HEPARAN SULFATE IS A CELLULAR RECEPTOR FOR PURIFIED INFECTIOUS PRIONS

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Summary

Prions replicate in the host cell by the self-propagating refolding of the normal cell surface protein, PrP\(^c\), into a \(\beta\)-sheet rich conformer, PrP\(^Sc\). Exposure of cells to prion-infected material and subsequent endocytosis can sometimes result in the establishment of an infected culture. However, the relevant cell surface receptors have remained unknown. We have previously shown that cellular heparan sulfates (HS) are involved in the ongoing formation of PrP\(^Sc\) in chronically infected cells. Here we studied the initial steps in the internalization of prions and in the infection of cells. Purified prion “rods” are arguably the purest prion preparation available. The only proteinaceous component of rods is PrP\(^Sc\). Mouse neuroblastoma N2a, hypothalamus GT1-1, and Chinese hamster ovary CHO cells efficiently bound both hamster and mouse prion rods (at 4° C) and internalized them (at 37° C). Treating cells with bacterial heparinase III or chlorate (a general inhibitor of sulfation) strongly reduced both rods binding and uptake, whereas chondroitinase ABC was inactive. These results suggested that the cell surface receptor of prion rods involves sulfated HS chains. Sulfated glycans inhibited both rods binding and uptake, probably by competing with the binding of rods to cellular HS. Treatments that prevented rods endocytosis also prevented the de novo infection of GT1-1 cells when applied during their initial exposure to prions. These results indicate that HS are an essential part of the cellular receptor used both for prion uptake and for cell infection. Cellular HS thus play a dual role in prion propagation, both as a co-factor for PrP\(^Sc\) synthesis and as a receptor for productive prion uptake.

Introduction

The transmissible spongiform encephalopathies (TSE), which comprise infectious, familial, and sporadic neurodegenerations such as Creutzfeldt-Jakob disease (CJD) of humans (1), scrapie of sheep and bovine spongiform encephalopathy (BSE) (2), are caused by prions (3). These proteinaceous agents are thought to propagate by refolding a normal cell surface glycoprotein of the host, the cellular prion protein PrP\(^c\), into an abnormal \(\beta\)-sheet rich (4,5) conformation (reviewed in 6). The resulting pathological conformer, PrP\(^Sc\), is in turn the only known component of the infectious prion. The formation of PrP\(^Sc\) is thought to involve a direct contact between “seed” PrP\(^Sc\) and “substrate” PrP\(^c\) (7,8), and probably involves cellular co-factors (9) including the laminin receptors (10-12) and cellular heparan sulfate proteoglycans (HSPG) (13-15).

Although several cell lines are susceptible to prion infection (16, reviewed in 17), the molecular mechanisms involved remain largely obscure. Infection is usually started by exposing cells to prion-infected material, such as brain homogenate. Many cell types (including cell lines (18) and primary dendritic cells (19)) can internalize prion-infected material, but the cellular receptors for prions have not yet been identified. One factor that is likely to complicate the study of how prions enter cells is their notorious association with heterogeneous cellular membranes and aggregates (which contain in addition other cellular components). Thus, prions in crude tissue homogenates are likely to ‘hitch-hike’ their way into the cell using aggregates and microsomes as vehicles, via a variety of cell-surface receptors. However, prions can be extracted from membranes to yield purer preparations. Prion “rods” are arguably the purest form of prions known (20,21). These infectious, unbranched amyloidic structures are prepared from prion-infected tissues by the combined action of detergents and proteases (22) (often supplemented by nucleases) and their only proteinaceous component is PrP27-30 (23), the protease-resistant core of PrP\(^Sc\). The size of rods is very heterogeneous, and they may contain up to several thousands PrP molecules (20). Although purified prion rods are efficiently taken up by a variety of cells and often lead to productive infections (18), even in this highly simplified situation the relevant cellular receptors have not yet been identified. Here we set out to characterize receptors for rods in two infectible mouse cell lines, the neuroblastoma N2a (24) and the hypothalamic cell line GT1-1 (25-27), and Chinese hamster ovary...
(CHO) cells, which seem to be refractive to prion infection.

Glycosaminoglycans (GAGs) such as heparan sulfate (HS) are long, unbranched side chains of proteoglycans that are found in several cellular compartments including the cell surface and endosomes (reviewed in 28). Three arguments put forward GAGs as candidates for cellular receptors for prion rods. First, PrP has several heparin-binding sites both in the N-terminal unstructured region and in the PrP27-30 core (13,29,30). Second, cellular HS are required for PrP\textsuperscript{Sc} formation in persistently infected mouse neuroblastoma ScN2a cells (15). Since these HS prion co-factors probably perform their task by binding the endogenous PrP isoforms, it is plausible that they can also bind PrP27-30 molecules found in exogenous rods. Another indication that GAGs may serve as prion receptors is the finding that certain soluble dextran-based heparan mimetics (HMs) (31) reduce the internalization of prion rods in both N2a and CHO cells (32). Conceivably, this inhibition could result from the competition of soluble HM molecules with putative HS receptors of rods.

To evaluate the role of cellular HS in the binding and endocytosis of prion rods, we used GAG-degrading enzymes, the sulfation inhibitor chlorate, and soluble glycans including several HM species. Our results indicate that heparinase III-sensitive HS on the cell surface are involved in both the binding and the uptake of rods in N2a, CHO and GT1-1 cells. Treatments that prevented the binding and internalization of rods also prevented the \textit{de novo} infection of GT1-1 cells. Cellular HS are thus an essential component of cellular receptors for the uptake of prions and the infection of cells.

**Experimental Procedures**

**Materials.** Cell culture reagents were purchased from Biological Industries (Beit Haemek, Israel). OptiMem was from Gibco BRL (Invitrogen, Rand-Island, NY). Dextran sulfate 500 (150821) was from ICN (Costa Mesa, CA). Heparinase I (heparinase EC 4.2.27) and heparinase III (heparitinase I, EC 4.2.2.8) were from IBEX Technologies (Montreal, Canada). Chondroitinase ABC (EC 4.2.2.4) was from Seikagaku Corp. (Tokyo, Japan). The dextran-based heparan mimetics (HMs) (32) were obtained from OTR\textsuperscript{3}Sarl (Creteil, France). Micrococcal nuclease (N-5386) was from Sigma (St Louis, Mo). Recombinant mouse PrP23-231 and recombinant R1 and D13 PrP Abs were from InPro (South San Franciscoco, CA). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Porcine mucosa HS was kindly provided by Dr. K. M. Shwann (KabiPharmacia, Stockholm, Sweden).

**Preparation of prion rods.** Prion rods were purified from the brains of Syrian hamsters and of C57/bl mice infected with experimental Sc237 and RML scrapie, respectively, using a procedure modified from Prusiner et al. (33) and Diringer et al. (34). All steps were performed at 4° C. One hamster brain or three mouse brains (a total of about 1g) were homogenized in 20 ml of 10% sucrose in PBS (buffer A). A 3220 g\textsubscript{av}, 10 min pellet was rehomogenized in 10 ml buffer A and then re-pelleted, and the supernatants were united and cleared by a 3220 g\textsubscript{av}, 30 min spin. The supernatant was made 1mM with each EDTA and dithiothreitol (DTT), and Triton X-100 and Na-deoxycholate were added to 4% and 2% final concentrations, respectively. The supernatant was then stirred for 30 min and the following reagents were added dropwise while stirring: Tris acetate (pH 8.3) to 30 mM, KCl to 100 mM, glycerol to 20%, and PEG10000 to 8% w/v. After an additional 30 min stirring, the homogenate was pelleted at 4500 g\textsubscript{av}, 30 min. The pellet was resuspended in 20 mM Tris acetate, pH 8.3, 0.02% TX-100, 1 mM DTT, and 2 mM CaCl\textsubscript{2}, and then digested with micrococcal nuclease (12.5 units/ml) for 16 h at 4° C. The nuclease reaction was stopped by the addition of 2 mM EDTA and 0.2% Sarkosyl. The homogenate was then subjected to proteolysis with proteinase K (100 µg/ml, 8 h, 4° C), and the reaction was stopped by incubating for 30 min with 100 mM phenylmethylsulfonyl fluoride (PMSF). Sarkosyl was added to 1%, and after 30 min incubation on ice, the homogenate was spun at 100,000 g\textsubscript{av}, for 1 h. The pellet, which contains the prion rods, was resuspended in 500 µl TNS (10 mM Tris pH 7.5, 150 mM NaCl, 1%
Sarkosyl) using a probe sonicator (Sonopuls, Dandelin Electronics, Germany; full power, 4x1 sec) and then repelleted (3x). Sarkosyl was removed by rinsing the pellet twice with 70% EtOH, (100,000 g, 30 min), and then resuspended by sonication in 600 µl of 30% sucrose in TN. A 5 µl sample of this rods preparation was analyzed by electrophoresis and silver staining (Fig. 1A, right lane), and compared to 10 ng recombinant mouse PrP (Fig. 1A, left lane). Hamster rods contained a single 27-30 kDa band (Fig. 1A) which is characteristic of Syrian hamster Sc237 PrPSc, whereas mouse rods had three characteristic glycoforms. The PrP concentration in rods preparations was determined by comparing with dilution of recombinant mouse PrP in western blots developed with the recombinant Fab, R1 (Fig. 1B). Rods preparation usually contained about 5 ng/ml of PrP27-30.

**Cells** Mouse neuroblastoma ScN2a-M are ScN2a cells (24) that stably expresses the MHM2-PrP chimera that reacts with the mAb 3F4 (35). An uninfected version (N2a-M) was obtained by curing ScN2a-M cells with pentosan polysulfate (5 µg/ml, 5 d) (36) and subsequently maintaining them without inhibitors for at least 1 month prior to use. GT1-1 are mouse hypothalamus cells (27). GT1-1-M stably express MHM2-PrP and react with 3F4. Cells were grown at 37°C in low glucose DMEM-16 (N2a and GT1-1) or F12 (CHO-K1, (37)) containing 10% fetal calf serum. In some experiments, cells were maintained in a 1:1 mixture of the above media and OptiMem (Gibco BRL). To assess the susceptibility of GT1-1-MHM2 cells to infection, cells growing on 12 well trays were treated with the relevant inhibitors for 24 h. Thereafter, the cells were inoculated either with purified mouse rods for 24 h or with cell supernatant of ScGT1-1 (the medium was frozen and thawed at least 3 times before addition to the cells) for 48 h at 37°C in DMEM-OptiMem (1:1) in the presence of the inhibitors. The cells were then rinsed and further grown in fresh medium (without inoculum or treatments) for either 5 or 10 d, as indicated in Fig. 5. Since the mouse inocula are not recognized by the mAb 3F4, successful infection was identified by the appearance in the cells of 3F4-reactive PrPSc (as depicted in Fig. 5B).

**PrP isoforms and analysis** PrPSc was defined as the PrP fraction resistant to proteinase K (PK, 20 µg/ml, 37°C, 30 min). Western blots (WB) were carried out as described (38). The protein content of parallel samples was normalized using a Bradford kit (500-006) from Bio-Rad (Hercules, CA) prior to electrophoresis. Cell lysates (in ice-cold lysis buffer: 0.5% Triton X 100, 0.25% Na-deoxycholate, 150 mM NaCl, 10 mM Tris-Cl, pH 7.5, 10 mM EDTA), were immediately centrifuged for 40 sec at 14,000 rpm in a microfuge and biochemical analyses were performed on the post-nuclear supernatant.

**Rods internalization and binding** assays were carried out on confluent monolayers grown in 24 wells trays. Cells were treated with enzymes or inhibitors for 24 h at 37°C prior to the assays, unless otherwise indicated. Rods (1µl/well, ca 5 ng of PrP, Fig. 1B) were then added either for 20 h at 37°C (internalization) or for 3 h at 4°C (binding assay; performed in PBS or in F12/OptiMem, 1:1, with identical results). The cells were then rinsed with ice-cold PBS, lysed, and the protein content was normalized using the Bradford method. The samples were incubated with 2 µg/ml PK (1 h, 37°C, stopped by 2 mM PMSF), and the level of cell-associated PrPSc (from the exogenous rods) was assayed by WB developed with 3F4 mAb (for Syrian hamster rods) or D13 Fab (for mouse rods).

**Fluorescence microscopy** For internalization experiments, cells growing on 8 well slides (Nunc, (Roskilde, Denmark) were pre-treated with the relevant inhibitors for 24 h, and then exposed for 20 h to purified Syrian hamster prion rods with or without inhibitors. At the end of the incubation the cells were rinsed and further incubated in normal medium for 4 h to reduce the rods signal on the cell surface. The cells were then fixed (8% formalin in PBS, 30 min, RT), denatured in situ (3 M GdnSCN, 0.1% TX-100, 50 mM Tris–HCl, pH 7.5; RT, 5 min) (18) to visualize rods, immunostained with 3F4, and examined by fluorescence microscopy.
Results

**Inhibition of rods uptake by soluble glycans correlates with their anti-prion activity**

It is well established that certain glycans reduce the ongoing formation of PrPSc in chronically infected cells. To see if cellular GAGs might be part of a cellular receptor for prions, we thus first studied the extent to which several glycans reduce the uptake of prion rods, as compared to their anti-prion efficacy. To this end we chose the “classical” anti-prion dextran sulfate (DS500) (36), and 3 members of the HM library of substituted dextrans with vastly different anti-prion potencies (32): B103, HM2102, and HM2602 (Fig. 2). Chronically infected ScN2a-M cells were treated for 5 days with these compounds, and protease-resistant PrPSc was then analyzed by WB developed with 3F4 (panel A). As expected, the more sulfated and/or benzylaminated compounds (DS500, HM2602 and B103) reduced PrPSc more efficiently than the non-sulfated HM2102 (32). Next, we turned to determine the extent to which these glycans decrease rods uptake in uninfected N2a-M cells. The cells were pre-incubated for 24 h with the polyanions, as in panel A. Purified Syrian hamster rods were then added to the cell medium for an additional 20 h in the presence of the inhibitors. The cells were then rinsed thoroughly, and their protease-resistant PrP was monitored in WBs with 3F4 (panel B). The ability of these compounds to reduce rods internalization correlated well with their anti-PrPSc potency (compare panels A and B and Fig. 6 we have previously reported that HMs are not general endocytosis inhibitors (32)). Interestingly, higher concentrations of inhibitors were required to reduce rods uptake than to reduce endogenous PrPSc (see Discussion).

**Anti-prion heparinase III and chlorate inhibit rods uptake**

This correlation suggested that there may be a mechanistic relationship between the two anti-prion activities of these polyanions, namely that the cellular HS that are needed for the ongoing synthesis of PrPSc (15) also form parts of internalization receptors for rods. To verify that HS are involved in rods uptake, we explored other treatments that inhibit or digest cellular HS (Fig. 3). N2a-M (panel A) and CHO-K1 (panels B and C) cells were pre-treated for 24 h with either heparinase I or heparinase III to digest away HS, or they were incubated with the metabolic sulfation inhibitor, Na chlorate (30 mM) (39), or with soluble glycans, as indicated in panel B. Rods were added to the cell medium and were allowed to enter the cells for 20 h. The cells were then harvested and PK-resistant PrP was detected using either WB (Fig. 3A-B) or immunofluorescence (IF, Fig. 3C). Heparinase III, Na-chlorate, dextran sulfate DS500 and HM2602 almost completely abolished rods uptake, while heparinase I was only slightly inhibitory and the inactive HM compound HM2102 failed to reduce the uptake of rods altogether. These results correlate perfectly with the anti-prion potency of these treatments (Fig. 2 and (15)). Similar inhibitory results were obtained when mouse rods were applied to GT1-1 cells (Fig. 5A). In contrast to heparinase III, chondroitinase ABC failed to reduce rods uptake (Fig. 3B), correlating with its inability to reduce PrPSc in chronically infected ScN2a (15). Whether this is caused by the paucity of chondroitin sulfate in these cells or by other mechanisms remains to be determined.

**Heparinase III-sensitive HS mediate both binding and internalization of rods**

These results strongly suggested that heparinase III-sensitive cellular HS are part of endocytosis receptors for purified prion rods in N2a, CHO-K1 and GT1-1 cells. Prions also bound to cell surface HS at 4ºC. N2a-M (not shown) or CHO-K1 cells (Fig. 4) were pre-treated for 24 h with heparinase I, heparinase III, Na-chlorate (panel A), or dextran-based polyanions (panel B). The plates were then cooled on ice (to prevent endocytosis) and further incubated with purified rods in the presence of the inhibitors (3 h on ice). At the end of the incubation, the cells were rinsed thoroughly and cell surface bound rods were monitored by WB. All the efficient anti-PrPSc treatments also effectively reduced the binding of purified rods to the surface of these cells. Thus, only heparinase I and HM2102 left this binding intact (Fig. 4A, B).

In addition to removing HS GAGs from the cell surface, heparinase III is also likely to release HS fragments to the cell medium. By
analogy with other sulfated glycans, these degradation products could also inhibit rods binding by competing with surface receptors. It was therefore necessary to verify directly that the presence of HS chains on the cell surface perform as a binding receptor for prion rods. For this purpose, we incubated CHO-K1 cells with heparinase III (0.1, 1 or 2 units/ml) for 24 h (37°C). Then the cells were rinsed with ice-cold PBS to remove any soluble heparinase products, supplemented with fresh medium without heparinase, and rods were added to all cells (3 h on ice). At the end of the incubation the cells were rinsed thoroughly and the cell surface bound rods were monitored in WB (Fig. 4C). The cells treatment with heparinase III inhibited rods binding to the cells (lane 3-4). Taken together, the results described above indicate that: (i) heparinase III-sensitive HS play a crucial role both in the binding and in the internalization of prion rods in N2a and in CHO-K1 cells, and (ii) that these HS molecules are similar or identical to those that serve as co-factors for the ongoing formation of PrP\textsuperscript{Sc} in ScN2a cells.

We next asked whether endogenous HS are required for rods binding, or whether exogenous HS can mediate binding of rods to another cellular receptor. For this purpose CHO-K1 cells were treated with chlorate for 24 h to stop the sulfation of cellular HS. Exogenous HS (50 µg/ml) were then added to the cell medium along with prion rods, the cells were further incubated for 3 h on ice, and then cell-bound rods were analyzed by WB. As shown in Fig. 4D, exogenous HS did not mediate rods binding to other cell surface receptors. These results demonstrate conclusively that cellular HS are needed for prion rods binding.

**Infection of GT1-1 cells requires cellular HS during the exposure to the inoculum**

Having found that cellular HS are required for the binding and uptake of prions in N2a and CHO-K1 cells, we next examined their implication for the establishment of a productive infection. To this end, we chose GT1-1 cells (27) since they are infected more readily than N2a, but this time we used mouse prions in order to avoid the hamster/mouse species barrier. Mouse PrP\textsuperscript{Sc} is easily recognized by its characteristic glycoform triplet (Fig. 5A). First, we confirmed that heparinase III, chlorate and the anti-prion polyanions also prevent the uptake of rods in these cells. GT1-1 cells were exposed to mouse rods for 20 h, and the uptake of rods was monitored by WB (Fig. 5A).

Next, we examined whether HS mediate long term infection by prion rods. In order to distinguish more easily PrP\textsuperscript{Sc} made *de novo* in the cells (which denote a productive infection) from the mouse prion inoculum, (which is endocytosed by the cells), we prepared GT1-1-MHM2 cells which stably express a 3F4-tagged mouse PrP. First, we exposed these cells to mouse rods (Fig. 5C, left panel). The cells were treated with either chlorate or HM2602 for 24 h, and then rods were added to the cell medium for another 24 h incubation (in the presence of the inhibitors). The cells were then rinsed, and further incubated in fresh medium (without rods or inhibitors) for 5 d to allow PrP\textsuperscript{Sc} to form in the event of an infection. Cells were then proteolyzed prior to WB analysis with 3F4. While untreated cells were clearly infected by the mouse rods (Fig. 5C, leftmost lane), the presence of both chlorate and HM2602 at the time of exposure prevented this infection.

While rods are arguably the purest preparation of PrP\textsuperscript{Sc} available, cell-to-cell propagation of prions is likely to involve more complex prion containing structures, such as vesicles. Prions can propagate among GT1-1 cells in culture, in part using the medium as vehicle (27). To see if cellular HS also play a role in the initiation of infection by this route, we thus repeated the experiment above using the cell medium of ScGT1-1 cells as a source of infectious prions, but this time the cells were exposed for 48 h and further incubated in fresh medium without inhibitors for 10 d. This medium efficiently infected GT1-1-MHM2 cells, as shown by the appearance of protease-resistant, 3F4-reactive PrP\textsuperscript{Sc}. In contrast, little or no infection was established in cells that were treated with chlorate or DS500 at the time of exposure to the infected medium.

Taken together, the results of this section show that establishing an infection in GT1-1 cells
requires that cellular HS be present during exposure to prions.

Discussion

The initial interactions of pathogens with the cellular receptors are an essential aspect of their life cycle. Prion receptors have remained unknown. We now show that cell surface HS participate in the binding and uptake of purified prion rods in N2a, CHO-K1 and GT1-1 cells, and that they are involved in the establishment of a prion infection in GT1-1 cells (Fig. 5). Purified prion rods thus join a long series of pathogens (such as herpes simplex and adenoviruses) and toxins that use HS to penetrate their target cells (see 40 for a review). Our studies reveal that unlike most pathogens, however, prions require HS both as a receptor (this work) and as a co-factor for ongoing PrP\textsubscript{Sc} formation and prion replication (in ScN2a and ScGT1-1 cells, (15)). The exact HS motifs that bind rods and additional putative molecular components of these receptors remain to be identified.

Heparan sulfate is a binding and uptake receptor for rods and is required for cell infection

Removing cellular HS with heparinase III, preventing its sulfation with chlorate, or competing with sulfated glycans prevented both the binding of rods (at 4°C) and their internalization (at 37°C) in N2a, CHO-K1, and GT1-1 cells. The biological significance of HS-mediated prion uptake is shown convincingly by the finding that chlorate or glycans, that inhibit prion binding, also prevented the infection of GT1-1 cells when applied during their exposure to either purified rods or to the conditioned medium of ScGT1-1 cells. Taken together, these results argue convincingly that HS are an essential component of prion receptors in these cells. Whether additional cellular molecules are also involved in this receptor remains to be seen. Among the few known cell surface proteins that bind PrP, the 37-kDa/67-kDa laminin receptor is a prominent candidate to take part in a prion receptor since it binds both PrP and HS (10). Because there is only one heparin-binding region in PrP27-30 (aa-110-118 (29)), our results suggest that this region is exposed on the surface of rods.

Rods receptors and prion co-factors

Our results broaden the role of cellular HS in prion biology, as these GAGs are now implicated in two aspects of prion metabolism which are not necessarily related mechanistically: (i) HS serve as co-factors in the propagation of PrP\textsubscript{Sc} (15) and (ii) they perform as receptors for both Syrian hamster Sc237 and mouse RML rods (Fig. 6). The exact sequences within HS that are involved in these two tasks remain to be determined and need not be identical in the two cases. Heparan sulfate chains have a complex structure that includes regions of hypersulfation that are separated by undersulfated spacers (reviewed in 28). Many functions of HS are performed by very specific oligosaccharide sequences within the chain. The tools used in this study were unable to address this specificity. Thus, heparinase III, which cleaves undersulfated regions, often removes the entire HS chain only to leave the tetrasaccharide linker attached to the core protein (41). Therefore, the finding that heparinase III inhibits both rods binding and PrP\textsubscript{Sc} formation does not prove that the same HS sequences are involved in both tasks. More detailed tools, such as synthetic low Mr oligosaccharides, will have to be used to determine the specific HS sequences involved in each of these two functions. Of note, these two roles may also be performed in different subcellular locations since, in contrast to cell surface binding of rods, the \textit{de novo} formation of PrP\textsubscript{Sc} may take place in endocytic compartments (42).

Although there was a strict correlation between the types of exogenous glycans that inhibited the two prion-related functions of HS, higher concentrations were needed to inhibit rods uptake. Several mechanisms could contribute to this result. First, since rods are very large assemblies of PrP27-30 molecules (up to several thousands (20)), their binding to the cell surface is likely to be mediated by many parallel interactions with HS chains, which may act synergistically and thus may need higher levels of soluble glycan to be competed out. Another mechanism that could explain the greater sensitivity of the \textit{de novo} formation of PrP\textsubscript{Sc} (Fig. 6, right panel) to soluble
inhibitors is that the latter process involves full length PrP molecules that contain the two N-proximal HS binding regions in addition to the one region within PrP27-30 found in rods (29). Further studies will be needed to answer this question.

**Other receptors**

Although this work pinpoints HS as a major receptor component for prion rods in three cell types (N2a, CHO-K1 and GT1-1), it is probable that other cell-surface molecules will be found that bind PrP27-30 in other cells. In preliminary experiments (not shown), we found that CHO pgs A-745 mutants cells (37), that fail to initiate synthesis of GAGs on proteoglycan core proteins, do still bind and internalize purified rods (albeit less efficiently than their parent line CHO-K1). As expected, this binding was insensitive to heparinases, and is thus mediated by a non-HS molecule which we are now trying to identify. Of note, many cell surface molecules, including scavenger receptors and integrins, have been shown to bind other amyloids such as Aß (43).

**Biological relevance**

Whether infection of cells with prions requires the internalization of these pathogens, or if only contact is sufficient has not yet been established. The provocative findings that prions associated to steel wires (44) are infectious suggest that productive interactions between membrane-bound PrP⁹ and exogenous PrP⁸ might perhaps also occur on the surface of the target cell. In any case, our GT1-1 infection studies indicate that cellular HS are required for the establishment of an infection.

The finding that cell surface HS molecules bind PrP⁸ molecules may also have important implications for the subcellular trafficking of this pathogenic isoform. Metabolic studies have shown that PrP⁸ traffics from its synthesis site (either the cell surface or endosomes (4,42)) to an acid hydrolytic compartment were it looses its N-terminus (45,46), and then back to the cell surface. The molecular details of this trafficking, such as the identity of endocytic adaptors, remain unknown. Our findings raise the possibility that transmembrane HS molecules may serve as such trafficking co-factors for both PrP isoforms.

It will be interesting to see if the initial interaction of prions with cells through HS plays a role in targeting prions to particular anatomical targets within the host. Prions strains, for instance, seem to target specific regions within the brain (47). While heparan sulfates are found in all tissues, the variations of their fine structure from cell to cell (reviewed in 48, see also 49-51) might conceivably contribute to the specific targeting of prion strains. Further studies will be required to see if various strains interact with different HS molecules.

To study the importance of HS in the development of prion diseases, we have undertaken bioassays using transgenic mice that overexpress the mammalian heparanase (52). Preliminary results indicate that scrapie incubation time is prolonged in these mice.

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Figure legends

**Figure 1. Purification of prion rods.** (A) Silver staining. 5 µl of purified Syrian hamster prion rods (right lane) and 10 ng of recombinant mouse PrP (left lane) were analyzed by electrophoresis and silver staining. PrP27-30 is the major peptide in the rods preparation (arrow). (B) Increasing volumes of hamster prion rods (as indicated) were compared with defined amounts of recombinant mouse PrP (as indicated) by WB developed with the recombinant anti-PrP Ab, R1. One µl of prion rods contains about 5 ng of PrP. M, markers are (A) 47, 34, 26, and 19 kDa, and (B) 33, 26, and 19 kDa.

**Figure 2. Ongoing PrPSc formation and rods endocytosis are inhibited by the same polyanions.** (A) ScN2a-M cells were treated with increasing concentrations of DS500 or the heparan mimetics HM-B103, HM-2102, or HM-2602 (as indicated) for 5 days at 37° C. At the end of the incubation, cells were harvested and treated with PK (20 µg/ml, 30 min, 37° C) to monitor the amount of PrPSc in the cells. (B) Uninfected N2a-M cells were treated with the same polyanions for 24 h. Prion rods were then added to the cells which were further incubated for 20 h. Cells were harvested and treated with PK (20 µg/ml, 30 min, 37° C) to analyze the amount of internalized prion rods. Samples (A-B) were normalized for their protein content and then analyzed by WB developed with the mAb 3F4. M, markers are (A) 33, 26, and 19 kDa, and (B) 33, 26, and 19 kDa.

**Figure 3. Heparinase III, chlorate and anti-prion glycans prevent rods endocytosis in N2a-M and in CHO-K1 cells.** N2a-M (A) and CHO-K1 (B) cells were incubated for 24 h with 0.1 unit/ml heparinase I, 0.1 unit/ml heparinase III, 30 mM chlorate or left untreated. In B, 1 µg/ml DS500, HM2102
or 0.1 unit/ml chondroitinase ABC, were also included. Prion rods were then added to the medium and the cells were further incubated for 20 h. Cells were then harvested and treated with PK (2 µg/ml, 30 min, 37º C) to analyze the amount of internalized prion rods. Samples were normalized by protein content and then analyzed by WB developed with the mAb 3F4. M, markers are 26, and 19 kDa. (C) Immunofluorescence. CHO-K1 cells were treated with chloride (c), heparinase I (d), heparinase III (e), DS500 (f), HM2602 (1 µg/ml) (g), HM2102 (h) at the same concentrations used in panels A and B for 24 h, or left untreated (a,b). Rods were then added to the cell medium (b-h) for 20 h. At the end of the incubation the cells were thoroughly rinsed and chased in fresh medium for 4 h (to reduce the signal from surface-bound cells) and then hamster rods were revealed by immunofluorescence using mAb 3F4 after in situ denaturation with 3 M guanidine thiocyanate.

Figure 4. HS mediate binding of hamster rods in CHO-K1 cells. (A-B) Cells were incubated for 24 h with 0.1 unit/ml heparinase I, 0.1 unit/ml heparinase III or 30 mM chlorate (A), or with 1 µg/ml of DS500 or HM2602 or HM 2102 (B) or left untreated. The cells were then cooled on ice and prion rods were added for further 3 h incubation on ice. (C) Cells were treated with increasing concentrations of heparinase III (0, 0.1, 1, or 2 unit/ml) for 24 h and then cooled on ice. They were then rinsed and rods were added in fresh ice-cold medium for 3 hours on ice. (D) Cells were treated with or without 30 mM chlorate for 24 h and then were cooled and rods were then added with or without 50 µg/ml exogenous porcine mucosa HS for 3 h on ice. (A-D) At the end of the incubations, cell lysates were treated with PK (2 µg/ml, 30 min, 37º C), normalized by protein content, and cell-bound rods were detected in WB developed with mAb 3F4. M, markers are 33, and 26 kDa.

Figure 5. HS mediate the infection of GT1-1 cells. (A) GT1-1 cells were incubated for 24 h with 0.1 unit/ml heparinase I, 0.1 unit/ml heparinase III, 30 mM chlorate, 1 µg/ml DS500 or HM2102 or left untreated. Mouse prion rods were then added to the medium and the cells were further incubated for 20 h. Cells were then harvested and treated with PK (2 µg/ml, 30 min, 37º C) to analyze the amount of internalized prion rods. Samples were normalized by Bradford and then analyzed by WB developed with the mAb D13. (B) Infection of GT1-1-MHM2 cells is indicated by the appearance of 3F4-reactive PrPSc. Mouse prions in the inoculum are not recognized by this antibody. (C) GT1-1-MHM2 cells were pre-treated for 24 h with 30 mM chlorate, 1 µg/ml HM2602 or 1 µg/ml DS500 (as indicated). Mouse rods (24 h, left panel) or ScGT1-1 medium (48 h, right panel) were then added in the presence of the inhibitors. Cells were then rinsed and further grown in fresh medium for 5-10 days. The cells were then lysed and analyzed for their PrPSc-MHM2 content by PK treatment (20 µg/ml, 30 min, 37º C) and WB developed with 3F4. M, markers are 36, 26, and 19 kDa.

Figure 6. Cellular heparan sulfates serve both as rods receptors and as prion co-factors: a model. (A) Left: rods, which comprise large aggregates of PrP27-30, can bind cell surface HS and use them for internalization. Because of their large dimension, rods are likely to bind cooperatively to a very large number of HS molecules. These HS receptors are similar to or identical with the cellular HS co-factors (right panel). However, whereas rods binding take place on the cell surface, PrPSc synthesis (right panel) may also occur in intracellular compartments. (B) Digesting with heparinase or preventing sulfation with chlorate prevents both rods binding and internalization (left) and PrPSc formation (right). (C) Identical polyanions prevent the binding of rods and the formation of PrPSc, but a larger concentration is required to compete out the presumably more numerous binding sites of rods (left).
Figure 3 - Horonchik et al.

A  treatment  -  -  hpa I  hpa III  chlorate
rods  -  +  +  +  +

B  treatment  -  -  hpa I  hpa III  chlorate  chond  ABC  DS 500  HM2102
rods  -  +  +  +  +  +  +  +

C  a  b  c  d
   e  f  g  h
Figure 4 - Horonchik et al.

A  treatment rods  - - hpa I hpa III chlorate  

B  treatment rods  - - DS 500 HM2602 HM2102  

C  hpa III (u/ml)  - 0.1 1 2  

D  rods chlorate HS  + + + - + + -
Figure 5 - Horonchik et al.

A  treatment rods  -  -  chlorate  hpa I  hpa II  DS 500  HM2602  HM2102
                  -  +  +  +  +  +  +  +  +  PK

B  GT1-1-MHM₂ cells

   Mouse rods

   PrP<sup>C</sup>-MHM₂  3F4  3F4  PrP<sup>Sc</sup>-MHM₂

   Successful infection

C  treatment rods  -  -  chlorate  HM2602
               -  -  +  +  +  +  ScGT1-1 medium  -  -  chlorate  DS 500
Figure 6 - Horonchik et al.

A. Rods binding

B. PrP$^{Sc}$ formation

C. Further details on the process.
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Lior Horonchik, Salit Tzaban, Olga Ben-Zaken, Yifat Yedidia, Alex Rouvinski, Dulce Papy-Garcia, Denis Barritault, Israel Vlodavsky and Albert Taraboulos

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