Limited understanding of the functional diversity of N-linked glycans as a major gap of prion biology

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ABSTRACT. Among a broad range of hypotheses on the molecular nature of transmissible spongiform encephalopathy or scrapie agents discussed in 1960s was a hypothesis of self-replicating polysaccharides. While the studies of the past 40 years provided unambiguous proof that this is not the case, emerging evidence suggests that carbohydrates in the form of sialylated N-linked glycans, which are a constitutive part of mammalian prions or PrPSc, are essential in determining prion fate in an organism. The current extra-view article discusses recent advancements on the role of N-linked glycans and specifically their sialylation status in controlling prion fate. In addition, this manuscript introduces a new concept on the important role of strain-specific functional carbohydrate epitopes on the PrPSc surface as main determinants of strain-specific biologic features. According to this concept, individual strain-specific folding patterns of PrPSc govern selection of PrPC sialoglycoforms expressed by a host that can be accommodated within particular PrPSc structures. Strain-specific patterns of functional carbohydrate epitopes formed by N-linked glycans on PrPSc surfaces define strain-specific biologic features. As a constitutive part of PrPSc, the individual strain-specific patterns of carbohydrate epitopes propagate faithfully within a given host as long as individual strain-specific PrPSc structures are maintained, ensuring inheritance of strain-specific biologic features.

KEYWORDS. carbohydrate epitopes, microglia, N-linked glycans, prion, prion diseases, sialylation, sialic acid, secondary lymphoid organs

Among a broad range of ideas on the molecular nature of transmissible spongiform encephalopathy or scrapie agents discussed in 1960s was a hypothesis of self-replicating polysaccharides. The studies of the past 40 years provided unambiguous proof that the scrapie agents or prions consist of misfolded, self-replicating states of a sialoglycoprotein called the prion protein or PrPC. Nevertheless, emerging evidence suggests that carbohydrates in the form of sialylated N-linked glycans, which are constitutive part of prions or PrPSc, are essential in determining prion fate in an organism. While the fact that PrP Sc N-linked glycans are...
sialylated has been known for more than 30 years,3 little has been discovered about the role sialylation plays in prion diseases until recently.

Our recent studies demonstrated that PrPSc with reduced sialylation status does not transmit prion disease to wild type animals.4,5 For producing PrPSc with reduced sialylation, Protein Misfolding Cyclic Amplification with beads (PMCAb) was conducted using PrP\textsuperscript{C} as a substrate that was partially desialylated by treatment with bacterial sialidases (dsPMCAb). All animals inoculated intracranially or intraperitoneally with brain-derived 263K developed scrapie with substantial amounts of PrPSc found in their brains and spleens. Remarkably, no animals inoculated intracranially or intraperitoneally with dsPMCAb-derived 263K developed the disease.4,5 Moreover, no PrPSc was detected in brains or spleens of animals from these groups by Western blot or by calibrated serial PMCAb that can detect a single PrPSc particle,6 arguing that these animals were free of scrapie.4,5 Control groups of animals inoculated with PMCAb-derived 263K developed disease at slightly longer incubation times relative to the control group that received brain-derived PrPSc. Such delay is attributed to a moderate shift in the sialylation pattern of PMCAb-derived 263K relative to that of brain-derived 263K.4 In the course of PMCAb, PrPSc molecules with reduced sialylation status were preferentially recruited into PrPSc, producing a shift in sialylation status of PMCAb-derived material.4 In subsequent experiments, trafficking of brain-, PMCAb-, and dsPMCAb-derived PrPSc to secondary lymphoid organs was monitored after intraperitoneal administration. Colonization and replication of prions in secondary lymphoid organs, including spleen and lymph nodes, typically occur before and are important for neuroinvasion (reviewed in.7,8). While brain- and PMCAb-derived PrPSc were found in spleen and lymph nodes, dsPMCAb-derived PrPSc was targeted predominantly to the liver.5 In summary, sialylation was found to be critical for effective trafficking of PrPSc to SLOs and infecting a host, whether it is administered via intraperitoneal or intracranial routes.

To establish a cause-and-effect relationship between sialylation of PrPSc and its infectivity and to test whether this effect is limited to 263K or not, the sialylation status of a strain of synthetic origin SSLOW generated in our laboratory9,10 was altered using a similar approach. First, the sialylation status of SSLOW PrPSc was reduced by replicating brain-derived SSLOW in serial PMCAb using sialidase-treated PrP\textsuperscript{C} and then restored to the original levels by replicating using non-treated PrPSc (rsPMCAb). Remarkably, all animals that received PMCAb- or rsPMCAb-derived products with original or restored sialylation levels, respectively, were infected and showed PrPSc in brains and spleens.11 None of the animals that received PrPSc with reduced sialylation (dsPMCAb) were infected. Again, the brains and spleens of animals from the dsPMCAb group were completely cleared of prions, as assessed by Western blot and quantitative serial PMCAb.11 Analysis of SSLOW PrPSc purified from brain and PMCAb/dsPMCAb/rsPMCAb reactions using FTIR revealed that the structures of PMCAb-, dsPMCAb-, and rsPMCAb-derived SSLOW were indistinguishable as much as this technique can detect, whereas only dsPMCAb SSLOW with reduced sialylation failed to infect the animals.11 Similarly, FTIR analysis of PMCAb- and dsPMCAb-derived 263K revealed that their structures were also indistinguishable, whereas only dsPMCAb-263K failed to induce disease.5 In summary, these studies revealed that prion infectivity could be switched off and on in a reversible manner via altering the sialylation status of PrPSc.

Our work suggests that sialylation of PrPSc is one of the key determinants underlying prion infectivity. Several hypotheses could explain the observed effects.12 According to one hypothesis, terminal sialic acid residues are required for PrPSc trafficking to SLOs and its long-term stability in the periphery and CNS.12 An alternative hypothesis proposes that it is the lack of terminal sialylation, i.e. exposed terminal galactose, that controls the life-time of PrPSc and its trafficking.12 Thus, asialo-PrPSc displays an “eat me” signal in the form of exposed galactose, which is recognized by professional and non-professional macrophages,
including Kupffer cells and microglia.\textsuperscript{13,14} According to the first hypotheses, the interacting partners of PrP\textsuperscript{Sc} involve proteins that recognize sialic acid residues, whereas the second hypothesis assumes that galactose-binding proteins are in control of PrP\textsuperscript{Sc} fate. A third possibility that both PrP\textsuperscript{Sc} life-time and trafficking are controlled by several classes of carbohydrate-binding proteins, including those that recognize sialic acids, galactose and possibly other carbohydrate residues found in PrP\textsuperscript{Sc} N-linked glycans (fucose, sulfate, mannose), should not be ignored.

In previous studies, highly infectious PrP\textsuperscript{Sc} was produced \textit{in vitro} using recombinant PrP and co-factors.\textsuperscript{15,16} Because entire N-linked glycans were missing in recombinant PrP\textsuperscript{Sc}, the “eat me” signal in the form of exposed galactose was also absent, preventing identification of recombinant PrP\textsuperscript{Sc} by the innate immune system in the same manner as it might deal with asialo-PrP\textsuperscript{Sc} with displays an “eat me” signal. Upon inoculation, recombinant PrP\textsuperscript{Sc} recruits glycosylated PrP\textsuperscript{C} expressed by the host, and therefore the fate of PrP\textsuperscript{Sc} seeded in vivo by recombinant PrP\textsuperscript{Sc} is likely to depend on its sialylation status.

Regardless of the specific mechanism behind the relationship between PrP\textsuperscript{Sc} sialylation and infectivity, our studies highlighted the important role of N-linked glycans in determining PrP\textsuperscript{Sc} fate. In intact PrP\textsuperscript{Sc} particles, the N-linked glycans are directed outwards exposing terminal residues that are accessible for interaction with other proteins. The range of proteins with which PrP\textsuperscript{Sc} could potentially interact is determined by the structural diversity of the functional carbohydrate epitopes on the PrP\textsuperscript{Sc} surface, which could be generated via several means. First, there are 2 types of sialic acid residues in mammals: N-acetylneuraminic acid or Neu5Ac and N-glycolylneuraminic acid or Neu5Gc.\textsuperscript{17} Humans and ferrets can synthesize only Neu5Ac, whereas the rest of mammalian species synthesize both Neu5Ac and Neu5Gc, with predominantly Neu5Ac produced in CNS and both Neu5Ac and Neu5Gc produced in peripheral tissues.\textsuperscript{12} Second, in PrP\textsuperscript{Sc} sialic acid residues can be attached to galactose via $\alpha2$–3 or $\alpha2$–6 linkages.\textsuperscript{18} The linkage type contributes to recognition specificity and selectivity by carbohydrate-binding proteins. Third, several natural modifications including O-acetyl, N-glycolyl, O-lactyl, O-sulfate, O-phosphate, hydroxyl, or O-methyl were found at several carbon positions in sialic acid residues.\textsuperscript{19} Whether any of these modifications are present in PrP\textsuperscript{Sc} has not been investigated; nevertheless, structural diversity of surface epitopes could be multiplied by these modifications. Fourth, combinations of sialic acid residues with other neighboring carbohydrate groups, including fucose and sulfate, result in a range of functional epitopes, some of which are found in PrP\textsuperscript{Sc}.\textsuperscript{18,20} Fifth, the N-linked glycans of PrP\textsuperscript{Sc} exhibit several types of branching patterns including bisected and nonbisected bi-, tri-, and tetraantennary types that determine the density of sialic acid and other residues on the surface of PrP\textsuperscript{Sc} particles.\textsuperscript{18,20,21} (Fig 1A). Tetraantennary structures that accommodate up to 4 sialic acid residues per glycan are the most branched glycans that were assigned to PrP\textsuperscript{C}/PrP\textsuperscript{Sc}.\textsuperscript{18,20,21} However, the fact that up to 5 sialic acid residues per glycan were found using mass spectrometry analysis\textsuperscript{21} and even higher number of sialic acid residues per glycan is predicted from analysis by 2D Western blots opens the possibility that a small fraction of PrP\textsuperscript{C}/PrP\textsuperscript{Sc} is modified with more complex and/or unconventional N-glycans (Fig 1B). Structural analysis of PrP N-linked glycans conducted almost 30 y ago using mass spectrometry described over 400 different PrP glycoforms.\textsuperscript{18} The actual diversity of glycoforms could be even greater considering that the assignment to specific structures could only be made for the glycans known at that time and that numerous new glycan structures including unconventional structures have been identified since then. However, not all PrP\textsuperscript{C} glycoforms are recruited proportionally into PrP\textsuperscript{Sc}. The size of N-linked glycans and electrostatic repulsion between sialic acid residues at the glycan terminal positions impose spatial and electrostatic constraints that control glycoform ratios and sialylation status within PrP\textsuperscript{Sc} in a strain-specific manner.\textsuperscript{4,22} (Fig 2A).

Here we propose a new hypothesis on the flow of information from species-specific
amino acid sequences of PrP\textsuperscript{C} to functional carbohydrate epitopes on the PrP\textsuperscript{Sc} surface that define strain-specific biologic properties. As was proposed previously, species-specific amino acid sequences of PrP\textsuperscript{C} define the range of strain-specific folding patterns of PrP\textsuperscript{Sc} accessible for individual sequences (Fig 2B).\textsuperscript{23} Individual folding patterns govern selection of sialoglycoforms that can be accommodated within strain-specific PrP\textsuperscript{Sc} structures and, as a result, define the strain-specific pattern of functional carbohydrate epitopes on PrP\textsuperscript{Sc} surfaces (Fig 2B). Using this information, prion strains could be classified into 2 large categories. The first one includes strains with minimal structural constraints. Strains of this group are predominantly diglycosylated, as their PrP\textsuperscript{Sc} structures can accommodate diglycosylated and highly sialylated PrP molecules (for instance, the majority of hamster strains and variant Creutzfeldt-Jakob Disease, Figure 2B). The second group exhibits much greater structural constraints. Strains of this group are predominantly monoglycosylated, as they selectively exclude diglycosylated and highly sialylated PrP molecules (the majority of mouse strains and sporadic Creutzfeldt-Jakob Disease, Figure 2B).\textsuperscript{22} PrP\textsuperscript{Sc} folding patterns are expected to be quite different for each of the above groups. In addition, because the range of N-linked glycans synthetized in different hosts is likely to be species-specific, the result of selection of PrP\textsuperscript{Sc} sialoglycoforms is not only determined by the constraints imposed by strain-specific PrP\textsuperscript{Sc} structures, but also shaped by the specific host. To illustrate, transmission of the same strain to hamsters and transgenic mice expressing hamster PrP\textsuperscript{C} might result in different patterns of functional carbohydrate epitopes. Moreover, we speculate that other characteristics of N-linked glycans, including their size, branching pattern, and the presence of other charged groups, such as sulfate, contribute to strain-specific selection of PrP\textsuperscript{Sc} glycoforms. Nevertheless, as a result of selective recruitment, unique strain-specific patterns of functional carbohydrate epitopes are formed on the surface of PrP\textsuperscript{Sc} particles. As a constitutive part of PrP\textsuperscript{Sc}, the strain-specific patterns of functional carbohydrate epitopes propagate faithfully as long as the host and individual strain-specific PrP\textsuperscript{Sc} structures are maintained, ensuring inheritance of strain-specific biologic features. Functional carbohydrate epitopes on the PrP\textsuperscript{Sc} surface determine the range of carbohydrate-binding molecules that can interact with PrP\textsuperscript{Sc} and, as a result, define the strain-specific biologic features, including cell-, neuro- and lymphotropisms. Several dozen if not hundreds of proteins that

FIGURE 1. (A) Structures of bi-, tri-, and tetraantennary N-linked glycans found in PrP\textsuperscript{C} and PrP\textsuperscript{Sc} that could be bisected (b) or nonbisected (n).\textsuperscript{18,20,21} Facultative fucosialtion and sialylation are shown within parenthesis with several sialic acid residues per glycan indicated. (B) Structures of pentatantennary and nonconventional N-linked glycans that can accommodate up to 5 sialic acid residues.\textsuperscript{28}
specifically recognize carbohydrate groups have been identified, including siglecs, selectins, galectins, asialoglycoprotein receptors, mannose receptors, and complement factors (reviewed in\textsuperscript{24,25}). Because the majority of carbohydrate-binding molecules have multivalent binding sites, the strength and selectivity of binding depends not only on the composition...
of functional carbohydrate epitopes but also their density and specific configuration of carbohydrate groups. To summarize, with the guidance of a strain-specific PrP\textsubscript{Sc} template, the information encoded in the amino acid sequence of PrP\textsubscript{C} is transformed into self-replicating patterns of functional carbohydrate epitopes on the PrP\textsubscript{Sc} surface that define strain-specific biologic properties.

Many questions need to be addressed in future studies. What factors control sialylation of PrP\textsubscript{Sc}? How can one manipulate PrP\textsubscript{Sc} sialylation status? In previous studies, modulating the activity of sialyltransferases was found to be more effective than targeting sialidases for modulating the sialylation status of PrP\textsubscript{C}.\textsuperscript{26} Moreover, in secondary lymphoid organs, PrP\textsubscript{Sc} was shown to be subject to enhanced post-conversion sialylation that might ensure extra-protection from the innate immune system.\textsuperscript{27} Therefore, identification of sialyltransferases responsible for sialylation of PrP\textsubscript{C} and PrP\textsubscript{Sc} might represent an important future goal. Additionally, does the difference in sialic acids expressed in human and other mammals contribute to the mammal-to-human transmission barrier? Does the age-dependent decline in sialic acid content contribute to the etiology of sporadic prion diseases? Moreover, the hypothesis on functional carbohydrate epitopes has to be tested. Can we decode the relationship between composition of carbohydrate epitopes and strain-specific biologic features? However, perhaps the most important question is whether new therapeutic strategies against prion diseases could be developed by manipulating PrP\textsubscript{Sc} sialylation status.

**ABBREVIATIONS**

PrP\textsubscript{C}  normal cellular isoform of the prion protein  
PrP\textsubscript{Sc}  abnormal, disease-associated isoform of the prion protein

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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