Modulation of Glycosaminoglycans Affects PrPSc Metabolism but Does Not Block PrPSc Uptake

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ABSTRACT

Mammalian prions are unconventional infectious agents composed primarily of the misfolded aggregated host prion protein PrP, termed PrPSc. Prions propagate by the recruitment and conformational conversion of cellular prion protein into abnormal prion aggregates on the cell surface or along the endocytic pathway. Cellular glycosaminoglycans have been implicated as the first attachment sites for prions and cofactors for cellular prion replication. Glycosaminoglycan mimetics and obstruction of glycosaminoglycan sulfation affect prion replication, but the inhibitory effects on different strains and different stages of the cell infection have not been thoroughly addressed. We examined the effects of a glycosaminoglycan mimetic and undersulfation on cellular prion protein metabolism, prion uptake, and the establishment of productive infections in L929 cells by two mouse-adapted prion strains. Surprisingly, both treatments reduced endogenous sulfated glycosaminoglycans but had divergent effects on cellular prion levels. Chemical or genetic manipulation of glycosaminoglycans did not prevent PrPSc uptake, arguing against their roles as essential prion attachment sites. However, both treatments effectively antagonized de novo prion infection independently of the prion strain and reduced PrPSc formation in chronically infected cells. Our results demonstrate that sulfated glycosaminoglycans are dispensable for prion internalization but play a pivotal role in persistently maintained PrPSc formation independent of the prion strain.

IMPORTANCE

Recently, glycosaminoglycans (GAGs) became the focus of neurodegenerative disease research as general attachment sites for cell invasion by pathogenic protein aggregates. GAGs influence amyloid formation in vitro. GAGs are also found in intra- and extracellular amyloid deposits. In light of the essential role GAGs play in proteinopathies, understanding the effects of GAGs on protein aggregation and aggregate dissemination is crucial for therapeutic intervention. Here, we show that GAGs are dispensable for prion uptake but play essential roles in downstream infection processes. GAG mimetics also affect cellular GAG levels and localization and thus might affect prion propagation by depleting intracellular cofactor pools.
tions (13). The importance of GAGs in prion pathogenesis is supported by the findings that HS colocalizes with abnormal prion protein deposits in vivo (24, 25). Furthermore, GAG modulators exhibit antiprion activity in animal models (21, 26–29). Studies addressing the question of whether cell-associated GAGs represent attachment factors that enable prion uptake have yielded inconsistent results (21, 30, 31). Importantly, most studies were performed with detergent-extracted or proteinase K–treated prions. Those treatments, however, have drastic effects on the structure and/or amino acid sequence of PrPSc (32) and can alter its cellular uptake and infectivity (33–35). So far, it is unclear if cell-type- and strain-specific differences in the GAG requirements for prion entry and the establishment of chronic infections exist.

Soluble GAGs, such as HS and heparin, as well as GAG-related sulfated polysaccharides, including dextran sulfate, pentosan polysulfate, and suramin, act as GAG mimetics with potent antiprion activity in vivo and ex vivo (12, 20, 26, 29, 31, 36–40). Sulfate moieties of GAG mimetics are required for the antiprion activity (40). Sodium chloride, a competitive inhibitor of the cellular 3′-phosphoadenosine 5′-phosphosulfate, prevents both HS and CS sulfation (41–43) and also decreases PrPSc accumulation in persistently infected cells (31, 44). GAG sulfation also affects PrPSc formation in in vitro assays and thus directly acts on PrPSc amplification (45). So far, a comparative analysis of the effects of GAG modulators on host cell PrPSc, on endogenous sulfated GAGs, and on the individual stages of infection by different strains has not been performed. In this study, we analyzed how the GAG mimetic DS-500 and sodium chloride (NaClO3) affect acute and persistent prion infections by the mouse-adapted prion strains RML and 22L. We analyzed in detail if cellular GAGs act as essential receptors for prion internalization. Our study demonstrates that both DS-500 and sodium chloride reduce endogenous sulfated GAGs but have divergent effects on cell surface and total PrPSc levels. Neither RML nor 22L prions require endogenous GAGs to gain entry into the cell. However, although PrPSc is efficiently taken up by cells, DS-500 or undersulfation during exposure to prions affects the establishment of productive infections and strongly reduces PrPSc in chronically infected cells. Our data underscore the important role of sulfated GAGs as general cofactors for prion replication, either by directly engaging in PrPSc formation or by modulating the cellular levels and distribution of PrPSc.

MATERIALS AND METHODS

Cell culture and reagents. This study was conducted under biosafety containment level 2 in accordance with the German Engineering Act of April 2008. The susceptibility of L929 cells (46) to prion strains was increased by several rounds of cloning and selection of susceptible clones. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. L929 cell clone 15.9 was used for the generation of cell populations persistently infected with 22L and RML prions. CHO cells (ATCC CCL-61) and mutant CHO cells that were variably deficient in GAGs were purchased from the American Type Culture Collection (ATCC) and maintained in Ham’s F-12K medium supplemented with 10% FCS and antibiotics. CHO pgsA-745 (ATCC CRL-2242) cells are defective in GAG synthesis, as they lack functional xylosyltransferase activity. CHO pgsD-677 (ATCC CRL-2244) cells are deficient in N-acetylgalactosaminytransferase and glucuronotransferase and do not produce HS (47). N2a cells were cultured in Opti-MEM supplemented with 10% FCS and antibiotics.

Unless otherwise noted, chemicals were from Sigma (Steinheim, Germany). Proteinase K was obtained from Roth (Karlsruhe, Germany) and Pefabloc from Roche (Mannheim, Germany). Cell culture media and supplements were purchased from Invitrogen (Darmstadt, Germany), and fetal bovine serum was from PAA Laboratories (Pausing, Austria). The ECL plus chemiluminescence kit was from GE Healthcare (Buckinghamshire, United Kingdom). Anti-PrP antibody 4H11 has been described previously and was used for detection of cellular PrP by Western blotting.

Prion infection. Standard brain homogenate preparations from terminally sick mice were used to circumvent potential artifacts due to detergent extraction of scrapie-associated fibrils (35). Brain homogenates are routinely used for in vivo and ex vivo infection (31, 46) and exhibit very high prion titers (34). The mouse-adapted scrapie strains RML and 22L were passaged in C57Bl/6 mice. Brain homogenates (10%) of healthy control mice and terminally sick mice were prepared in Opti-MEM using a Dounce homogenizer. Cell debris was pelleted by centrifugation at 872 × g for 5 min at 4°C, and the resultant brain homogenates were stored at −80°C. A total of 2 × 10⁵ to 1 × 10⁶ cells per well were plated in 24-well plates or on ibidi dishes (ibidi, Martinsried, Germany). The medium was replaced after 24 h or 48 h by 200 μl 1% brain homogenate in fresh medium with supplements. After 5 h, the brain homogenate was replaced by 1 to 2 ml of culture medium. For studying early events of a prion infection, cells were analyzed 18 h postexposure to brain homogenate. Cells were routinely split at a ratio of 1:8 to 1:10 every 3 to 4 days. Prion infection was determined by testing lysates of cells at passage 2 or 3 after prion exposure for protease-resistant PrPSc by Western blotting.

Dextran sulfate and sodium chloride treatment. For de novo infections, cells were plated in 24-well plates; 24 h later, the cells were preincubated with 50 mM NaClO₃ for 24 h or treated with 1 μg/ml DS-500 at the time of exposure to brain homogenates. The cells were exposed to 1% brain homogenates of uninfected or RML or 22L prion–infected mice in the presence or absence of compounds. Five hours postexposure, the medium containing brain homogenate was replaced by fresh medium with or without compounds. The cells were passaged three times before PrPSc formation was assessed by Western blotting. To determine the effects of the compounds on PrPSc accumulation in persistently infected cells, subconfluent monolayers were exposed to 0.01 to 1 μg/ml DS-500 or 5 to 50 mM NaClO₃-containing medium. Seventy-two hours postexposure, the cells were lysed, and the lysates were tested for PrPSc content by Western blotting.

Viability assay (XTT). The viability of a cell population upon treatment with different compounds was determined with the XTT assay (Roche, Mannheim, Germany). L929 cells were plated at a density of 1.5 × 10⁴ cells per well in 96-well plates. The following day, the cells were treated with 50 mM NaClO₃ for 24 h or treated with 1 μg/ml DS-500 at the time of exposure to brain homogenates. The cells were exposed to 1% brain homogenates of uninfected or RML or 22L prion–infected mice in the presence or absence of compounds. Subsequently, 50 μl of the XTT reagent was added to each well. After incubation for 4 h, absorption at 450 nm was measured with a Fluostar Omega plate reader (BMG Labtech, Offenburg, Germany). The average absorption of four control wells was set as 100% viability. The viability of treated cells was compared to the viability of the control cells.

Blyscan sulfated glycosaminoglycan assay. The Blyscan sulfated glycosaminoglycan assay kit (Biocolor, Carrickfergus, United Kingdom) quantitatively measures sulfated proteoglycans and GAGs in biological samples. The assay was performed according to the manufacturer’s instructions. Briefly, 4 × 10⁴ cells were plated in 24-well plates; 48 h later, the cells were treated with 1 μg/ml DS-500 or 50 mM NaClO₃. Twenty-four hours after treatment, the medium was aspirated, and the cells were rinsed with phosphate-buffered saline (PBS). The cells were lysed in papain extraction reagent added to the cell monolayer for 3 h at 65°C. The total cell extract containing total GAGs was harvested, and samples were
centrifuged at 10,000 × g for 10 min. A total of 100 μL of the supernatant was used for the assay.

Flow cytometry. For cell surface PrPSc detection, cells were rinsed with PBS and detached with 1 mM EDTA in PBS. The cells were pelleted for 2 min at 4°C and 314 × g. The cells were resuspended in fluorescence-activated cell sorter (FACS) buffer (2.5% FCS, 0.05% sodium azide in PBS) and incubated with primary antibody for 45 min on ice. After rinsing the cells three times with FACS buffer, they were incubated for 45 min on ice with the appropriate fluorophore-conjugated secondary antibody diluted in FACS buffer. The cells were rinsed three times and resuspended in FACS buffer. To detect dead cells, 7-aminoactinomycin D (7-AAD) was added to all samples. 7-AAD-positive cells were excluded from the analysis. To measure the total PrPSc content in the cells, they were detached and fixed with Roti-Histo fix (Roth, Karlsruhe, Germany) for 10 min at room temperature (RT). The cells were rinsed with PBS and quenched with 50 mM ammonium chloride, 20 mM glycine for 10 min at RT. After rinsing with 0.1% saponin in FACS buffer, the cells were incubated with the primary antibody diluted in saponin buffer for 45 min at RT. After three rinsing steps with saponin buffer, the fluorophore-conjugated secondary antibody was added to the cells for 45 min at RT. The cells were again rinsed three times and resuspended in saponin buffer. Control samples incubated without the first antibody were included in each experiment. Nonspecific fluorescence signals of the secondary antibody (background) in these control samples were subtracted from the measurements of all other samples. Experiments were performed using a Gallios flow cytometer (Beckman Coulter, Krefeld, Germany). A total of 15,000 events per sample were measured using Gallios software (Beckman Coulter, Krefeld, Germany).

Detection of abnormal prion protein by confocal microscopy. Cells plated on ibidi dishes were rinsed thoroughly with PBS and fixed with 4% paraformaldehyde (PFA) for up to 20 min and permeabilized with 0.1% Triton X-100 for 10 min. For detection of PrPSc, cells were incubated with 6 M guanidine hydrochloride (GdnHCl) and thoroughly rinsed with PBS. Samples were blocked with 0.2% gelatin for 30 to 60 min, followed by incubation with primary antibodies diluted in 0.2% gelatin for 45 to 60 min. After three rinsing steps, the cells were incubated for 45 min with fluorophore-conjugated secondary antibodies. Nuclei were counterstained with Hoechst (1:10,000 in PBS). In some instances, the cytoplasms were stained with HCS CellMask blue stain (1:5,000 in PBS) (Life Technologies, Darmstadt, Germany) for 30 min. Confocal laser scanning microscopy was carried out using an LSM 700 laser-scanning microscope (Zeiss, Jena, Germany). Uninfected (mock) cells were included for every individual experiment. Non-specific fluorescence signals of the secondary antibody (background) in these control samples were subtracted from the measurements of all other samples. Experiments were performed using a Gallios flow cytometer (Beckman Coulter, Krefeld, Germany).

RESULTS

DS-500 or NaClO3 decreases endogenous GAG levels and divergently affects PrPSc levels. Sulfation inhibition and the GAG mimicic DS-500 have been shown to strongly impact PrPSc formation in several ex vivo models of prion replication, but their precise effects on the establishment of prion infections remain unclear (20, 37, 39, 40). The effects of DS-500 and NaClO3 treatment on endogenous levels of sulfated GAGs in L929 fibroblasts was first studied with the Blyscan sulfated glycosaminoglycan assay. The assay revealed that the total amount of sulfated GAGs was significantly decreased upon treatment with either DS-500 or NaClO3 (Fig. 1a). To estimate the reduction of cellular HS, immunofluorescent staining of cells using the HS-specific antibody 10E4 (50) was performed (Fig. 1b). In untreated L929 control cells, HS was predominately localized to the cell membrane, with some punctate staining present in the cytoplasm. Upon NaClO3 treatment, cell surface HS was drastically decreased and was redistributed to intracellular vesicular structures. Interestingly, the GAG mimetic DS-500 also reduced cell surface HS. Automated image analysis revealed a significant reduction of HS per cell upon DS-500 and NaClO3 treatment (Fig. 1c). Doses up to 1 μg/mL of DS-500 or 50 mM NaClO3 for 72 h did not negatively affect cell viability (Fig. 1d). Western blot analysis of L929 cells treated with DS-500 or NaClO3 demonstrated that PrPSc was properly glycosylated (Fig. 1e).

Image data analysis. Z-stacks of confocal images were converted to two-dimensional (2D) data by maximum-intensity projection (MIP) using Fiji (http://rsbweb.nih.gov/ij/). The detection of cells with PrPSc uptake was conducted with CellProfiler cell image analysis software (Broad Institute). All further calculations were performed with Matlab 2010b (Mathworks GmbH, Ismaning, Germany). Single cells were identified on the basis of nucleus detection. The nucleus image was smoothed with a Gaussian filter (sigma = 2.97 μm), and nucleus positions were defined by local intensity maxima. Cell regions were subsequently defined by applying a morphological watershed (Matlab function, watershed), where the maximal cell radius was restricted to 21.0 μm. Cell regions touching the image border were excluded from analysis. Subsequently, the mean intensity of the 10E4 signal per cell was determined. The same processing routine was used for determining PrPSc intensities in cells exposed to prions. Here, only cells detected as positive for PrPSc uptake, defined by an intensity threshold, were selected for image analysis. To analyze HS and PrPSc spatial distributions within the cell, we adopted a method for averaging of cell images, as previously described (49). In brief, the orientation of each cell was defined by the angle where the spatial spread of the fluorescence signal was maximal. On the basis of this angle and the nucleus position, each cell was transformed into a coordinate system with the nucleus located at the system’s origin and the cell’s orientation along the major axis. This allowed the calculation of “average cells” (see Fig. 1c, 3d, and 4c).

Statistics. Data sets were analyzed with one-way analysis of variance (ANOVA). To evaluate statistically significant differences between control sample data and treated-sample data, Dunnett’s or Kruskal-Wallis multiple-comparison post hoc tests were used. P values of less than 0.05 were considered significant. The sample size was three or more.
DS-500 or NaClO₃ reduces PrPSc accumulation in chronically infected L929 cells. To test the antiprion activities of both compounds in our cellular model, L929 cells persistently infected with mouse-adapted prion strain RML or 22L were exposed to DS-500 and NaClO₃ for 3 days. Western blot analyses of PrP Sc revealed a robust, dose-dependent decrease in PrP Sc abundance (Fig. 2a and b). Thus, in agreement with previously published data (31), our data confirm that the GAG mimetic DS-500 and NaClO₃ exert general antiprion effects in chronically infected cells independent of the scrapie strain. The fact that both treatments reduced PrPSc content but had divergent effects on total and cell surface PrP⁰⁰ levels argues that PrP⁰⁰ and PrPSc levels are not necessarily correlated.

Internalization of PrPSc does not require sulfated GAGs. To test the effects of DS-500 and NaClO₃ on PrPSc uptake, uninfected L929 cells pretreated with compounds were exposed to prion strain RML or 22L or uninfected control brain homogenate for 5 h. The rinsed cells were subsequently cultured for an additional 13 h in the absence of inoculum. Western blot analyses of cells lysed 18 h after exposure to prions demonstrated that the PrPSc signal in inoculum-exposed cells differed in both glycosylation profile and molecular mass from that in persistently infected L929 cells loaded as a control (Fig. 3a, top). This strongly argues that PrPSc associated with the cells represents PrPSc from the inoculum and not de novo formed PrPSc. The result was further confirmed by exposing prion-susceptible L929 cells expressing antibody epitope-tagged mouse PrP (3F4-L929 cells) (46) to prion strains RML and 22L. No 3F4-positive PrPSc could be detected within 18 h (Fig. 3a, bottom), strongly arguing that PrPSc detected at this time point originates from the inoculum. For detection of internalized PrPSc,
fixed cells were treated with GdnHCl prior to immunofluorescence staining, a method known to drastically increase immunostaining for PrPSc (Fig. 3b) (51, 52). Image analysis revealed that approximately 63.92% ± 25.71% of L929 cells had taken up exogenous RML PrPSc. 22L PrPSc was taken up by approximately 81.5% ± 10.29% of the cells. For statistical analysis between independent experiments, the numbers of cells that had taken up PrPSc were normalized to the untreated control cells, set to 100% (Fig. 3c). No significant difference in uptake was observed for RML PrPSc in the presence or absence of the compounds. Interestingly, significantly fewer cells internalized 22L PrPSc when exposed to NaClO3, suggesting that 22L PrPSc uptake is more sensitive to sulfation perturbation (Fig. 3c). We further quantitatively assessed the amount of PrPSc taken up per cell in the presence or absence of either compound (Fig. 3d and e). No significant difference in RML PrPSc was detected upon DS-500 treatment, whereas 22L PrPSc was slightly but significantly decreased. Assumption of at least 176 cells per treatment revealed that undersulfation by NaClO3 slightly but significantly increased total levels of PrPSc per cell, independent of the scrapie strain (Fig. 3d and e). The reason for the increased PrPSc signal per cell is unclear but might be related to a slight change in cell size upon NaClO3 treatment (Fig. 3b). Overall, while slight differences in uptake efficiency exist, L929 cells treated with DS-500 or NaClO3 are still capable of internalizing RML or 22L PrPSc. Efficient PrPSc uptake was also observed in N2a cells treated with DS-500 or sodium chloride, arguing that the results were not dependent on the fibroblast line (Fig. 3f). Thus, the antiprion effects of DS-500 and NaClO3 are not due to blockage of PrPSc internalization.

The experiments were repeated in CHO cells deficient in GAG synthesis (Fig. 4). CHO cells are not permissive to prions but can be used to monitor GAG-independent uptake of pathogens and cargo from the extracellular space (21). CHO pgsD-677 cells lack functional N-acetylglucosaminyltransferase and glucuronyltransferase and thus do not synthesize HS. CHO pgsA-745 cells are defective in xylosyltransferase and do not produce any GAGs (47). Lack of GAG or HS synthesis did not drastically affect PrPSc uptake or even increased intracellular PrPSc levels (Fig. 4a to c). CHO cells lacking HS expression appeared to be efficient in internalization of both RML and 22L PrPSc, as the numbers of cells that had taken up PrPSc were significantly increased compared to wild-type CHO cells (Fig. 4a and b). Deficiency in HS synthesis also increased PrPSc levels per cell (Fig. 4c and d). The fact that PrPSc was efficiently endocytosed by prion-permissive L929 cells with reduced sulfated-GAG expression and by CHO cells that did not
FIG 3 Initial uptake of RML and 22L prions is not inhibited by DS-500 and NaClO₃. (a) PrPSc detected 18 h after exposure to brain homogenate originates from the inoculum. Uninfected L929 cells (top) or uninfected 3F4-L929 cells (bottom) were exposed to RML or 22L prions for 5 h. Eighteen hours postexposure, the cells were lysed. Total PrPSc and de novo generated PrPSc were detected following PK treatment of cell lysates using MAb 4H11 or MAb 3F4, respectively. Experiments were performed in triplicate. Additional lanes were excised for presentation purposes. The loading control (ldg ctrl) for L929 cells was PK-treated lysate of persistently infected L929RML and L92922L cells. The loading control for 3F4-L929 cells was PK-treated lysate of persistently infected 3F4-L92922L cells. Note that the glycosylation profile and size of cell-derived PrPSc in the loading controls differed from those of brain-derived PrPSc detected 18 h postexposure.
express GAGs strongly argues for a GAG-independent mode of attachment and internalization of PrP<sub>Sc</sub>.

**DS-500 and NaClO<sub>3</sub> inhibit initiation of prion infection.** Next, we tested if internalization of PrP<sub>Sc</sub> in the presence of compounds results in productive infections. For DS-500 exposure, cells were incubated for 5 h with infectious brain homogenate in the presence or absence (control) of the compound. Subsequently, brain homogenate was replaced with fresh medium or

(b) Confocal microscopy analysis of immunofluorescence staining of PrP<sub>Sc</sub> in compound-treated cells 18 h after exposure to prions. Uninfected L929 cells were pretreated with 1 μg/ml DS-500 or 50 mM NaClO<sub>3</sub> for 24 h prior to exposure to prions in the presence of compounds for 5 h. Inoculation was performed in the presence of compounds for 18 h. Cells exposed to mock brain homogenate and untreated cells served as controls. PrP<sub>Sc</sub> was detected by antigen retrieval using MAb 4H11. Nuclei were counterstained with Hoechst. Scale bars, 10 μm.

c) Fluorescent signal of PrP<sub>Sc</sub> (red channel in panel a) averaged over a minimum of 620 cells per cell line/treatment, which were defined as PrP<sub>Sc</sub>-positive. The cells were aligned along the vertical axis by an automated image-processing routine. The PrP<sub>Sc</sub> intensities of PrP<sub>Sc</sub>-positive cells in panel c were calculated using an automated image-processing routine. The PrP<sub>Sc</sub> intensities in pgsA-745 and pgsD-677 cells were normalized to CHO-K1 control cells, set to 100%. The box plots illustrate the median of PrP<sub>Sc</sub> intensity, and the whiskers display the 5th to 95th percentiles (***, P ≤ 0.001; *, P < 0.05; ns, not significant; Kruskal-Wallis test).
with medium containing DS-500 for an additional 19 to 43 h (Fig. 5a). Cells were expanded and tested for PrPSc production two passages postinfection by Western blot analysis (Fig. 5b). Interestingly, even short exposure of cells to DS-500 during the infection process drastically impaired PrPSc formation. The effect was more pronounced for strain RML, where 5-h treatment during infection decreased PrPSc levels in the infected cultures to approximately 8% of that in controls (Fig. 5c). PrPSc levels in cells exposed to 22L prions were approximately 15% of that in control cells after 24-h treatment with DS-500.
Cells pretreated with NaClO₃ for 24 h to inhibit endogenous GAG sulfation were subsequently exposed to prions in the presence of the inhibitor for 5 h. Drug treatment was terminated or continued for an additional 19 to 43 h (Fig. 5d). Again, drug treatment had more pronounced effects on the establishment of RML prion infections (Fig. 5e and f). Here, pretreatment for 24 h and subsequent infection in the presence of the sulfation inhibitor reduced PrPSc levels to less than 30% of those of untreated controls (Fig. 5e). Interestingly, NaClO₃ treatment during the first 5 h of infection (29-h total drug treatment) did not significantly impair the establishment of 22L infections (Fig. 5f). Only after extended incubation with NaClO₃ (i.e., a total of 48 h) was a reduction in the PrPSc signal observed in 22L-infected cells (Fig. 5f). Thus, both DS-500 and NaClO₃ significantly impair the establishment of prion infections when administered within the first hours of infection, albeit to different degrees. In summary, DS-500 and NaClO₃ do not do antagonize initial prion-host cell interactions, but rather, target downstream events following prion uptake.

DISCUSSION

Interference with endogenous GAG metabolism by GAG mimetics or impairment of cellular sulfation has been shown to drastically interfere with PrPSc formation in prion-infected cells (21, 30, 31, 37, 39, 40, 44), but the effects of the substances on different stages of the infection process have not been thoroughly assessed. In this study, we have examined in detail the effects of the GAG mimetic DS-500 and GAG undersulfation on endogenous GAG and PrPSc levels, as well as on PrPSc uptake and PrPSc accumulation during acute and persistent infection. We also compared the effects of the compounds on two different prion strains to assess any potential differences in prion strain infections. While slight differences in sensitivities of strains RML and 22L to DS-500 and undersulfation exist, both treatments impaired the establishment of productive infections and significantly reduced PrPSc levels in chronically infected cells. The observed antiprion effects of the GAG mimetic or GAG undersulfation underline the importance of GAGs as general factors for prion biogenesis (14).

Cellular pathogens, such as viruses and microorganisms, commonly bind to GAG moieties on the cell surface to adhere to and gain entry into their target cells (15). The affinity of intracellular pathogens for certain GAGs that serve as coreceptors can be an important determinant of organ tropism and pathogenesis (53) and could also affect the internalization of different prion strains. Indeed, several studies argue that GAGs are essential coreceptors for PrPSc uptake (30, 54, 55). Recent studies even suggest that GAGs also mediate cellular internalization of misfolded protein aggregates in several neurodegenerative diseases (56, 57). Of note, however, quantitative assessment of PrPSc internalization was exclusively performed by Western blot analysis (21, 30, 31), a method that cannot accurately discriminate between cell surface-bound and internalized PrPSc. We therefore performed detailed analysis of immunolabeled cells, which allowed us to determine both the number of cells that had taken up PrPSc and the PrPSc intensity per cell. Our studies showed that treatment of L929 cells with DS-500 or sodium chloride significantly reduced endogenous sulfated-GAG levels but did not inhibit PrPSc internalization. Similarly, the mouse neuroblastoma cell line N2a also efficiently took up PrPSc under the same treatment conditions, arguing that endogenous GAGs might not be required for prion invasion. In line with these findings, both RML and 22L PrPSc were efficiently taken up by CHO cells deficient in GAG expression. Interestingly, PrPSc uptake by CHO cells appeared to be significantly increased when the cells did not synthesize HS. One possible explanation for this finding is that bypassing PrPSc attachment to HS on the cell surface could allow direct binding to functional prion receptors, such as the laminin receptor precursor LRP/LR (54, 55, 58) or low-density lipoprotein-related protein 1 (LRP1) (59), both of which are expressed by L929 cells (data not shown). Our findings are consistent with results by Paquet and colleagues, who demonstrated efficient sheep scrapie PrPSc association with rabbit kidney epithelial cells in the presence of sodium chloride or the GAG mimetic DS-500 (31). Likewise, another study demonstrated that DS-500 concentrations that inhibit PrPSc accumulation in RML-infected N2a cells were ineffective at inhibiting PrPSc binding to the cell (30). The conflicting results for PrPSc binding and uptake might, at least in part, be explained by prion strain differences. Some indication of this comes from *in vitro* studies using human enterocytes that readily take up bovine spongiform encephalopathy (BSE) PrPSc but do not appear to internalize mouse-adapted scrapie (55). While we did not observe such drastic strain-specific effects, slight differences in strain-dependent uptake were apparent upon undersulfation of endogenous GAGs. Thus, the presence of endogenous sulfated GAGs might still slightly influence the uptake of certain prion strains.

Importantly, however, different PrPSc preparations could also have an impact on cellular uptake. Several studies have been performed using PK-treated prion brain homogenate or PK-treated, detergent-extracted PrPSc rods (21, 30). As PK also eliminates approximately 90 amino-terminal PrP residues, including GAG-binding motifs comprising amino acid residues 23 to 52 and 53 to 93 (16), binding affinities of PrPSc to putative cell surface receptors might be altered. Furthermore, detergent extraction has been shown to drastically change PrPSc infectivity and the kinetics of PrPSc uptake, possibly by artificially altering its aggregation state upon extraction (32–35, 55). The effect of prion preparations on PrPSc uptake was nicely demonstrated in human Caco-2/TC7 enterocytes that internalized BSE PrPSc from brain homogenate but did not take up PK-treated scrapie-associated fibrils (55). Of note, components other than PrPSc present in the brain homogenate could also influence prion uptake. However, even highly purified fractions of PrPSc still contain substantial amounts of non-PrP components (60–62). Clearly, the still undefined exact composition and structure of infectious prions add another layer of complexity to prion cell biology.

Our data suggest that GAG mimetics and prevention of endogenous GAG sulfation affect events downstream of the initial PrPSc attachment and internalization. Antiprion effects were apparent when drugs were administered during the early phases of prion infection and in persistently infected L929 cells, arguing that sodium chloride and DS-500 target both initiation and maintenance of productive prion infections. Interestingly, higher concentrations of drugs were required for reducing PrPSc levels in L929 cells acutely or persistently infected with prion strain 22L. Whether this is related to higher levels of PrPSc in 22L-infected than in RML-infected L929 cells or strain-specific differences in sulfated-GAG dependence is so far unclear. However, the fact that reduced PrPSc levels were detected in both 22L- and RML-infected L929 cells strongly suggests that sulfated GAGs are generally required for efficient prion infection and PrPSc accumulation.

The effect of GAG undersulfation or GAG mimetics on PrPSc ac-
cumulation and prion infection could also be due to changes in substrate PrP\textsuperscript{C} and GAG metabolism (18). Both sodium chloride and DS-500 treatments significantly reduced cellular HS levels and total sulfated GAGs. PrP\textsuperscript{C} can interact with endogenous GAGs through specific binding sites comprising amino acid residues 23 to 52, 53 to 93, and 110 to 128 (16), and interactions of PrP\textsuperscript{Sc} with GAGs is required for proper intracellular trafficking (18, 20, 44). Thus, any change in cellular GAG levels and distribution likely also affects PrP\textsuperscript{C} metabolism. Changes in endogenous sulfated-GAG levels could affect transport of PrP\textsuperscript{Sc} to subcellular sites of conversion and thereby modulate PrP\textsuperscript{Sc} metabolism. The conformational transition of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} is believed to involve direct contact between the two PrP isoforms. As RNA (22). If and where PrP associates with nucleic acid in the cell and how GAGs modulate this interaction remain to be established.

We conclude that DS-500 and sodium chloride could affect PrP\textsuperscript{Sc} biogenesis downstream of PrP\textsuperscript{Sc} uptake by (i) altering the subcellular distribution and levels of PrP\textsuperscript{Sc} (18), (ii) changing the levels and localization of endogenous sulfated glycosaminoglycans, or (iii) directly interfering with the conversion process. A better understanding of the subcellular compartments of prion propagation and the molecular process of PrP\textsuperscript{Sc} formation will help to define the exact mechanism by which GAGs affect prion biogenesis.

ACKNOWLEDGMENTS

We thank Ireen König for assistance with microscopy and Dino Dimonte and Dan Ehninger for helpful comments on this work. Financial support was provided by the Deutsche Forschungsgemeinschaft (VO1277/1-3, HAI-Neurodegeneration, and APRI Exploration III grant Cell Tropism and Prion Strains).

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