppm, which is also consistent with substitution at C-3 of the penultimate Gal residues by KDN [cf. Kanamori et al. (1990)]. Based on the above data, the structure of a-2 was unequivocally established as shown in Figure 5.

a-1 and a-2 had virtually idential carbohydrate compositions except that a-1 was found to contain a small peptide covalently linked to the glycan chain, i.e., glycopeptide, whereas a-2 was

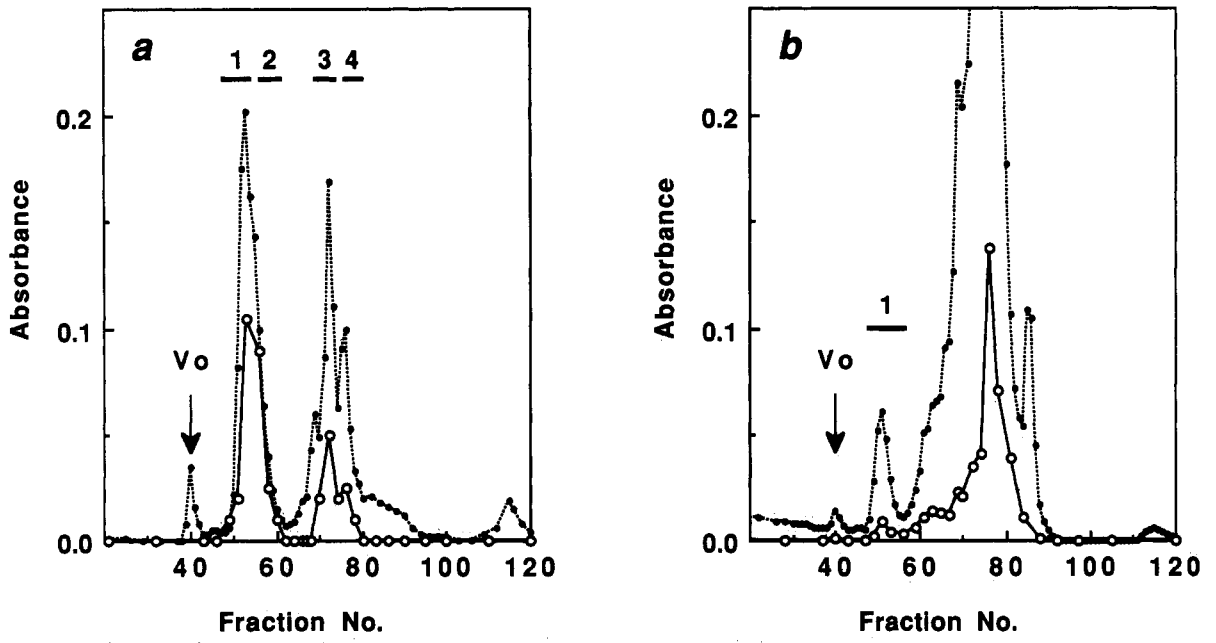


FIGURE 3: Purification of KDN-containing N-linked glycan chains by Bio-Gel P-4 chromatography. The fractions (a) a and (b) d, obtained from the DEAE-Toyopearl 650S chromatography shown in Figure 2, were applied on a column (1.3 × 105 cm) of Bio-Gel P-4 and eluted with 0.1 M NaCl. 1.1-mL fractions were collected and monitored by the absorbance at 230 nm (●) and at 549 nm (○) as described in Figure 2. Vo, void volume. The fractions of a-1, a-2, and d-1 were pooled as indicated.

Table 2: Methylation Analysis of a-2 and d-1 and Their KDN-Depleted Forms

	a-2	KDN-depleted a-2	d-1	KDN-depleted d-1
2,3,4-tri-*O*-Me-Fuc	0.52	0.57	0.21	+[a]
3,4,6-tri-*O*-Me-Man	2.3	1.6	1.0	1.0
2-*O*-Me-Man	0.89	0.75	0.94	1.1
2,4-di-*O*-Me-Man	0.19	0.18		
3,6-di-*O*-Me-Man			0.94	1.1
2,4,6-tri-*O*-Me-Gal	2.0		2.5	
2,3,4,6-tetra-*O*-Me-Gal		2.0		2.8
3,6-di-*O*-Me-GlcN(Me)Ac	3.8	3.7	5.3	4.5
3-*O*-Me-GlcN(Me)Ac	0.53	0.53	+	0.42
3,4,6-tri-*O*-Me-GlcN(Me)Ac	0.50	0.62	0.70	0.72

[a] Plus sign (+) indicates not quantitated because of the overlapping nature of the partially methylated alditol acetates in question with unidentified contaminating materials.

a free glycan. This indicated that a-1 was possibly resulting from the incomplete hydrazinolysis of KDN-gp. The amino acid composition of a-1 was determined as included in Table 1, and the NH_2-terminal analysis showed Ala. One-dimensional proton NMR spectra of a-1 and a-2 displayed the superimposed resonances, except that the proton resonanes due to the reducing terminal GlcNAc[1] in a-2, including H-1 (δ 5.19 ppm) in the α-configuration, were not seen in a-1, and the most obvious differences were the presence of the H-1 resonance (δ 5.04 ppm) of the Asn-bound GlcNAc residue and the Asn methylene and Ala methyl proton resonances in a-1 at 2.83, 1.38, and 1.44 ppm in the area ratios of 2:3:3, respectively. Combined data obtained by carbohydrate composition analysis, methylation analysis, and 400-MHz ^{1}H NMR spectroscopy indicated that both a-1 and a-2 have the identical biantennary glycan structure.

(ii) d-1. The elution position from a DEAE-Toyopearl 650S column (Figure 1) indicated the presence of a triKDNosylated structure. d-1 was also formed by the incomplete hydrazinolysis of KDN-gp (Table 1). The sugar analysis supported this indication, showing approximately three residues of KDN per three Man residues (Table 1). Thus, the triantennary structure appeared to be fully substituted by KDN. The methylation analysis showed Man derivatives (3,6-di-*O*-Me-Man, 2-*O*-Me-Man, and 3,4,6-tri-*O*-Me-Man) commonly found in bisecting GlcNAc-substituted triantennary types of structures (Table 2). d-1 was partially hydrolyzed to remove the KDN residues. The KDN-depleted d-1 was then subjected to methylation analysis (Table 2). By this procedure, the KDN residues were exclusively linked to the 3-position of Gal. d-1 contained a small amount of Fuc, and about 40% of these triantennary oligosaccharide molecules appeared to be fucosylated. The methylation analysis was consistent with the presence of a 4,6-di-O-substituted GlcNAc residue, indicating the substitution of this residue in the 6-position by Fuc. By analogy with a-1, d-1 was anticipated to have an α1→6-linked L-fucose residue at the Asn-bound GlcNAc, and evidence for this structure was provided by ^{1}H NMR spectroscopy (Table 3). The anomeric proton signals at 4.62 and 4.66 ppm, whose peak areas are comparable, were attributable to the GlcNAc[2] H-1 of the glycan chains, one substituted at the Asn-bound GlcNAc by an α1→6-linked Fuc residue and the other not fucosylated on the GlcNAc[1] residue, respectively. These assignments were based on previous literature values summarized by Vliegenthart et al. (1983); a CH_3 doublet at 1.22 ppm attributable to a Fuc residue-linked α1→6 to the reducing terminal GlcNAc[1] was in close agreement with previously reported data. Considering all the data, d-1 was concluded to be an almost equimolar mixture of the two molecular species, one fucosylated and the other not fucosylated as shown in Figure 5.

DISCUSSION

The results presented here showed the occurrence of the KDN residues on N-linked glycan units in KDN-gp. The N-linked oligosaccharides consisted of bi- and triantennary complex-type structures. Most of them contained a bisecting GlcNAc residue (80–90%) in the β1→4 linkage to the β-linked Man residue of the core, and about half of them were fucosylated at C-6 of the proximal Asn-linked GlcNAc residue.

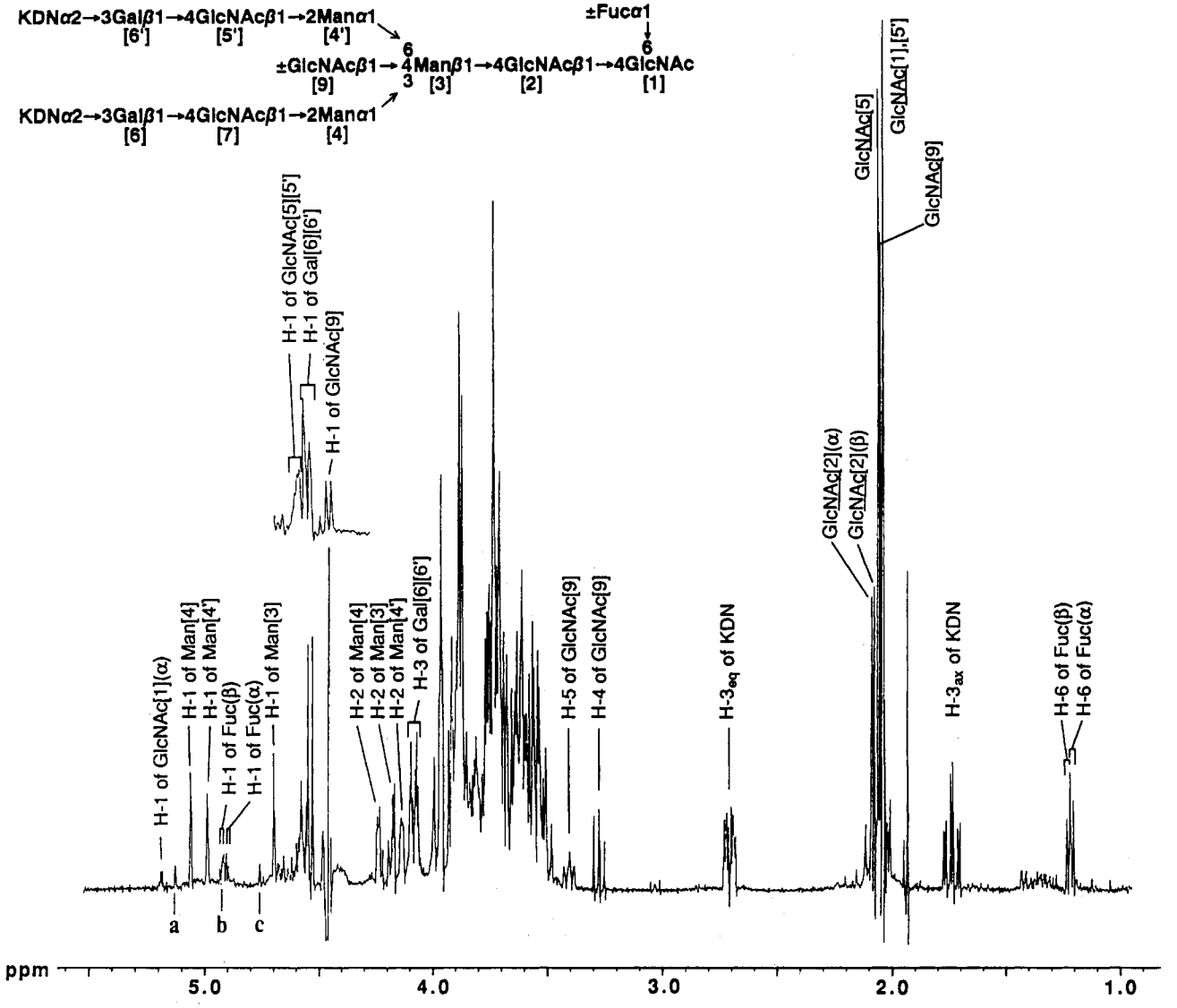


FIGURE 4: 400-MHz ^1H NMR spectrum of a-2 in D_2O at 50 °C. The spectrum in the region of 4.3–4.7 ppm at 25 °C is also shown. The finally established structure and residue numbering are also included. Signals indicated by a, b, and c are respectively assignable to anomeric protons of Man[4], Man[4'], and Man[3] residues in the glycan devoid of a bisecting GlcNAc residue.

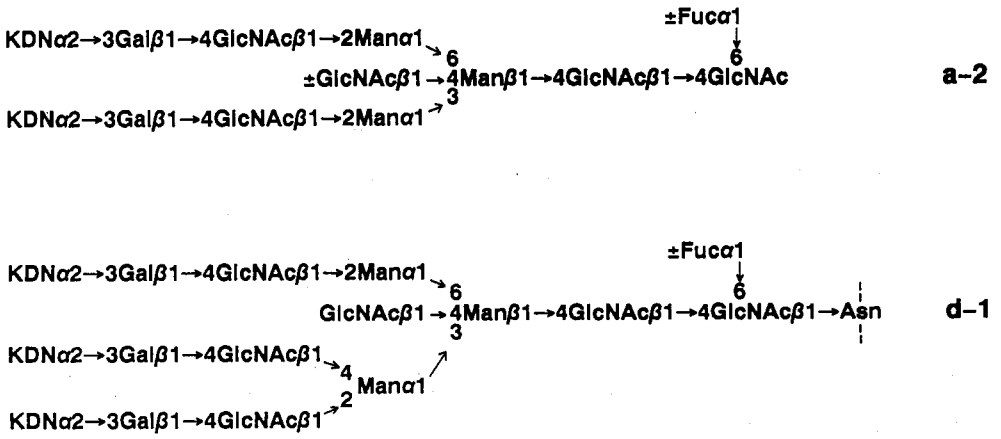


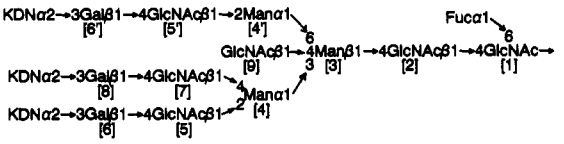
FIGURE 5: Proposed structures of KDN-containing N-linked oligosaccharides a-2 and d-1.

The Gal residues of both bi- and triantennary structures were fully substituted by KDN in a form of KDNα2→3Galβ1→(Figure 5).

Although the occurrence of O-linked KDN-containing glycan chains in glycoproteins had been identified in several animal species including fish (Inoue et al., 1988; Iwasaki et al., 1987, 1990; Kanamori et al., 1989, 1990; Nadano et al., 1986) and amphibians (Strecker et al., 1990, 1992a,b), this is the first report of the occurrence and structure of N-linked KDN-containing glycan units present in glycoproteins. When one combines the previous findings of the occurrence of KDN-containing glycosphingolipids (Yu et al., 1991, 1993) and KDN-containing capsular polysaccharides (Knirel et al., 1989), the results of this study can be considered to imply the

general existence of KDN in a wide variety of glycoconjugates as presently known for sialic acid.

KDN-rich glycoprotein (KDN-gp) derived from rainbow trout vitelline envelope is a unique glycoprotein in which KDN comprises more than 50% of the weight of the molecule. KDN-gp is therefore a highly charged polyanionic and very polydisperse (10^6 to 4×10^6 Da; $<M_r>_{av}$, 2.9×10^6). Previously, we demonstrated that KDN-gp is heavily O-glycosylated, and each molecule was believed to possess on average nearly 1000 O-linked oligoKDN-containing glycan chains (Inoue et al., 1988; Kanamori et al., 1990) attached laterally to the polypeptide core, so that carbohydrate comprises about 80% of the weight of the molecule:



Our earlier analytical study of KDN-gp indicated the presence of a minute amount of N-linked glycan units in the KDN-gp (Inoue et al., 1988). In this study, the N-linked oligosaccharides were released by hydrazinolysis and separated from serine- or threonine-linked glycan chains, which were also released by this treatment by combination of anion-exchange chromatography and gel filtration. Although the yield of N-linked oligosaccharides (17 mg/g of KDN-gp) appeared to be at least 1 order of magnitude lower than that of O-linked oligosaccharides, it was necessary to isolate enough quantities of the N-linked oligosaccharides to biochemical purity for determination of their detailed structure by the chemical methods. Such information will be required for further studies on their biosynthetic mechanisms. The most important result obtained in this study is the finding that KDN-gp also contains N-linked glycan chains.

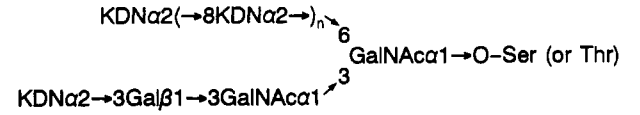
The proportion of N-linked complex-type glycan chains we have found to the total KDN-glycan chains of the KDN-gp molecule is small, *viz.* about 6%. Thus, the current data demonstrate that a single vitelline envelope KDN-gp contains complex-type N-linked KDN-glycan chains and a multiple number of O-linked KDN-glycan units. The unusually high apparent molecular weight of KDN-gp is due to the presence of a number of O-linked oligosaccharides closely spaced on the polypeptide backbone of the KDN-gp. This characteristic feature of the structure of KDN-gp is apparently very similar to major sialoglycoproteins of human hematopoietic cells such as glycophorin A (the major sialoglycoprotein of erythrocytes) and leukosialin or leukophorin (the major sialoglycoprotein of leukocytes) in having a large number of O-glycosidic oligosaccharides with a limited number of N-glycosidic chains.

Although the physiological roles of such heavily O-glycosylated sialoglycoproteins have remained poorly understood, it may be assumed that this class of sialoglycoproteins normally functions as a receptor for growth or differentiation factors (Gahmberg et al., 1988). It can be also assumed that the O-glycosidic glycan chains play an important role for the putative cell surface receptor functions, as is the case for glycophorin A, human low density lipoprotein (LDL) receptor (Cummings et al., 1983), and interleukin-2 receptor (Leonard et al., 1984; Nikaido et al., 1984). In fact the LDL receptor contains a cluster of O-glycosidic oligosaccharides near the membrane (Cummings et al., 1983) and the interleukin-2 receptor contains a large number of O-glycosidically linked oligosaccharides (Leonard et al., 1984; Nikaido et al., 1984). A group of mucin-like sialoglycoproteins are known to be present in cell surfaces directly associated with the plasma membranes such as episialin (Hilkens et al., 1992), ascites sialoglycoprotein 1 (Carraways & Hull, 1991), and cancer-associated mucins and mucin-type glycoproteins [for a review, see Bhavanadan (1991)].

Table 3: Chemical Shift Values of Selected Proton Signals in 400-MHz ^{1}H NMR Spectra of a-2 and d-1[a]

	residue	a-2	d-1
H-1	GlcNAc[1]	5.19(α)	nd[b]
	GlcNAc[2]	4.66,4.62[c]	4.66,4.62[c]
	Man[3]	4.70[4.77][d]	4.70
	Man[4]	5.07[5.12]	5.07
	Man[4']	5.01[4.92]	5.00
	GlcNAc[5]	4.59	4.58
	GlcNAc[5']	4.58	4.58
	Gal[6]	4.56	4.55
	Gal[6']	4.55	4.55
	GlcNAc[7]		4.53
	Gal[8]		4.55
	GlcNAc[9]	4.47 (25 °C)	4.47 (25 °C)
	Fuc	4.90(α)	nd[b]
		4.91(β)	
H-2	Man[3]	4.18[4.25]	4.15
	Man[4]	4.25[4.20]	4.28
	Man[4']	4.15[4.11]	4.15
H-3	Gal[6]	4.11	4.11
	Gal[6']	4.11	4.11
	Gal[8]		4.11
H-3ax	KDN	1.76	1.75
	KDN	1.75	1.75
	KDN		1.75
H-3eq	KDN	2.72	2.72
	KDN	2.71	2.72
	KDN		2.71
H-4	GlcNAc[9]	3.29	3.29
H-5	GlcNAc[9]	3.42	3.42
	Fuc	4.09(α)	4.1[e]
		4.12(β)	
CH_3	Fuc	1.23(α)	1.22
		1.24(β)	
NAc	GlcNAc[1]	2.05	2.05
	GlcNAc[2]	2.10(α)	2.09
		2.09(β)	
	GlcNAc[5]	2.07	2.07
	GlcNAc[5']	2.05	2.05
	GlcNAc[7]		2.07
	GlcNAc[9]	2.06	2.06

[a] Chemical shift values are given in ppm at 50 °C as referenced to the sodium 2,2-dimethyl-2-silapentane-5-sulfonate methyl signal set to 0 ppm. Numbering of the monosaccharide residues is shown below. α and β represent the anomeric configuration of GlcNAc[1].



[b] The H-1 signals of GlcNAc[1] and Fuc were observed at ~5.05 and ~4.9, respectively. [c] Values for the GlcNAc[2] residue in the unfucosylated glycan unit. [d] Values in [ ] represent chemical shifts for the glycans not having a bisecting GlcNAc residue. [e] The signal was observed at ~4.1 ppm but partially overlapped by the signals of Gal H-2's.

A possible physiological role for KDN-gp in the sperm–egg interaction has been suggested by the sperm binding properties of this glycoprotein, and the transenvelope nature of KDN-gp has been discussed previously in this context (Kanamori et al., 1990; Inoue, 1993). Immunohistochemical methods revealed that KDN-gp molecules are localized around the micropyle of the unfertilized egg and the second layer of the surface of vitelline envelope (Inoue et al., 1993). It was also observed that FITC-labeled KDN-gp specifically bound to sperm surface (Inoue et al., 1993) where we found KDN-containing glycosphingolipid, (KDN)GM3 (Yu et al., 1991). Whether or not the interaction between KDN-gp and (KDN)-GM3 is actually related to egg and sperm interaction awaits

further studies. Nevertheless, because of their marked tendency to associate most likely through carbohydrate–carbohydrate interaction, our current efforts are being directed toward an understanding of the possible biological role of these gamete cell surface-derived KDN-glycoconjugates. It is interesting to point out that the N-linked fucosylated and bisecting GlcNAc-substituted bi- and triantennary glycan chains found on KDN-gp are essentially similar to those found on the human erythrocyte glycophorin A molecule and on some other glycoproteins (Yoshima et al., 1980). In spite of the rarity of the N-linked glycan chains when compared to the O-linked glycan units in these molecules, they could have specific functions that are distinct from those mediated by the more prevalent O-linked glycan chains. Thus, the role of such N-linked complex-type sugar chain(s) in cell surface highly acidic glycoprotein functions will have to continue to be an important subject of study.

In addition to such studies, our current efforts have been directed toward an understanding of the mechanism of the biosynthetic incorporation of KDN residues in KDN-glycoconjugates (Terada et al., 1993a,b). In KDN-gp there are four different types of KDN linkages, i.e., KDNα2→3Galβ1→ in N-glycan units and KDNα2→3Galβ1→, KDNα2→6GalNAcα1→, and KDNα2(→8KDNα2→)$_n$ in *O*-glycan chains. In analogy with the biosynthesis of the sialic acid residues, it is likely that the KDN residue is incorporated into a glycan chain according to the following pathways: KDN + CTP → CMP-KDN + PPi (reaction I) and CMP-KDN + acceptor glycan → KDN-glycan + CMP (reaction II). In support of this view, recently we have shown the occurrence of CMP-KDN synthetase, which catalyzes reaction I, in testis of rainbow trout (Terada et al., 1993a). Most recently, we have identified and characteristized CMP-KDN: KDN-transferases, which have different acceptor specificities and are necessary for the biosynthesis of different KDN-glycoconjugates in trout testis and ovary (Terada et al., 1993b).

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