Sialylation of Glycosylphosphatidylinositol (GPI) Anchors of Mammalian Prions Is Regulated in a Host-, Tissue-, and Cell-specific Manner*

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Prions or PrP\textsuperscript{Sc} are proteinaceous infectious agents that consist of misfolded, self-replicating states of the prion protein or PrP\textsuperscript{C}. PrP\textsuperscript{C} is posttranslationally modified with \(N\)-linked glycans and a sialylated glycosylphosphatidylinositol (GPI) anchor. Conformational conversion of PrP\textsuperscript{C} gives rise to glycosylated and GPI-anchored PrP\textsuperscript{Sc}. The question of the sialylation status of GPIs within PrP\textsuperscript{Sc} has been controversial. Previous studies that examined scrapie brains reported that both sialo- and asialo-GPIs were present in PrP\textsuperscript{Sc}, with the majority being asialo-GPIs. In contrast, recent work that employed cultured cells claimed that only PrP\textsuperscript{C} with sialo-GPIs could be recruited into PrP\textsuperscript{Sc}, whereas PrP\textsuperscript{C} with asialo-GPIs inhibited conversion. To resolve this controversy, we analyzed the sialylation status of GPIs within PrP\textsuperscript{Sc} generated in the brain, spleen, or cultured N2a or C2C12 myotube cells. We found that recruiting PrP\textsuperscript{C} with both sialo- and asialo-GPIs is a common feature of PrP\textsuperscript{Sc}. The mixtures of sialo- and asialo-GPIs were observed in PrP\textsuperscript{Sc} universally regardless of prion strain as well as host, tissue, or type of cells that produced PrP\textsuperscript{Sc}. Remarkably, the proportion of sialo- versus asialo-GPIs was found to be controlled by host, tissue, and cell type but not prion strain. In summary, this study found no strain-specific preferences for selecting PrP\textsuperscript{C} with sialo- versus asialo-GPIs. Instead, this work suggests that the sialylation status of GPIs within PrP\textsuperscript{Sc} is regulated in a cell-, tissue-, or host-specific manner and is likely to be determined by the specifics of GPI biosynthesis.

Prions or PrP\textsuperscript{Sc} are proteinaceous infectious agents that consist of misfolded, self-replicating states of a sialoglycoprotein called the prion protein or PrP\textsuperscript{C} (1, 2). Prions replicate by recruiting and converting PrP\textsuperscript{C} molecules expressed by a host into misfolded PrP\textsuperscript{Sc} states (3). In the PrP\textsuperscript{Sc} state, the prion protein can acquire conformationally distinct self-replicating states, referred to as prion strains, that elicit different, strain-specific disease phenotypes (4–10). Accumulation of PrP\textsuperscript{Sc} in the CNS leads to prion diseases, a family of transmissible neurodegenerative maladies that are 100% lethal (11).

PrP\textsuperscript{C} is posttranslationally modified with two \(N\)-linked glycans and a glycosylphosphatidylinositol (GPI) anchor that is attached to the C-terminal residue Ser-230 (the residue number is provided for mouse PrP\textsuperscript{C}) (12–16). Upon conversion of PrP\textsuperscript{C} into PrP\textsuperscript{Sc}, the \(N\)-linked glycans and GPI are carried over, giving rise to glycosylated and GPI-anchored PrP\textsuperscript{Sc} (17, 18). Like amyloids formed by other amyloidogenic proteins or peptides, PrP\textsuperscript{Sc} displays a cross-\(\beta\) folding pattern (19, 20), a key structural feature of amyloid states. However, unlike other amyloidogenic proteins linked to neurodegenerative diseases, PrP\textsuperscript{Sc} recruits PrP\textsuperscript{C} anchored via the GPI to the cell surface and replicates its cross-\(\beta\) structure while also being attached to the membrane via its GPI anchors (21–24). For that reason, the mechanism of PrP\textsuperscript{Sc} replication appears to be unique.

Multiple lines of evidence indicate that the GPI anchor plays an important role in PrP\textsuperscript{Sc} transmission and toxicity (reviewed in Ref. 25). Using the GPI anchor, PrP\textsuperscript{C} could be efficiently transported from cell to cell (26). GPI anchoring of PrP\textsuperscript{Sc} was found to be important for its binding to and replication on the cell surface (27) and might be also involved in intercellular transmission of PrP\textsuperscript{Sc} via exosomes (28). Upon inoculation with prions, transgenic mice expressing GPI anchorless PrP\textsuperscript{C} supported prion replication and accumulated prion infectivity; however, the disease onset in these mice was substantially delayed or was lacking (29). Mice that overexpressed GPI anchorless PrP\textsuperscript{C} developed neurological dysfunction and generated infectious prions spontaneously in the absence of exposure to prions (30). Previous studies suggested that neurotoxic signaling is dependent on PrP\textsuperscript{C} attachment to the plasma membrane via its GPI anchor (31, 32). In particular, experiments using primary neuronal cultures suggested that toxicity triggered by PrP\textsuperscript{Sc} is dependent on the sialylation status of the GPI anchor within PrP\textsuperscript{C}, as clustering of PrP\textsuperscript{C} molecules with sialylated GPIs led to activation of cytoplasmic phospholipase A2 and synapse damage (31).

PrP\textsuperscript{C} is one of very few proteins in which GPI anchors are sialylated (33, 34). In fact, previous studies revealed that GPIPs of PrP\textsuperscript{C} are structurally heterogeneous, with a subtraction of GPIs modified with sialic acids (12). GPIs are synthesized in the endoplasmic reticulum, and then posttranslational modification of polypeptide chains with preformed GPIs occurs in both
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FIGURE 1. Two-dimensional analysis of the sialylation status of brain- and spleen-derived PrPSc from 22L-infected animals. A, representative two-dimensional Western blots of 22L brain homogenate (BH), 22L BH treated with A. ureafaciens sialidase (BH + AU), 22L spleen homogenate (SH), and 22L SH treated with A. ureafaciens sialidase (SH + AU). Black and white arrowheads mark diglycosylated and monoglycosylated glycoforms, respectively, whereas arrows mark the unglycosylated form. The homogenates were treated with PK, and Western blots were stained with Ab3531 antibody. B, intensity profiles of unglycosylated isoforms of brain-derived (top plot, solid thick lines) or spleen-derived PrPSc (bottom plot, solid thick lines) from animals infected with 22L (n = 3 independent animals). Intensity profiles of unglycosylated isoforms of 22L brain or spleen homogenates treated with A. ureafaciens sialidase are provided as a reference (dotted lines). Three major spots corresponding to unglycosylated isoforms are marked as 1, 2, and 3. Profiles were built as described under “Experimental Procedures” based on two-dimensional Western blots.

Although the biological function of sialylation of GPIs is not known, learning whether PrPSc with both sialo- and asialo-GPIs can be recruited into PrPSc is important. If prion replication is supported only by PrPC with either but not both sialo- or asialo-GPIs, then new strategies for therapeutic intervention against prion disease that would control GPI sialylation could be considered. The question of the sialylation status of GPIs within PrPSc has been controversial. The classic study by Stahl et al. (12) reported that GPIs within PrPSc consisted of six structural isoforms, 30% of which were sialylated. Moreover, the composition of GPIs within PrPSc was found to be similar to that of PrPC (12). In contrast, a recent work by Bate et al. (37) claimed that only PrPC with sialylated GPIs could be recruited into PrPSc, whereas PrPC with asialo-GPIs inhibits conversion of PrPC with sialo-GPIs into PrPSc.

To resolve the controversy, we analyzed the sialylation status of GPIs within PrPSc of five prion strains from two hosts. In addition, we also assessed the sialylation status of GPIs of brain- and spleen-derived PrPSc as well as PrPSc generated in neuroblastoma N2a cells or terminally differentiated C2C12 myotube cells cultured in vitro. This study reports that PrPSc can recruit PrPC with both sialo- and asialo-GPIs, as the mixtures of sialo- and asialo-GPIs were observed universally regardless of prion strain or host, tissue, or cell type in which PrPSc was generated. However, the proportion of sialo forms of GPIs was variable and depended on the host, tissue, and cell type but not prion strain. In summary, these findings suggest that the proportion of the two sialo forms of GPIs within PrPSc is determined by cell- or tissue-specific rates of synthesis of sialo-GPIs but not strain-specific structural constraints.

Results

GPI Anchors of Brain-derived Mouse PrPSc Consist of Sialylated and Asialylated Forms—To assess the sialylation status of GPI anchors, PrPSc was denatured into monomers, and then individual PrP molecules were analyzed using two-dimensional gel electrophoresis followed by Western blotting. In the vertical dimension of two-dimensional blots, PrP molecules are separated according to their glycosylation status. Diglycosylated PrPs are of the highest and unglycosylated PrPs of the lowest molecular weight. In the horizontal dimension of two-dimensional blots that separates molecules according to their pl, all three PrP glycoforms display multiple charge isoforms. For diglycosylated molecules, multiple charge isoforms are largely attributed to heterogeneity in the sialylation status of N-linked glycans and, to a lesser degree, heterogeneity of the GPI anchors (38, 39). However, in unglycosylated molecules, the multiple charge isoforms report on the structural heterogeneity of the GPI anchors, including their sialylation status (12). Because the sialylation status of N-linked glycans was analyzed in previous studies (39, 40), this work focuses on the GPI anchors.

To assess the sialylation status of GPI anchors, we analyzed the charge distribution of unglycosylated molecules using two-dimensional blots. In brain material from mice infected with prion strain 22L, unglycosylated PrP showed three major spots (referred to as 1, 2, and 3; Fig. 1, A and B). To find out whether any of these spots could be attributed to sialo-GPIs, 22L brain material was treated with Arthrobacter ureafaciens sialidase, which exhibits a broad specificity with respect to sialylation...
linkages. Cleavage of negatively charged sialic acid residues is expected to shift the distribution of charge isoforms toward basic pH (to the right in two-dimensional blots). Indeed, upon sialidase treatment, the relative intensity of spot 1 increased at the expense of spots 2 and 3 (Fig. 1A). The relative presentation of sialo-GPIs versus asialo-GPIs is difficult to estimate because the yields from cleavage of sialic residues from GPI anchors by sialidases are not known. As judged from the substantial shift of di- and monoglycosylated PrP molecules toward basic pl, the treatment with sialidase was very effective yet less than 100%. Nevertheless, these shifts argue that spot 2 consists of unglycosylated PrPs with both sialo- and asialo-GPIs, whereas spot 3 consists predominantly of PrPs with sialylated GPIs. The lack of appearance of any new spots to the right of spot 1 upon sialidase treatment argues that spot 1 consists only of PrPs with asialo-GPIs. These results demonstrated that brain-derived 22L scrapie consisted of PrPs with both sialo- and asialo-GPIs.

**Figure 2.** Two-dimensional analysis of the sialylation status of brain-derived PrPSc from animals infected with RML, ME7, or 22L. A, representative two-dimensional Western blots of BH from animals infected with RML, ME7, or 22L. Black and white arrowheads mark diglycosylated and monoglycosylated glycoforms, respectively, whereas arrows mark the unglycosylated form. The homogenates were treated with PK, and Western blots were stained with Ab3531 antibody. B, intensity profiles of unglycosylated isoforms of brain-derived PrPSc from animals infected with RML (top plot) or ME7 (bottom plot) (n = 3 independent animals). Three major spots corresponding to unglycosylated isoforms are marked as 1, 2, and 3. Profiles were built as described under “Experimental Procedures” based on two-dimensional Western blots.

To investigate whether the sialylation status of GPIs in PrPSc is species-specific, we compared brain- and spleen-derived PrPSc from animals infected with 22L. The profiles of unglycosylated charge isoforms were markedly different in spleen versus brain (Fig. 1A and B). In contrast to brain-derived PrPSc, the charge isoforms corresponding to asialo-GPIs (spot 1) was predominant in spleen-derived PrPSc (Fig. 1A and B). The isoforms corresponding to sialo-GPIs (spot 3) were low populated in spleen-derived PrPSc (Fig. 1A and B). Upon treatment with sialidase of spleen-derived PrPSc, the relative intensities of both spots 2 and 3, which contain sialo-GPIs, decreased; however, to a lower degree than the decrease observed upon treatment of brain-derived PrPSc. Together, these results show that, although spleen-derived 22L contained sialo-GPIs, the proportion of asialo-GPIs versus sialo-GPIs in spleen was substantially higher than that in brain-derived PrPSc.

The Sialylation Profile of GPIs of Brain-derived PrPSc Is Strain-independent—Previously, we showed that the sialylation status of N-linked glycans on PrPSc are strain-specific, illustrating that prion strains recruit PrPC sialoglycoforms selectively according to their strain-specific structural constraints (39). To test whether the sialylation status of GPIs is strain-specific, brain-derived scrapie material for three mouse prion strains 22L, RML, and ME7, were analyzed (Fig. 2A). As judged from two-dimensional blots and corresponding density profiles, the distribution of charge isoforms of non-glycosylated PrPs were very similar for all three strains (Fig. 2A). In contrast, significant differences in sialylation patterns of N-linked glycans were observed for these three strains, as judged from the profiles of mono- and diglycosylated isoforms. In full agreement with previous studies (39, 40), the highest level of sialylation of N-glycans was observed for ME7 and the lowest for RML.

Species-specific Variations in Sialylation Status of GPIs in PrPSc—To determine whether the sialylation status of GPIs in PrPSc is host-dependent, unglycosylated isoforms of brain-derived PrPSc of two hamster strains, HY and 263K, were analyzed by two-dimensional blots (Fig. 3A). The profiles of unglycosylated charge isoforms were very similar for both hamster strains but different from those of mouse strains. In hamster strains, the charge isoforms corresponding to asialo-GPIs (spot 1) were predominant, whereas the isoforms corresponding to sialo-GPIs (spot 3) were the least populated (Fig. 3B). All three mouse strains contained sialo-GPIs.
strains showed clear prevalence of spot 2 (Figs. 3B and 2B). Interestingly, after treatment with sialidase, the profiles of mouse and hamster strains looked similar (Fig. 3B), supporting the claim that the species-specific differences in GPIs were due to different ratios of sialo- versus asialo-GPI forms. These results argue that the sialylation status of GPIs was largely determined by the species or host, whereas strain-specific variations were very minor.

The Sialylation Status of GPIs in PrPSc Depends on Cell Type—To test whether the sialylation status of GPIs depends on cell type, we analyzed unglycosylated charge isoforms of PrPSc generated in N2a and myotube C2C12 cells, both infected with the RML strain. N2a is a neuroblastoma cell line that can be stably infected with mouse strains, including RML (41). Myotubes were also susceptible to the RML strain when generated via differentiating C2C12 myoblast cells (42). The two cell lines displayed significantly different profiles of unglycosylated charge isoforms, suggesting that a cell-specific difference in the sialylation status of GPIs exists. In N2a, spot 1 was clearly predominant, suggesting that the majority of N2a-generated PrPSc had asialo-GPIs (Fig. 4A). Indeed, upon treatment of N2a-generated PrPSc with sialidase, the profile for unglycosylated isoforms did not change, supporting the claim that PrPSc had predominantly asialo-GPIs (Fig. 4, A and B). Remarkably, the dramatic shift of di- and monoglycosylated isoforms toward basic pH upon treatment with sialidase is attributed to a removal of sialic acids from N-linked glycans and confirms that this enzymatic procedure for desialylation was highly effective (Fig. 4A). Meanwhile, in myotubes examined on day 15 post-infection, the unglycosylated charge isoforms showed at least five major spots (Fig. 4A). In addition to spots 1–3, at least two more spots (designated A and B, Fig. 4A) that were not readily observed in other samples were observed in myotube-derived material. Sialidase treatment of myotube-produced PrPSc revealed that the unusually broad charge heterogeneity of GPIs was attributable to sialylation (Fig. 4A). Notably, both the N2a- and myotube-specific sialylation profiles of GPIs for RML were different from that of brain-derived RML (Fig. 4A). In summary, these results argue that the sialylation status of GPIs within PrPSc is controlled by cell type but not strain.

Time-dependent Changes in the Sialylation Status of GPIs in PrPSc in Differentiating Myotubes—To test whether the status of GPIs in PrPSc changes over the course of PrPSc formation, we first examined the kinetics of PrPSc accumulation in myotubes. During the first week after infection, the amounts of PrPSc dropped considerably, reflecting clearance of PrPSc in myotubes. The amount of PrPSc dropped considerably, reflecting clearance of PrPSc in myotubes. During the first week after infection, the amounts of PrPSc increased steadily,
reflecting PrPSc newly produced by myotubes (Fig. 5A). Two-dimensional analysis of cells collected on days 10 and 15 post-infection revealed significantly different profiles of unglycosylated charge isoforms (Fig. 5, B and C). On day 10, three main spots (1, 2, and 3) were clearly visible, with spot 2 being predominant. On day 15, the profile becomes more complex, with the relative contribution of spots 3, A, and B increased at the expense of spot 2 (Fig. 5, B and C). This experiment illustrates that although sialo- and asialo-GPIs are present in PrPSc at different stages of PrPSc accumulation and cell differentiation, a shift toward sialo-GPIs could be observed over time. A more complex composition of GPI isoforms was observed with the progression of cell differentiation.

The Sialylation Profiles of GPIs of PrPSc—

We found previously that PrPC charge isoforms were poorly resolved on two-dimensional blots, presumably because of peculiar properties of the octarepeat region (43). Multiple attempts to improve the resolution using different strategies have not been successful. However, sialoglycoforms of the C-terminal proteolytic fragment C1, which is produced by cleavage of PrPc at amino acid residue 111, were well resolved on two-dimensional blots (43). In the absence of a reliable method for resolving full-length PrPC isoforms, we used C1 as a reporter for assessing the sialylation status of the GPIs. In C1 fragments, multiple positively charged amino acid residues within residues 99–110 are lacking in comparison with PK-treated PrPSc, which consists of residues 90–230. As a result, the C1 charge isoforms are shifted toward acidic pH on two-dimensional blots relative to those of PK-treated PrPSc (compare Fig. 6A to Figs. 1 and 2). Two-dimensional analysis of mouse brain-derived C1 revealed three spots for unglycosylated isoforms, with spots 1 and 2 being predominant (Fig. 6, A and B). This profile resembled those of brain-derived PrPSc, in which spot 2 was predominant. In N2a-derived C1, the profiles for unglycosylated isoforms showed one main spot (1) and was very similar to the profiles seen for N2a-derived PrPSc (compare Figs. 6A and B, and 4A and B). In summary, although the sialylation profiles for GPIs of C1 and PrPSc were not identical, N2a-derived PrPSc and C1 were considerably less sialylated relative to the brain-derived PrPSc and C1, respectively, reflecting the same ranking order.

Discussion

GPI anchors represent a structurally diverse class of post-translational modifications that anchor proteins in the outer leaflet of the cellular membrane and are involved in a number of functions, including protein trafficking into lipid rafts, signal transduction, cellular communication, targeting of protein to
apical membranes, and others (35, 44). It is believed that GPI-containing proteins are sorted toward glycosphingolipid- and cholesterol-rich rafts, a sorting that already occurs in the Golgi (34). PrPC is one of the GPI-anchored proteins associated with lipid rafts and detergent-resistant membranes. Sialylated GPI anchors are rare in mammals (33, 34). Apart from PrPC, only pig kidney dipeptidase and human CD59 (a membrane inhibitor of reactive lysis) were found to carry sialic acids on their GPIs (33). The biological roles of sialylation of GPIs are not known.

Determining whether PrPSc recruits PrPC with either sialo-GPIs, asialo-GPIs, or both is important for several reasons. Selective recruitment of PrPC with either sialo-GPIs or asialo-GPIs would limit the population of PrPC molecules that can support prion replication. Moreover, if molecules with sialo-GPIs and asialo-GPIs are trafficked to different sites on cell surfaces (reviewed in Ref. 25), then prion replication would be restricted to those sites. Finally, selective recruitment of PrPC as a function of the sialylation status of their GPIs would open new opportunities for therapeutic intervention against prion diseases. For instance, via targeting the enzymatic pathways that are in charge of sialylation of GPIs (48–50). The total sialyltransferase activity in the spleen is higher than in the brain (48). However, in part, the sialyltransferase activity in the spleen is attributed to extracellular sialyltransferases, which are involved in modulating the differentiation and activation of cells of the immune system.

Although the ability to incorporate both forms appears to be universal, the ratio of sialo-versus asialo-GPIs was found to depend on host, tissue, and cell type. All three prion strains propagated in mice showed a higher proportion of sialo-versus asialo-GPIs in comparison with hamster strains. Thus, these differences are likely to be attributed to the host-specific ratios of sialo-versus asialo-GPIs in PrPC rather than strain-specific preferences for selecting a particular type of GPI. Within the same strain, brain-derived PrPSc showed a significantly higher ratio of sialo-versus asialo-GPIs than spleen-derived PrPSc. This result argues that the sialylation status of GPI is controlled in a tissue-specific manner. Tissue-specific differences in the sialylation status of GPI could be due to differences in the expression levels of sialyltransferases and sialidases in cells of different origin and/or tissue-specific differences in the rates of trafficking of PrPSc through the Golgi. In the spleen, only a small subpopulation of cells, specifically follicular dendritic cells (45–47), express PrPC and supports prion replication (45–47). The relative levels of expression of sialyltransferases and sialidases in follicular dendritic cells versus brain are not known. It is also not known which sialyltransferases are responsible for sialylation of GPIs. Twenty sialyltransferases have been identified in mammals (48–50).
which N2a belongs, than in differentiated cells such as myotubes (reviewed in Refs. 58, 59). If the sialylation status of GPIs within PrPSc reflects that of PrPSc, then the cell-specific differences in sialylation of PrPSc GPIs might reflect differences in the speed of PrPSc trafficking through the Golgi and/or the life time of PrPSc in myotubes versus N2a cells. Notably, parallel changes toward more sialylated isoforms of GPIs and N-linked glycans were observed in myotubes with the progression of differentiation to a terminal stage (Fig. 5). These changes are consistent with an increase in the expression levels of sialyltransferases during differentiation of myoblasts (60). RML derived from brain, N2a, and myotubes showed differences in sialylation patterns of N-linked glycans, a topic that will be examined in detail in other studies.

We showed previously that prion strains recruit PrPSc selectively according to the sialylation status of N-linked glycans (39, 61). Some strains are able to accommodate PrPSc molecules with heavily sialylated glycans, whereas others preferentially select PrPSc with low levels of glycan sialylation at the expense of heavily sialylated ones (39, 61). It appears that this is not the case with respect to the sialylation status of GPI. This study established that, within PrPSc, the sialoforms of GPIs are determined by host, tissue, cell type, and cell differentiation status but not strain-specific structures of PrPSc. Indeed, the GPI sialylation status was very similar if not identical in the three mouse strains or the two hamster strains. At the same time, the GPI sialylation status was significantly different for the same scrapie strain derived from brain or spleen (Fig. 4) or produced in N2a cells, myotubes, or brain (Fig. 4). In fact, within the same strain, the tissue-specific differences in GPI sialylation status were considerable enough to determine the tissue origin of PrPSc. Notably, upon treatment with sialidase, the profiles of asialo-GPIs were found to be very similar between mouse and hamster PrPSc or between brain- and spleen-derived PrPSc. These results illustrate that the differences in the GPI profiles between different species or tissues mainly result from alterations in the ratio of sialo-GPIs versus asialo-GPIs.

Previous work by Stahl et al. (12) that employed mass spectrometry demonstrated that PrPSc and PrPSc have a similar GPI composition (12). Because of the poor resolution of PrPSc isoforms on two-dimensional gels (43), we were unable to assess the sialylation status of GPI within PrPSc directly. Instead, proteolytic fragment C1, which is well resolved on two-dimensional blots, was used as a reporter of sialylation of GPIs in PrPC. The extent to which the sialylation status of C1 reflects that of PrPSc is not known, these results should be considered with caution. The sialylation profiles of GPIs in N2a-derived C1 and PrPSc were very similar, with asialo-GPI being predominant in both forms. However, the GPIs of brain-derived PrPSc appeared to be more sialylated than brain-derived C1. This difference could be due to an increase in the expression levels of sialyltransferases in scrapie-infected brain relative to normal brain (62). Nevertheless, N2a-derived C1 and PrPSc were considerably less sialylated in comparison with the brain-derived C1 and PrPSc, respectively, suggesting that the pattern of GPI sialylation in PrPSc is reflective of GPI sialylation of a substrate.

The heterogeneity in isoelectric points for unglycosylated PrPSc attributed to sialo- and asialo-GPIs in this study is con-
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consistent with the two-dimensional patterns for PrPSc of diverse origin, including human prion diseases reported in previous work (39, 40, 63–67). Multiple charge isoforms attributable to several structural isoforms of GPls were also observed upon removal of N-linked glycans using PNGase F treatment (63–67). However, interpretation of charge isoform patterns on two-dimensional blots upon removal of N-glycan by PNGase F is difficult because the asparagine residues to which glycans are linked are converted to aspartic acid residues upon PNGase F treatment. Such a transformation adds negative charges to the proteins and charge heterogeneity to two-dimensional profiles.

A previous study by Stahl et al. (12) analyzed GPI structures of PrPSc of two hamster strains, 139H and Sc237, where the latter is of the same origin as the strain 263K used in this work (68). Using mass spectrometry analysis, Stahl et al. (12) identified six structural isoforms of GPls in brain-derived PrPSc that belong to two classes, sialo- or asialo-GPls, where the sialylated forms contributed to ~30% of the total GPls. In sharp contrast to the work by Stahl et al. (12), recent studies by Bate et al. (37) claimed that PrPC with asialo-GPls were not converted into PrPSc and, even more, inhibited conversion of PrPSc with sialo-GPls into PrPSc. To arrive at this conclusion, Bate et al. (37) used N2a cells and primary neurons infected with PrPSc of an unspecified strain, whereas a cell painting technique was used to administer PrPC with sialylated or desialylated GPls to cultured cells. The results of this work are in excellent agreement with the work by Stahl et al. (12), as we demonstrated that PrPSc consists of PrP molecules with both sialo- and asialo-GPls. Moreover, our two-dimensional analysis revealed that, in hamster strains, the majority of PrPSc anchors were asialo-GPls, which is again in excellent agreement with the study by Stahl et al. (12). Our conclusions regarding the composition of GPls are not limited to the brain-derived scrapie material but are also applicable to PrPSc produced in terminally differentiated and non-differentiated cultured cells, including the N2a cell line used by Bate et al. (37). Remarkably, in sharp contrast to the results of Bate et al. (37), we found that, when N2a cells are stably infected with prions, the vast majority of GPls within PrPSc were asialo-GPls. If the hypothesis introduced by Bate et al. (37) is correct, N2a would not support prion replication.

Several factors could contribute to the discrepancy between this work and the study by Bate et al. (37). First, it is not clear whether PrPSc administered to cells using the cell painting technique recapitulates all the features of PrPSc expressed by a cell (37). Adding PrPSc exogenously can cause nonspecific aggregation of PrPSc that depends on the sialylation status of its GPls or altered cellular localization, leading to the exclusion of a subpopulation of PrPSc from conversion. Second, the experiments with the cell painting technique employed PrPSc that was deglycosylated by treatment with PNGase F (37). Not only does PNGase F treatment alter the amino acid sequence of PrPSc by transforming asparaginyl residues to aspartic acid residues, the absence of glycans in PrPSc is known to promote formation of self-replicating, PK-resistant states that are not PrPSc (69–73). Third, the question of whether PK-resistant material generated by the cell painting technique in cultured cells is infectious was not addressed in the study by Bate et al. (37). In contrast, the infectivity of PrPSc produced in N2a cells stably infected with prions, the method used in this work, was documented in numerous previous studies (41, 74–77). In summary, this study found no strain-specific preferences for selecting PrPSc with sialo- versus asialo-GPls. Instead, this work suggests that the sialylation status of GPls within PrPSc is regulated in a cell-, tissue-, or host-specific manner and is likely to be determined by the levels of biosynthetic enzymes that control the sialylation status of GPl.

Experimental Procedures

Ethics Statement—This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore (assurance no. A32000-01, permit no. 0215002).

Preparation of Brain and Spleen Material for Two-dimensional Electrophoresis—Brains or spleens were collected individually from each animal at the terminal stage of the disease. 10% (w/v) scrapie brain or spleen homogenates were prepared separately for each animal in PBS using glass/Teflon homogenizers attached to a cordless 12-V compact drill (Ryobi) as described previously (40). For two-dimensional electrophoresis of brain-derived material, an aliquot of 10% (w/v) homogenate was diluted with 9 volumes of 1% (v/v) Triton X-100 in PBS, sonicated for 30 s inside a Misonix S-4000 microplate horn (Qsonica), and treated with 20 μg/ml PK (New England Biolabs) for 30 min at 37 °C. For two-dimensional electrophoresis of spleen-derived material, 250 μl of 10% (w/v) homogenate was diluted 1:1 with PBS, aliquoted into 0.2-ml, thin-wall PCR tubes, sonicated for 30 s inside a Misonix S-4000 microplate horn, and then combined into one tube, which was subjected to a 30-min centrifugation at 16,000 × g at 4 °C. The pellet was resuspended in 25 μl of 1% (v/v) Triton X-100 in PBS and treated with 20 μg/ml PK for 30 min at 37 °C. The resulting brain or spleen samples were supplemented with 4× SDS loading buffer, heated for 10 min in a boiling water bath, and processed for two-dimensional electrophoresis as described below. For two-dimensional analysis of age matched non-infected mouse brains, 200 μl of 10% mouse normal brain homogenate was diluted 2-fold in PBS buffer supplied with protease inhibitors (catalog no. 1836145, Roche) and sonicated for 30 s in a water bath at 37 °C. The sample was subsequently centrifuged at 16,000 × g at 4 °C for 30 min. The supernatant was discarded, and the pellet was dissolved in 100 μl of 1% Triton X-100 in PBS. 18 μl of brain material was mixed with 6 μl of 4× SDS sample buffer, incubated for 10 min at 95 °C, and subsequently used for two-dimensional gel electrophoresis.

Culturing of N2a Cells—N2a cells were cultured at 37 °C, 5% CO2 in minimum essential medium (catalog no. 10-010-CV, Corning) supplemented with 10% FBS (catalog no. 10437, Life Technologies), antibiotics (1% v/v penicillin/streptomycin, catalog no. 15140, Life Technologies), and 1% GlutaMAX. To produce scrapie-infected N2a cells, they were grown to 50% confluence and then incubated for 24 h with 0.5% scrapie brain homogenate from RML-infected mice. After incubation, the medium was removed and replaced with fresh medium. Cells...
were then split and passaged 8 times to obtain a stable infection. N2a cells stably infected with RML were collected in 200 μl of BS, lysed by 30-s sonication (Misonix S-4000 microplate horn, Qsonica) in the presence of 1% v/v Triton X-100, and treated with 10 μg/ml PK (New England Biolabs) for 30 min at 37 °C. After adding 4× SDS loading buffer, the sample was heated for 10 min in a boiling water bath and analyzed by two-dimensional electrophoresis. Non-infected N2a cells were cultured to 70% confluence, lysed with M-PER Mammalian Protein Extraction Reagent (catalog no. 78501, Thermo Fisher Scientific), supplemented with 4× SDS sample buffer, incubated for 10 min at 95 °C, and used for two-dimensional analysis.

* Culturing of Myotubes—C2C12 cells were differentiated into murine myotube cells as described previously with minor modifications (42). Differentiation was initiated by replacing proliferating medium (DMEM (catalog no. 10-013-CV, Corning-Collgro), 10% fetal bovine serum (catalog no. 10082-147, Life Technologies), and 1× antibiotics (catalog no. 15240-062, Life Technologies)) to differentiating medium (DMEM (catalog no. 10-013-CV, Corning-Collgro), 1% horse serum (catalog no. 26050-088, Life Technologies), and 1× antibiotics (catalog no. 15240-062, Life Technologies)) in confluent myoblast cells. After differentiation for 4 days, cells were incubated overnight with 0.05% scrapie brain homogenate from RML-infected mice. Cells were then washed with PBS and cultured in differentiating medium for up to 15 days as indicated. The differentiating medium was changed every day. Cells were lysed in 1 ml of M-PER (catalog no. 78501, Thermo Scientific). 200 μl of lysate was digested by proteinase K (10 μg/ml) for 30 min at 37 °C with gentle shaking and then concentrated by overnight acetone precipitation. The resulting pellet was dissolved in 50 μl of 1× SDS loading buffer, heated for 10 min in a boiling water bath, and analyzed by two-dimensional electrophoresis.

* Treatment of PrPSc with Sialidase—10% (v/v) scrapie brain and spleen materials were diluted 10-fold in 1× PBS supplied with 1% (v/v) Triton X-100 and supplemented with 25 μg/ml PK. After 30 min at 37 °C, PK digestion was stopped by addition of 5 mM PMSF. Then the samples were denatured by incubating for 10 min at 95 °C. The subsequent treatment with *A. ireae-faciens* sialidase (catalog no. P0722L, New England Biolabs) was as follows. After addition of 10% (v/v) sialidase buffer GlycoBuffer1 supplied by the enzyme manufacturer, 200 units/ml sialidase were added, followed by incubation on a shaker at 37 °C for 10–12 h. After treatment, 4× SDS buffer was added to the samples, and they were boiled for 10 min at 95 °C.

* Two-dimensional Electrophoresis—Samples of 25 μl (50 μl in the case of sialidase-treated spleen material or N2a- and myo-tube-produced PrPSc) volume were prepared in loading buffer as described above, solubilized for 1 h at room temperature in 200 μl of solubilization buffer (8 μl urea, 2% (w/v) CHAPS, 5 mM tributylphosphine (cat no. 1632101, Bio-Rad), and 20 mM Tris-HCl (pH 8.0)), alkylated by adding 7 μl of 0.5 M iodoacetamide, and incubated for 1 h at room temperature. Then 1150 μl of ice-cold methanol was added, and samples were incubated for 2 h at −20 °C. After centrifugation for 30 min at 16,000 × g at 4 °C, the supernatant was discarded, and the pellet was resolubilized in 200 μl of rehydration buffer (7 M urea, 2 M thiourea, 1% (w/v) DTT, 1% (w/v) CHAPS, 1% (w/v) Triton X-100, 1% (v/v) ampholyte, and a trace amount of bromphenol blue). Fixed, immobilized, precast immobilized pH gradient (cat no. ZM0018, Thermo Fisher Scientific) strips with a linear pH gradient of 3–10 (catalog no. ZM0018, Life Technologies) were rehydrated in 155 μl of the resulting mixture overnight at room temperature in IGP Runner cassettes (catalog no. ZM0003, Life Technologies). Isoelectrofocusing (first dimension separation) was performed at room temperature with rising voltage (175 V for 15 min, then a 175- to 2000-V linear gradient for 45 min, then 2000 V for 30 min) on a Life Technologies Zoom Dual Power Supply using the XCell SureLock Mini-Cell Electrophoresis System (catalog no. EI0001, Life Technologies). The IGP strips were then equilibrated for 15 min consecutively in (a) 6 mM urea, 20% (v/v) glycerol, 2% SDS, 375 mM Tris-HCl (pH 8.8), and 130 mM DTT and (b) 6 mM urea, 20% (v/v) glycerol, 2% SDS, 375 mM Tris-HCl (pH 8.8), and 135 mM iodoacetamide and then loaded on 4–12% BisTris SDS-PAGE precast gels (catalog no. NP0330BOX, Life Technologies). For the second dimension, SDS-PAGE was performed for 1 h at 170 V. Immunoblotting was performed as described elsewhere, and blots were stained using 3F4 antibody for hamster-derived material or Ab3531 antibody for mouse- or cell-derived material.

* GPI Sialylation Profile Analysis—Two-dimensional Western blotting signal intensities were digitized for densitometry analysis using AlphaView software (ProteinSimple, San Jose, CA). Charged isoform profiles of the non-glycosylated glycoform were built using the “Lane profile” function in the AlphaView program. Normalization was performed with the highest curve signal value taken as 100%.

**Author Contributions**—E. K., S. S., and N. K. performed the experiments. E. K. analyzed the data. E. K. and I. V. B. conceived the study. I. V. B. and E. K. wrote the manuscript. All authors read and approved the final manuscript.

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