

Diversity in sialic and polysialic acid residues and related enzymes*

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Abstract: The occurrence of extensive diversity in the family of sialic acids is now well recognized but biological significance of this diversity has been clarified only partially. In 1986 we showed natural occurrence of deaminated form of neuraminic acid, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN) as a capping residue of polysialyl chain of rainbow trout egg polysialoglycoprotein. Subsequent studies of our own and by others showed ubiquitous occurrence of KDN from bacteria to human although expression of this residue in large amounts was limited to certain species of animal. KDN was different from other 'families' of sialic acids usually denoted as modified *N*-acetylneuraminic acids of which *O*-acetyl substitution is most frequently found. The definition of sialic acid has to be changed by the finding of KDN.

Prior to the finding of poly *N*-acetylneuraminic acid group in mammals, in 1978 we discovered poly *N*-glycolylneuraminic acid in rainbow trout eggs. This was the first report of the occurrence of polysialic acid structure in animal glycoproteins. We also found the occurrence of polyKDN chains. All these findings made significant contribution to show that diversity of sialic acid family and especially diversity in polysialic acid structure are much more extensive and more ubiquitous than generally believed before. We showed that diversity of polysialic acid is not only originated from the diversity in building block but also the type of interresidue linkage. The diversity of sialic acid and polysialic acid is reflected in diversities in molecules that recognize these structures. We studied some of these molecules that specifically recognize different types of sialic acids and polysialic acids, and biosynthetic mechanism of polysialic acids.

INTRODUCTION

More than 40 compounds are now counted as naturally occurring members of sialic acids (Sia). In polysialic acids (polySia), naturally occurring polymeric forms of Sia, a large number of new structures has been reported in the past several years. The occurrence, structure, biochemistry and possible physiological significance of diversity found in Sia and polySia have been documented in excellent review articles [1–5]. In this article we will present our contribution in this area of studies through the discovery of new family of sialic acid (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid; KDN) [6], and identification of diverged numbers of polySia structures originated from not only the difference in monosialic acid structure [7–12] but also new linkage type [13,14] and specific sulfation [14–16]. In view of the importance of the discovery of KDN as a compound not derived by mere modification of known Sia, our recent studies on the identification of this residue in human cells and tissues will be emphasized [17]. We also address our view on the mechanism of polysialylation in animal cells based on our own structural studies.

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DIVERSITY IN SIALIC ACID RESIDUES

Sia are a family of 9-carbon carboxylated sugars having the nonulosonate skeletal structure (Fig. 1). More than 40 different molecular species of Sia are known to occur in Nature and the most common sialic acids are Neu5Ac ($R = -\text{NHCOCH}_3$ in Fig. 1) and Neu5Gc ($R = -\text{NHCOCH}_2\text{OH}$ in Fig. 1). The most recent example is 9-*O*-sulfated Neu5Gc in the egg receptor for sperm in sea urchin [14,15]. These residues are generally found at the terminal position on vertebrate oligosaccharide chains of a variety of glycoconjugate molecules, and are known often to play key roles in biological events. Sia residues are also present in glycosphingolipids and glycoproteins as their oligomeric and polymeric forms with different degree of polymerization (DP). Here, a special emphasis is placed on the structural features of Sia and polySia epitopes, and we will try to highlight recent progress in our nascent understanding of some of the unusual forms of sialylation and polysialylation.

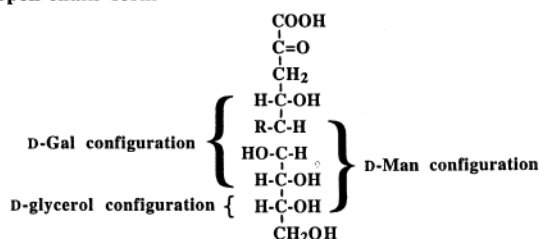
Sialic Acids (Sia)

Neu5Acyl ($R = -\text{NH-Acyl}$) and KDN ($R = -\text{OH}$)

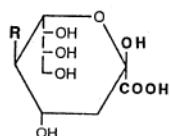
Neu5Acyl: 5-Acylamido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid

KDN: 2-Keto-3-deoxy-D-glycero-D-galacto-nononic acid

(A) open-chain form



(B) pyranoid form in Haworth formula



(C) chair conformation

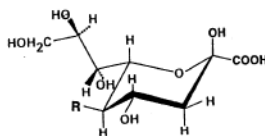


Fig. 1 Structure of sialic acid (free Sia in its hemiketal form is present as an equilibrium mixture of α - and β -anomers, the latter being the major form): (A) open-chain form; (B) pyranoid form in Haworth formula; (C) chair conformation. $R = -\text{NHAcyl}$, 5-acylamido-3,5-dideoxy-D-glycero-D-galacto-nonulosonic acid (Neu5Acyl); $R = -\text{OH}$, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN).

During our studies on fish egg polysialoglycoproteins (PSGP), in 1986 we reported the unexpected finding of the occurrence of KDN residues [6]. As shown in Fig. 1, the stereochemistry of KDN is identical with that of Neu5Acyl and only difference is the replacement of *N*-acyl groups by a hydroxyl group, so that we added KDN and its derivatives as a new family of Sia. Subsequently, the presence of the KDN residues was found in our and other laboratories in both *O*- and *N*-linked glycan chains of glycoproteins [9–11,18–24] and in bacterial polysaccharide chains [25,26]. We also found a series of KDN-containing glycosphingolipids [27–29]. As we anticipated, the list is continuously growing, and more recently we have identified KDN as a minor component in mammalian tissues and cells including human [30], and showed the elevated expression of KDN in fetal cord blood and ovarian cancer cells of human as is discussed later [17].

KDN appears not to be formed from Neu5Ac but a separate route is more likely responsible for biosynthesis of this new type of Sia [31,32]. Thus, Neu5Ac and KDN are the independent metabolic precursors of a diverged number of modified Sia (for KDN see, for example [33,34]). Such diversity in the

structure of Sia may be relevant to their individual roles in biological functions. We have already begun to learn that minor modifications of carbohydrates often play major biological roles. In this regard, we should pay more attention to their natural occurrence, structure, biosynthesis and functional significance as was previously pointed out [35].

DIVERSITY IN POLYSIALIC ACID CHAINS

It should be emphasized that the first finding of polySia structure in eukaryotic cells was made in 1978 by one of us (S. Inoue) [7,8]. She discovered the occurrence of $\alpha 2 \rightarrow 8$ -linked oligo/polyNeu5Gc groups in PSGP derived from rainbow trout eggs and actually this represents the first discovery of the occurrence of polySia glycotopes in animals. Prior to her discovery three different polySia structures had been identified as the neuroinvasive bacterial capsular polysaccharides. All of these are homopolymers of Neu5Ac, i.e. $(\rightarrow 8\text{Neu5Ac}\alpha 2 \rightarrow)_n$, $(\rightarrow 9\text{Neu5Ac}\alpha 2 \rightarrow)_n$, and $(\rightarrow 8\text{Neu5Ac}\alpha 2 \rightarrow 9\text{Neu5Ac}\alpha 2 \rightarrow)_n$. Subsequent to her finding, an increasing number of polySia-containing glycoproteins has been identified in a wide variety of animal species ranging from insect to human (see for review [2,4,5]). Thus, it can now be said that the $\alpha 2 \rightarrow 8$ -linked polySia glycotopes occur in glycoproteins from a variety of living species ranging from bacteria to human. Particularly, a remarkable degree of progress has been made since polySia chains were shown to be expressed on the embryonic form of neural cell adhesion molecule (N-CAM) in developmentally regulated manner [36–38].

Subsequent studies from our laboratories have demonstrated the diversity of polySia (Fig. 2). As anticipated, in 1990 we found a polymeric form of KDN in KDN-rich glycoprotein isolated from vitelline envelope of trout eggs and the structure of $\alpha 2 \rightarrow 8$ -oligo/polyKDN was established. We also found a new type of polySia chains in polySia-gp isolated from the extract of the jelly coat of sea urchin eggs [13]. In the latter, the interketosidic linkages were elucidated by analysis of the product obtained by strong alkali (2N NaOH) treatment and subsequent *N*-acetylation. Strong alkali treatment of polyNeu5Gc chains in polySia-gp depolymerized and gave rise to Neu5Ac $\alpha 2 \rightarrow \text{OCH}_2\text{COOH}$ upon *N*-acetylation (Fig. 3), whereas $\alpha 2 \rightarrow 8$ -linked polyNeu5Acyl would not cause depolymerization but give rise to $\alpha 2 \rightarrow 8$ -linked polyNeu5Ac upon re-*N*-acetylation because the inter-residue linkages are stable to strong alkali. Combining chemical, biochemical and instrumental methods, we have established a complete chemical structure as illustrated in Fig. 4. These polySia chains found in sea urchin eggs are thus a homopolymer of Neu5Gc unusually glycosidically linked through the glycolyl group. Therefore, until now there exist at least five distinct forms of polySia structures in animal-derived polysialoglycoproteins as shown in Table 1. In addition to these homopolymers structural studies also revealed the occurrence of $\alpha 2 \rightarrow 8$ -linked copolymer of Neu5Ac and Neu5Gc [12]. There is also considerable oligo/polydispersity in the length of the polySia chains. Diversity in the structure of Sia and polySia strongly indicates a broad

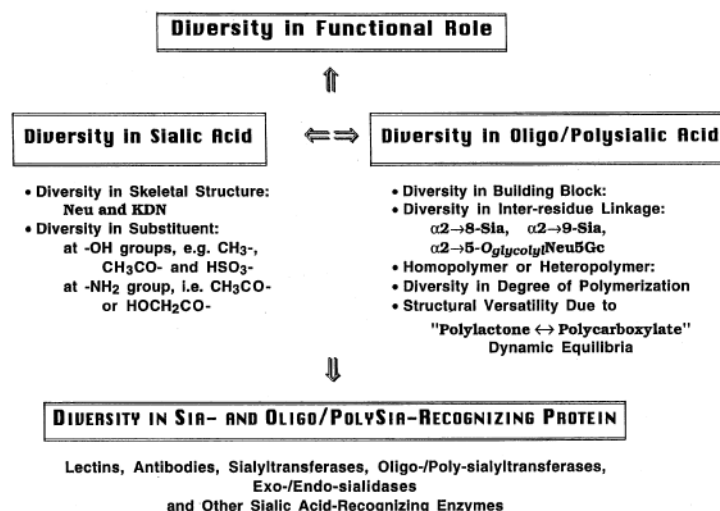
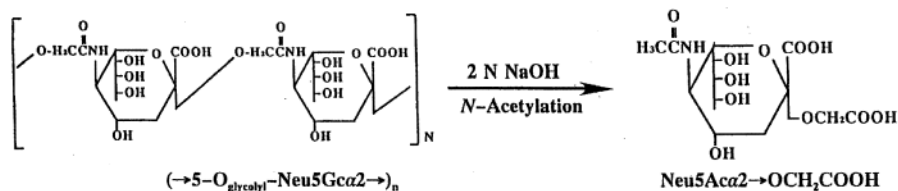


Fig. 2 Interrelationship of diversities in Sia, polySia and their recognizing proteins.

(A)



(B)

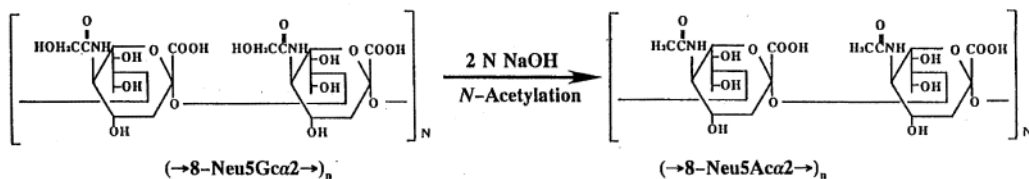
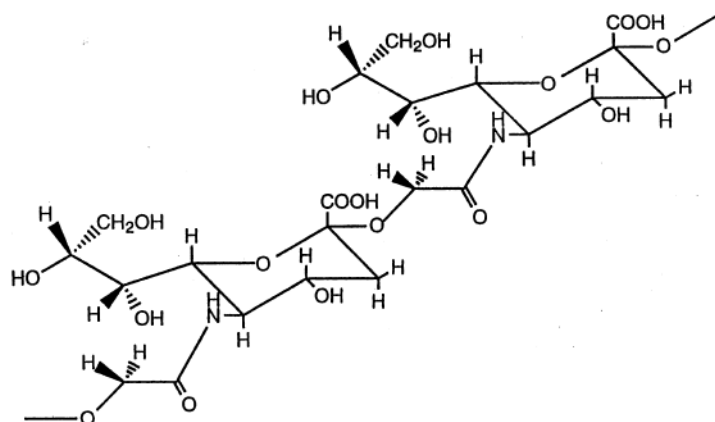
Alkali treatment: 2 N NaOH containing NaBH_4 , 105°C, 6hN-Acetylation: Acetic anhydride in saturated NaHCO_3 solution

Fig. 3 Strong alkaline treatment of a pair of linkage isomers of Neu5Gc homopolymer (A) $(\rightarrow 5\text{-O}_{\text{glycolyl}}\text{-Neu5Gca2}\rightarrow)_n$ and (B) $(\rightarrow 8\text{-Neu5Gca2}\rightarrow)_n$.



$\alpha 2 \rightarrow 5\text{-O}_{\text{glycolyl}}\text{-linked polyNeu5Gc}$

Fig. 4 Structural representation of a novel type of polySia chains established for those present in polySia-gp isolated from the egg jelly coat of sea urchin.

Table 1 Molecular diversity in polySia chain structures and their partially acetylated forms reported for animal-derived glycoproteins

- | | |
|---|--|
| (A) Trout egg (Cortical alveolar glycoproteins; S. Inoue, 1978, 1980) | $\alpha 2 \rightarrow 8\text{-linked polyNeu5Gc}$; |
| (B) Rat brain (pronase digests of delipidated brain; [36]) | $\alpha 2 \rightarrow 8\text{-linked polyNeu5Ac}$ |
| (C) Trout egg (cortical alveolar glycoproteins; [12]) | $\alpha 2 \rightarrow 8\text{-linked poly(Neu5Ac, Neu5Gc)}$ |
| (D) Rainbow trout egg (vitelline envelope glycoprotein; [11]) | $\alpha 2 \rightarrow 8\text{-linked polyKDN}$ |
| (E) Sea urchin egg (jelly coat glycoprotein; Egg receptor for sperm; [13–15]) | $\alpha 2 \rightarrow 5\text{-O}_{\text{glycolyl}}\text{-linked oligo/polyNeu5Gc}$ |

spectrum of functional versatility of these cell surface components. We believe that new polySia isoforms, i.e. lactones, indicate that dynamic equilibria *in vivo* may possibly contribute to both structural and functional versatility.

During our collaborating work with Prof. Lennarz's laboratory, we identified that oligoNeu5Gc chains present in the sea urchin egg receptor for sperm are capped by the chain terminating signal, 9-*O*-sulfated Neu5Gc residues (Fig. 5B; [14,15]). And these 9-*O*-sulfated Neu5Gc capping residues appear to have multiple roles such as: (a) protection of oligo/polyNeu5Gc from the attack by bacterial sialidases, (b) prevention of further chain extension, and (c) facilitation of lactone formation of the inner part of oligo/polyNeu5Gc. The sulfated oligoSia chains were found to inhibit fertilization in a dose-dependent manner, showing that these sulfated oligoSia chains participate in an initial sperm-egg binding event [15].

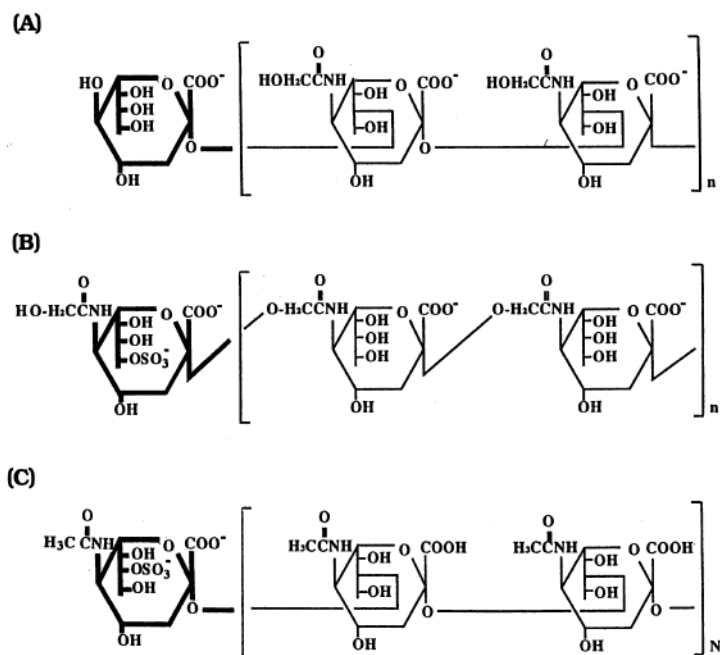


Fig. 5 Three different nonreducing terminal residues identified for chain termination signals for polysialyltransferases: (A) KDN, (B) 9-*O*-sulfated Neu5Gc, and (C) 8-*O*-sulfated Neu5Ac residues.

Until now, we have identified three examples of terminal capping of oligo/polySia chains which serve as chain termination signals for polysialyltransferases: the nonreducing terminal KDN [6], 9-*O*-sulfated Neu5Gc [15] and 8-*O*-sulfated Neu5Ac [16] residues as shown in Fig. 5. *O*-Acetylation at the non-reducing terminal Sia residues could also be a possible candidate for a termination signal, although its identification in a long polySia chain must be greatly hampered by the lability of ester group.

UNIQUE PROPERTIES OF OLIGO/POLYSIALIC ACID EPITOPES

It has become realized that sialic acid residues tend to exhibit unusual chemical and biochemical properties upon polymerization, particularly on going from lower oligomers to highly polymerized form. Basic physicochemical studies such as determination of precise values of ionization constant of the carboxyl groups of oligo/polySia and determination of linkage conformation of oligo/polySia chains besides α 2 $\rightarrow$ 8-linked Neu5Ac are needed to understand the chain-length dependent properties of oligo/polySia chains such as lability of α 2 $\rightarrow$ 8-linked Neu5Ac linkages under mild acidic conditions by accelerated spontaneous depolymerization of polySia chains (e.g. [39,40] for intrinsic difference in reactivity of different interresidue linkages, see [41]) and the conformational versatility. Perhaps, it is also interesting to study change in avidity of oligo/polySia toward divalent cations with DP.

The net negative charges of a polySia chain can be reduced by intramolecular esterification even though the number of sialic acid residues remains unchanged. In the above and other contexts it can be

conceivable that the possible presence of dynamic equilibria between multiple forms of partially lactonized polySia formed either by spontaneous nonenzymatic reaction or by an enzyme (PSA-lactonase)-catalyzed reversible lactonization of polySia chains *in vivo*, thereby possibly mediating protein ligand–receptor interactions. Biological significance of lactone formation in oligo/polySia chains particularly *in vivo* should be paid more attention.

We have studied Ca^{2+} binding properties of three different types of oligo/polysialic acid chains by equilibrium dialysis using ^{45}Ca as a tracer and circular dichroism [42]. Analysis by equilibrium dialysis of the binding of Ca^{2+} to colominic acid was biphasic, and the high-affinity interaction was shown to change with the degree of polymerization [42].

DIVERSITY IN SIA- AND POLYSIALIC ACID-RECOGNIZING PROTEINS – LECTINS, SIALIDASES, TRANSFERASES, ANTIBODIES, AND MODIFYING ENZYMES

As you can see from Table 2, there are a large number of Sia/polySia-recognizing proteins [3]. Here we will discuss only some of the unusual examples. Exo-sialidases are a class of glycosyl hydrolases that release terminal sialic acid residues (*N*-acylneuraminate or KDN) from glycoproteins, glycolipids, and polysaccharides. Although the substrate specificity is relatively broad, most of the commercially available bacteria-derived sialidases were found not to cleave KDN ketosidic linkages. Enzyme catalyzing the hydrolytic removal of KDN residues from their α -ketosidic linkage was anticipated to be an important reagent to complement immunochemical probes in identification of KDN-glycoconjugates and studying their function. We found a KDNase-inducing microorganism, *Sphingobacterium multivorum* [43,44]. This bacterium induced KDNase that hydrolyzes specifically KDN-ketosidic linkage but not *N*-acylneuraminyl linkages (Table 3). We were successful in purification of this enzyme to homogeneity and designated the purified enzyme 'KDNase Sm'. The specific term, KDNase, is defined as a new class of sialidase which is devoid of *N*-acylneuraminidase activity, so that KDNase should be differentiated from 'KDN-sialidase', the enzyme hydrolyzes not only KDN-but also Neu5Acyl-linkages (e.g. [45]). Because KDNase Sm is a new type of sialidase, we carried out a quantitative investigation of the equilibria and kinetics involved in KDNase Sm-catalyzed hydrolysis by the steady-state enzyme kinetics and ^1H NMR spectroscopy of 4-methylumbelliferyl-KDN (KDN α 2MeUmb) [46]. ^1H NMR spectroscopic studies clearly demonstrated that the thermodynamically less stable α -form is preferentially formed as the first product of the cleavage reaction, and that isomerization rapidly follows, leading to an equilibrium mixture of the two isomers; the β -isomer being the major species at equilibrium.

Table 2 Diversity in Sia-/polySia-recognizing proteins

-
- | | |
|---|---|
| 1 | Sia-/polySia-specific lectins (Limulus, Limax, Scylla lectins, etc.) |
| 2 | Sia-/polySia- degrading enzymes (exo- and endo-sialidases) |
| 3 | Sia-/polySia-forming enzymes (CMP-Sia synthetase; sialyltransferases; polysialyltransferases) |
| 4 | Sia-/polySia-specific antibodies |
| 5 | Sia-/polySia-modifying enzymes (acety-CoA-Sia-/polySia- <i>O</i> -acetyl-transferases; S-adenosylmethionine-Sia-/polySia- <i>O</i> -methyltransfer-ases; CMP-N-acetylneuraminic acid monooxygenase; PAPS-Sia- <i>O</i> -sulfotransferases; lactonases(?); esterase) |
-

Biological importance of modifications of the parent sialic acid has been demonstrated or suggested in a number of reports. For example, 9-*O*-acetylation of sialic acids has significant effects on bindings with enzymes, viruses and other bioactive proteins (e.g. [35]). It is thus interesting to identify specific enzymes which are responsible for formation of KDN-glycan chains. *N*-Glycolylneuraminic acid probably cannot be synthesized in normal human man tissues and cells. Evidence for a role of the multiple forms of sialic acids as differentiation antigens is also accumulating [47,48].

EVIDENCE FOR THE OCCURRENCE OF KDN AND KDN RESIDUES IN MAMMALS

Antibodies against a variety of oligo/polySia chains are useful immunochemical probes to detect their

Table 3 Substrate specificity of affinity-purified KDNase Sm toward various deaminoneuraminyl and *N*-acetylneuraminyl linkages

Compound	Linkage	Cleavage
4-MU-KDN	KDN α 2→Me-umbelliferyl	+
KDN dimer	KDN α 2→8KDN	+
di-KDN-biantennary <i>N</i> -glycan	KDN α 2→3Gal	+
KDN-oligosaccharide alditols	KDN α 2→8KDN, KDN α 2→3Gal, KDN α 2→6GalNAcol	+
KDN-gp	KDN α 2→8KDN, KDN α 2→3Gal, KDN α 2→6GalNAcol	+
(KDN) G_{M3}	KDN α 2→3Gal	+
4-MU-Neu5Ac	Neu5Ac α 2→Me-umbelliferyl	—
Neu5Ac dimer	Neu5Ac α 2→8Neu5Ac	—
Neu5Gc dimer	Neu5Gc α 2→8Neu5Gc	—
<i>N</i> -acetylneuraminylactose	Neu5Ac α 2→3(6)Gal	—
di-Neu5Ac-biantennary <i>N</i> -glycan	Neu5Ac α 2→3Gal	—
Neu5Ac α 2→6(Gal β 1→3)GalNAcol	Neu5Ac α 2→6GalNAcol	—
human transferrin	Neu5Ac α 2→6Gal	—
fetal calf serum fetuin	Neu5Ac α 2→3(6)Gal	—
porcine submaxillary mucin	Neu5Gc α 2→6GalNAc	—
toad egg jelly glycoprotein	Neu5Ac α 2→6GalNAc	—
colominic acid	(→8Neu5Ac α 2→) _n	—
PSGP(<i>Sn</i>)	(→8Neu5Ac α 2→) _n	—
PSGP(<i>Om</i>)	(→8Neu5Gc α 2→) _n	—
PSGP(<i>Slp</i>)	(→8Neu5Ac α 2→, →8Neu5Gc α 2→) _n	—
sea urchin egg jelly polySia-gp	(→ <i>O</i> _{glycolyl} Neu5Gc α 2→) _n	—
(Neu5Ac) G_{M3}	Neu5Ac α 2→3Gal	—
(Neu5Ac) G_{M1}	Neu5Ac α 2→3(Gal β 1→3GalNAc β 1→4)Gal	—

occurrence in animal cells and tissues. Several different mono- and polyclonal antibodies are now available (see, e.g. [49]). However, characterization of their antigen specificities was prerequisite for their use as reliable probes. We determined the immunospecificity of each of the available antioligo/polySia antibodies by the ELISA method using lipidated oligo/polySia samples [49]. Immunochemical probes for new glycotopes, KDN, were needed for detection of KDN-glycan chains expressed in very minute amounts in cells. So far, we developed two different monoclonal antibodies, mAb.kdn3G and mAb.kdn8kdn, which specifically recognize KDN α 2→3Gal β 1→ and α 2→8-linked oligoKDN, respectively [50,51]. The two useful reagents, mAb.kdn8kdn and KDNase Sm, were used in our collaborating studies for immunohistochemical detection of α 2→8-linked oligoKDN epitopes in different organs of rat [51,52]. The results obtained were the first indication of the presence of KDN residues in mammalian tissues. What remained to be determined was the unambiguous demonstration of the occurrence of KDN in mammalian species. This rare carbohydrate component was isolated from pig submaxillary gland, and chemical and instrumental methods were used for unequivocal assignment of the KDN residues in mammals [17,30]. Thus, we became now quite confident in use of the highly sensitive fluorescent-assisted HPLC method for detecting an extremely small amount of KDN residues expressed in mammals, particularly in human. The sensitive chemical probe for tagging Sia is 1,2-diamino-4,5-methylenedioxybenzene (DMB; [53]) which produces fluorophoric derivatives, and analysis of the products by HPLC allowed us to separate, identify and quantitate KDN and other Sia at picomole level.

Because, in our previous study [30,52], KDN was shown to be expressed in all of human lung cancer cell lines examined, we initiated a systematic survey of KDN in white blood cells, blood plasma, ascites fluid, and ascites cells of patients of different types of cancer. The objectives of these studies are two-fold: one is to develop a possible utilization of levels of KDN as a reliable and useful method for monitoring

and diagnostic purposes; the other is to understand the molecular mechanism underlying such regulatory expression of KDN or KDN residues by quantitative determination of the expression level of KDN and identifying enzymes involved in the KDN formation. It is of interest to point out a markedly elevated expression of KDN relative to Neu5Ac in ascites cells [17]. These preliminary data showed that levels of KDN are somewhat related to malignancy and metastatic stages of human ovarian tumor.

Next, we analyzed red blood cells obtained from 14 pairs of maternal blood and fetal cord blood for sialic acids. KDN levels of fetal cord blood are markedly higher than maternal blood as shown in Fig. 6 [17]. Interestingly, 98% of KDN in the human red blood cells were in free form. While the reason for higher free KDN levels in fetal cord red blood cells and metastasizing human ovarian tumors still remains open, the present findings can be considered to be intriguing in view of the possible role of KDN and KDN-glycoconjugates in normal development and malignancy.

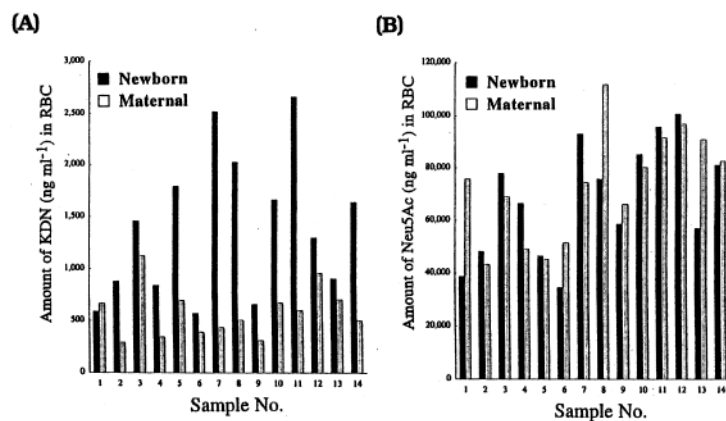


Fig. 6 Differential determination of (A) KDN and (B) Neu5Ac in 14 matched pairs of fetal cord and maternal red blood cells. A 20- μ L-aliquot of each red blood cell sample was hydrolyzed in 200 μ L of 0.1 M HCl for 1 h at 80 °C. The hydrolysate was passed through a small column of Bio-Rad AG-1 $\times$ 8 (formate) and the Sia mixture that was eluted with 0.7 M formic acid was subjected to HPLC analysis after reacting with DMB reagent as described [17,30]. RBC denotes red blood cells.

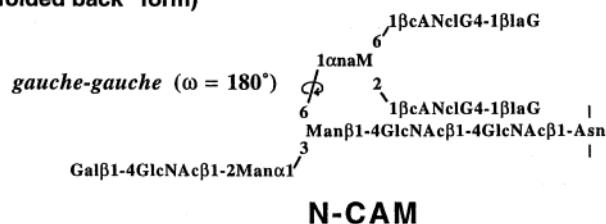
WHAT DO WE NEED TO KNOW ABOUT MOLECULAR MECHANISM OF POLYSIALYLATION IN ANIMALS

Studies in the past several years have increased our understanding of the polysialic acid biosynthesis and regulation. Of particular interest is the cloning of the genes encoding polysialyltransferases (polySTs [54–60]). However, until now only polySTs responsible for synthesis of α 2 $\rightarrow$ 8-linked polyNeu5Ac have been studied rather extensively. Much current debate centers on how many enzymes are needed to confer polysialylation to N-CAM. Enzymatic activities synthesizing other different types of polySia chains have not been described. Recently, a developmentally regulated expression of α 2 $\rightarrow$ 8-polyNeu5Ac transferase activity was detected in the sea urchin embryos by Lennarz and his co-workers [61] although endogenous α 2 $\rightarrow$ 8-polyNeu5Ac chains have not yet been identified until now by biochemical or immunochemical method. It is unclear why α 2 $\rightarrow$ 8-linked polyNeu5Ac chains are formed *in vitro* in a developmentally regulated manner. Several hypotheses may be offered. One of them is a possible catalytic versatility of sea urchin sialyl and polysialyltransferases. In other words, the polyST may act on both CMP-Neu5Gc and CMP-Neu5Ac, but the linkage specificity of this enzyme is controlled by the nature of the donor molecule used (study on this topic is being underway in our laboratory).

Because biosynthetic mechanism of N-CAM polysialylation is one of the major current topics in polySia studies, we would like to discuss just a little bit about our understanding of molecular mechanism of polysialylation. Jukka Finne is the first who attempted to determine its structure, and he proposed a triantennary structure as best explaining the chemistry of the glycan chain of N-CAM [36]. Since then nothing was known about the detailed structure of the core glycan chains present in N-CAM.

Biosynthetically there exist two isomeric forms of triantennary glycan chains: one is the complex-type triantennary chain having the 2,6-Man residue on the $\alpha 1 \rightarrow 6$ arm and the other is the glycan chain having the 2,4-Man on the $\alpha 1 \rightarrow 3$ arm. To gain more insight into the question of what factor determines the acceptor specificity, we elucidated the exact structure of triantennary glycan chain in N-CAM derived from embryonic chick brain [62]. We also carried out the detailed analysis of 2-D proton NMR spectral data of these two different types of triantennary glycan chains [63,64]. The results are depicted in Fig. 7 [62]. This figure represents how these two types of triantennary chains adopt different conformations ('open form' in fetuin and 'folded back form' in N-CAM; see also [65]). Thus, a comparative study has revealed that one of these glycan chains adopts a folded conformation and the other an extended conformation, and the extended form of an triantennary glycan isoform may have an unfavorable backbone conformation for sialylation and/or polysialylation as judged from the available experimental data. Therefore, taken all available experimental data together and cutting a long story short, polysialylation mechanism can be formulated as shown in Fig. 8. The highlighted arrows indicate that the reaction proceeds most likely under nonsaturating conditions. A by-route indicates that the polyST may act on a poor acceptor, monosialylated chains, under saturating conditions, but the reaction proceeds less

(A) ("folded back" form)



(B) ("extended" form)

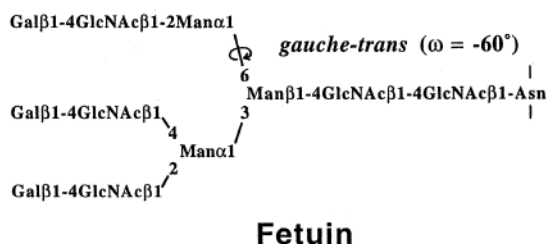


Fig. 7 Conformational difference indicated by the dihedral angle (O6-C6-C-5H5) in Man $\alpha 1 \rightarrow 6$ Man $\beta 1 \rightarrow$ between the two discrete types of triantennary N-linked glycan chains: (A) 'folded back form' in N-CAM (chick embryo) and (B) 'open or extended form' in fetuin (bovine).

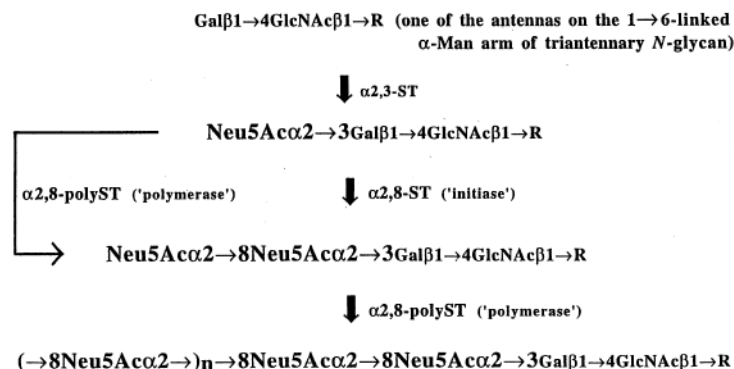


Fig. 8 Postulated polysialylation mechanism in N-CAM molecule based on available experimental data and some enzyme-kinetic consideration.

efficiently via this by-route. Although we cannot completely rule out the possibility that the monosialylated species would be the acceptor substrate *in vivo* too, we favor the two-enzyme mechanism as the preferred route for polysialylation. It seems to us that extensive and heated debates seem to continue in the study of biosynthetic mechanism of polysialylation for some time to come until we obtain quantitative data not only on the differential profiles of the development of diSia and polySia formation in N-CAM by the biochemical approach but also on the rate processes of the enzyme-catalyzed reactions. However, as we pointed out here, their results seem not to violate our hypothesis of two-enzyme mechanism (cf. [66–68]).

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