The Journal of Biological Chemistry 280(17):17057-17061, 2005
Published, JBC Papers in Press, January 24, 2005, DOI 10.1074/jbc.M411314200

PrPSc Incorporation to Cells Requires Endogenous Glycosaminoglycan Expression

Nuha Hijazi†, Zehavit Kariv-Inbal‡, Maria Gasset§, and Ruth Gabizon¶

From the §Department of Neurology, The Agnes Ginges Center for Human Neurogenetics, Hadassah University Hospital, Jerusalem 91120, Israel and ¶Insto de Quimica-Fisica Rocasolano, Consejo Superior de Investigaciones Cientificas, Serrano 119, 28006 Madrid, Spain

Many lines of evidence suggest an interaction between glycosaminoglycans (GAGs) and the PrP proteins as well as a possible role for GAGs in prion disease pathogenesis. In this work, we sought to determine whether the PrP-GAG interaction affects the incorporation of PrPSc (the scrapie isoform of PrP) to normal cells. This may be the first step in prion disease pathogenesis. To this effect, we incubated protease K-digested hamster scrapie brain homogenates with several lines of Chinese hamster ovary (CHO) cells in the presence or absence of heparin. Our results show that over a wide range of PrPSc concentrations the binding of PrPSc to wild type CHO cells, which do not express detectable PrP, was equivalent to the binding of PrPSc to CHO cells overexpressing PrP. A significant part of PrPSc binding to both lines could be inhibited by heparin. Additional evidence that PrPSc binding to cells was dependent on the presence of GAGs could be concluded from the fact that the binding of PrPSc to CHO cells missing GAGs on the cell surface was significantly reduced. Interestingly, preincubation of scrapie brain homogenate with heparin before intraperitoneal inoculation into normal hamsters resulted in a significant delay in prion disease manifestation.

During prion disease, an abnormal protease-resistant isoform of cellular PrP (PrPSc),1 denominated PrPSc, which is considered to be the major component of the prion, accumulates in the central nervous system (1). It has been postulated that β-sheet-rich PrPSc is produced from mostly α-helical PrPSc by a conformational-dependent conversion process (2). Although the detailed steps of such conversion as well as the mechanisms of infection and pathogenesis leading to death from prion disease are as yet unknown, the absolute requirement of endogenous PrPSc in prion disease pathogenesis has been solidly demonstrated (3).

Studies in transgenic mice suggest that a host factor, designated protein X, is involved in the conversion of the normal cellular prion protein, PrPC, into the scrapie isoform, PrPSc (4). Consequently, considerable effort has been devoted to the identification of natural receptors for the prion proteins, both to understand the prion pathogenesis process and to reveal potential targets for therapeutic intervention. Among the proteins that were shown to bind PrPSc were amyloid precursor-like protein 1 (5), stress-induced proteins, and the laminin receptors (6, 7). Interestingly, antibodies to laminin receptors were shown to reduce the accumulation of PrPSc in ScN2a cells (8). Both PrP and laminin receptors have been shown to interact with glycosaminoglycans (GAGS), and especially with heparan sulfate (HS), supporting the possibility that these sugar polymers function as possible cofactors for 37-kDa laminin receptors mediating PrP binding (9).

In addition to PrPSc, GAGS are by far the most important host molecules connected so far to prion pathogenesis and to the metabolism of the prion proteins (10). HS accumulates in cerebral prion amyloid plaques as it does in Alzheimer disease or other amyloidotic diseases (11, 12). Also, addition of HS promotes the reconstitution of infectivity of dimethyl sulfoxide-solubilized PrPSc (13). In addition, several lines of evidences connect GAGS, and especially HS, to the metabolism of the PrP isoforms as well as to the accumulation of PrPSc in prion-infected cells. A variety of sulfated glycans, such as low molecular weight heparin (14), suramin (15), pentosan polysulfate, and dextran sulfate (16, 17), were shown to reduce the accumulation of PrPSc in ScN2a cells and in some cases prolong the incubation time of experimental prion diseases. In addition, long-term incubation of ScN2a cells with sulfate, which affects the sulfation of GAGS as well as of proteins and glycolipids, reduces PrPSc accumulation (14). Finally, ScN2a cells treated with heparinase III showed a significant decrease in PrPSc accumulation (18).

In this work, we investigated whether endogenous GAGS play a role in the first step of prion disease pathogenesis, i.e. the binding of PrPSc, the main if not the only prion component, to normal cells. We show here that, over a wide range of PrPSc concentrations, the binding of PrPSc to mutant cells in which expression of total GAGs or only HS was abolished was significantly reduced when compared with PrPSc binding to wt Chinese hamster ovary (CHO) or to CHO cells overexpressing PrP. Most of this binding resulted in the internalization of PrPSc to the cells as can be seen by the fact that only a small part of PrPSc was bound to the cells at 4 °C. PrPSc binding to both wt CHO or to CHO cells overexpressing PrP was significantly inhibited when the cells were preincubated either with heparin or with copper. Because PrPSc is an insoluble molecule, it is unclear whether it binds directly to the GAGS on the cell surface or through some closely associated molecule.

EXPERIMENTAL PROCEDURES

Heparin, polyaspartic acid, and heparin-agarose were purchased from Sigma. Cell culture media and serum were purchased from Beit Haemek, Biological Industries.
of cell homogenates with 40 bated for 1 h with 25 or 50 I
17058
of brain homogenate from either normal or scrapie-infected an-
100 mM Nacl, 1% Nonidet P-40. Beads were boiled in SDS to elute bound
containing 10 mM Tris-HCl, pH 7.4, 300 mM sucrose, and 5 mM EDTA in
/streptomycin (100
CHO cells were grown in Ham's F12 medium supplemented
with fetal calf serum (10%), glutamine (2 mM), penicillin (100 units/ml), and
CHO cells were transfected with a chimeric mouse hamster PrP, denominated MHM2 PrP (20, 21).
CHO cells were grown in Ham's F12 medium supplemented with fetal
calf serum (10%), (23–232) was produced as insoluble inclusion bodies, purified,
appropriate bands (Fig. 1
a) mutant cells, which do not express chondroitin sulfate and HS or only HS, respectively (for explanation

Cell Culture—Several cell lines were used: 1) wt CHO cells, 2) mutant
CHO cells lacking xylosyltransferase (XT−/−), incapable of initi-
ing the synthesis of both HS and chondroitin sulfate chains, or
3) CHO cells lacking glucuronic acid/N-acetylgalactosaminetransferase
( HS−/−), selectively deficient for HS (19), and 4) CHO cells transfect-
ated CHO cells lacking glucuronic acid/N-acetylgalactosaminetransferase
( HS−/−), selectively deficient for HS (19), and 4) CHO cells transfected
with a chimeric mouse hamster PrP, denominated MHM2 PrP (20, 21).
CHO cells were grown in Ham's F12 medium supplemented with fetal
calf serum (10%), (23–232) was produced as insoluble inclusion bodies, purified,
appropriate bands (Fig. 1
a) mutant cells, which do not express chondroitin sulfate and HS or only HS, respectively (for explanation

Preparation of Scrapie Brain Homogenates—Normal or scrapie-in-
fected hamster brains were homogenized in 10% (wt/v) cold buffer
containing 10 mM Tris-HCl, pH 7.4, 300 mM sucrose, and 5 mM EDTA in
phosphate-buffered saline, pH 7.4. The homogenates were centrifuged
at 800 g (2000 rpm) for 15 min at 4 °C. The supernatants were frozen
in aliquots for future experiments.

Binding Experiments—Binding experiments were performed as re-
ported previously (22). Briefly, after reaching 80% of confluence in 25-cm2
flasks, cells were preincubated with Ham's F12 medium supplemented
with or without 4 mg/ml heparin for 2 h at 37 °C. After the preincubation,
PK-digested (40 μg/ml PK for 30 min at 37 °C) scrapie hamster brain
homogenate at the different concentrations used was added for each flask
to the designated flasks for an additional 2 h. Subsequently, the cells were
washed four times with medium and twice with phosphate-buffered sa-
ine. Cells were lysed in 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1%
N-acetylgalactosaminetransferase
( HS−/−), selectively deficient for HS (19), and 4) CHO cells transfected
with a chimeric mouse hamster PrP, denominated MHM2 PrP (20, 21).
CHO cells were grown in Ham's F12 medium supplemented with fetal
calf serum (10%), (23–232) was produced as insoluble inclusion bodies, purified,
appropriate bands (Fig. 1
a) mutant cells, which do not express chondroitin sulfate and HS or only HS, respectively (for explanation

Binding of Brain Homogenates to Heparin-Agarose—Equal amounts
of total protein (0.37 mg) from normal or scrapie hamster brain homo-
genate digested with PK (40 μg/ml PK for 30 min at 37 °C), were incu-
bated for 1 h with 25 or 50 μg/ml of heparin or with 50 μg/ml polysac-
charic acid or with no additions. Subsequently, the different aliquots
were incubated with heparin-agarose beads for 1 h at 4 °C before washing
the beads five times with a buffer containing 10 mM Tris-HCl, pH 7.4,
100 mM NaCl, 1% Nonidet P-40. Beads were boiled in SDS to elute bound
material that was then immunoblotted with aPrP mAb 3F4.

In Vivo Experiments—Syrian hamsters (six animals in each group) at
the age of 4 weeks were inoculated intraperitoneally (IP) with 100 μl
(0.1%) of brain homogenate from either normal or scrapie-infected an-
imals that were preincubated with or without heparin (2 or 4 mg/ml) for
2 h before injection. Animals were followed closely through the incuba-
tion time and sacrificed when showing distinct scrapie symptoms.

Results—The Binding of PrPSc to CHO Cells Is GAGs-dependent and
PrPSc-independent—Because PrPSc can only be purified as an
insoluble aggregate, which may not exist as such in vivo, we used
PK-digested scrapie brain homogenate from Syrian ham-
ster brains for our binding experiments. In this context, PrPSc
may be associated to components of membrane rafts (23).
Therefore, we should be aware that the binding of PrPSc to cells
or heparin resins (see below) may be either direct or occur
through other molecules. We have shown before (22) that this
preparation can be used to study PrPSc binding and internal-
ization to cells. To determine the concentration of PK-resistant
PrP in these preparations, we immunoblotted serial dilutions of the
PK-treated brain homogenates as well as serial dilutions of
purified recombinant hamster PrP and compared the appro-
priate bands (Fig. 1a). Recombinant Syrian hamster (SHa)PrP
(23–232) was produced as insoluble inclusion bodies, purified,
and refolded as previously described (24). As can be seen in Fig.
1a, 1 μg of purified recombinant PrP represents the PrPSc
present in ~45 μg of total brain homogenate (after digestion
with PK).

To examine the role of PrPSc and cellular GAGs on the bind-
ing of PrPSc to cells, we incubated PK-digested Syrian hamster
scrapie brain homogenate (comprising ~7.5 μg of PrPSc) with
several types of CHO cells. These include wt CHO cells, which
have been shown previously not to express detectable levels of
endogenous Chinese hamster PrP (25) as well as CHO cells
overexpressing MHM2 PrP (21). In addition, we used CHO
( wt) and ( HS−/−) mutant cells, which do not express chon-
droitin sulfate and HS or only HS, respectively (for explanation
on mutant CHO cells, see “Experimental Procedures”) (19). As can be seen in Fig. 1b, only the extract of CHO-MHM2 PrP cells reacted with the 3F4 antibody. Similar results were obtained with other αPrP antibodies known to react with Chinese hamster PrP (not shown).

Following incubation with the scrapie brain homogenate (see “Experimental Procedures”), cells were extracted, digested with PK, and immunoblotted with αPrP mAb 3F4 to assess the binding of PrPSc to the diverse cells. Regardless of the big difference in PrPC expression, similar concentrations of PrPSc were bound to both wt and MHM2 CHO cells (Fig. 1b, III). Contrary to the extensive binding of PrPSc to wt CHO cells, PrPSc binding to both mutant lines, lacking only HS or total GAGs, was very poor. These results suggest that the initial binding of PrPSc to CHO cells is independent of PrPC concentration but dependent on the presence of GAGs, and especially HS, on the cell surface.

As opposed to PK-resistant PrP from either mouse or human, PK-resistant hamster PrP always shows a predominant band representing fully glycosylated PrP. As can be seen in the figures throughout this report, this pattern did not change after the binding of the scrapie protein to cells or heparin beads, indicating there was no preference for any glycosylated isoform. Similar results were obtained for PK-resistant mouse PrP (not shown).

**Heparin Inhibits the Binding of PrPSc to CHO Cells**—To further assess the interaction of PrPSc with GAGs on the cell surface, we examined the effect of heparin (a naturally occurring analog of HS) on the binding of PrPSc to wt and mutant CHO cells. To this effect, cells were cultured in the presence of heparin for 2 h before the addition of PK-digested brain homogenate, as described above. Fig. 1b, IV, shows that heparin inhibited most of the PrPSc binding to wt and MHM2 PrP CHO cells but had no effect on the residual binding of PrPSc to HS−/− or XT−/− cells. These results suggest that PrPSc or a molecule closely associated with PrPSc may bind directly to cell-associated HS and that soluble heparin inhibits this interaction in a competitive manner. Moreover, Fig. 1b, IV, shows that heparin inhibited the binding of PrPSc to wt and PrP-overexpressing CHO cells to the same extent, consistent with the possibility that the presence of PrPSc is not required for the heparin-dependent binding of PrPSc to cells. Heparin also inhibited the binding of PrPSc to neuroblastoma cells (not shown), which have been shown to support prion replication (26).

**The Association of PrPSc with Cells Is Concentration- and Temperature-dependent**—To test further the specificity of the PrPSc-GAG interaction on the cell surface, we performed the same binding experiments described in the previous section at several concentrations of PrPSc (as present in brain homogenate). As can be seen in Fig. 2a, in the absence of heparin, PrPSc binding to wt CHO or to MHM2 CHO cells increased with the increasing concentration of homogenate until reaching saturation at about 45 μg of PrPSc (see also Fig. 2b). In the cells in which either HS or total GAGs were absent, the binding of PrPSc also increased with homogenate concentration but at a much lower rate. When heparin was added to the cells in culture before the scrapie homogenate, the binding of PrPSc was very similar in all four CHO cell lines regardless of the presence of either PrP or GAGs. These results suggest that at high PrPSc concentra-
PrPSc Binding and GAGs

binding of PrPSc to cells at 4 °C, indicating sulfated sugars may result in the absence of GAG-dependent binding of PrPSc to all CHO cells at this temperature, the results were not significant enough to assure this conclusion.

To determine whether the GAG-PrPSc interaction affects the internalization of the prion protein, we incubated increasing concentrations of scrapie homogenate with cells at both 4 and 37 °C in the presence and absence of heparin (27, 28). Cells were processed as described in the previous experiments. Concentrations of added or bound PrPSc were calculated by comparing PK-resistant PrP bands on gels with serial dilutions of recombinant PrP (Fig. 1a). Because the amount of PrPSc associated with the cells at 4 °C at a range of PrPSc concentrations was very low (Fig. 2a), we conclude most of the PrPSc was rapidly internalized into the CHO cells. As suggested from the results in Fig. 2a, although heparin (at 37 °C) inhibited very strongly the incorporation of PrPSc to cells at low PrPSc concentrations, it was less efficient in higher PrPSc concentrations, when the binding of PrPSc reached saturation. This suggests the existence of a low affinity mechanism for PrPSc incorporation into cells that is GAG-independent. The internalization of PrPSc into CHO cells was also shown by immunocytochemistry methods to be inhibited by heparin-like reagents (29).

The results in Fig. 2b may suggest heparin inhibited the binding of PrPSc to cells at 4 °C, indicating sulfated sugars may affect not only the internalization of PrPSc but also the initial binding of PrPSc to the CHO cells. However, because of the low binding of PrPSc to all CHO cells at this temperature, the results were not significant enough to assure this conclusion.

PrPSc Binds to Immobilized Heparin—Although the binding of PrPSc to heparin has been shown previously, this is not the case for PrPSc (14). To test this effect, we tested whether the protease-resistant core of PrPSc present in our protease-digested scrapie brain homogenate can bind to heparin-agarose. Homogenates from normal or scrapie-infected hamster brains were incubated in the presence of heparin-agarose, washed extensively, and boiled in SDS-PAGE buffer before immunoblotting with anti-PrP mAb 3F4 (see “Experimental Procedures”). Fig. 3 depicts the results of such an experiment. Both PrPSc and PrPc bind to heparin specifically because this binding activity could be abolished when the incubation of the homogenates with the beads was performed in the presence of soluble heparin. As opposed to soluble heparin, polyaspartic acid, another negatively charged polymer, did not inhibit PrPSc or PrPc binding to heparin-agarose. As stated above, although these results are consistent with the possibility that PrPSc binds directly to heparin molecules, the insoluble nature of PrPSc prevents us from claiming a direct binding between PrPSc and any other molecule.

Copper Also Inhibits the Binding of PrPSc to Cells in the Absence of PrPc—In a previous report (22), we showed that the binding of PrPSc to N2a cells was inhibited when N2a cells were first cultured in the presence of copper ions, which have been shown to bind to PrPc. Incubation of such cells with copper resulted in increased accumulation of PrPc, probably because of its internalization and delayed degradation. We speculated that at the time that the inhibition by copper of PrPSc binding to N2a cells may result from the absence of PrPc or another copper-internalized molecule from the cell surface. To test whether the presence of PrPc is required for copper to inhibit the binding of PrPSc to cells, we cultured wt and MHM2 PrP CHO cells in the presence or absence of 300 μg/ml heparin for 24 h (Fig. 4) and subsequently added PK-digested scrapie brain homogenate to the cell culture as described above. Fig. 5 shows the results of such an experiment. As was shown for N2a cells (22), whereas the concentration of PrPc in the presence of copper was largely increased in the MHM2 CHO cells (Fig. 4a), PrPSc binding was inhibited in a similar manner in both cell lines regardless of the presence of detectable PrPc on the cell surface. These results indicate that the copper-related inhibition of PrPSc binding to cells may not result only from the binding of copper to PrPc but rather from the binding of copper to cell surface GAGs, which have shown here to be essential for PrPSc binding to cells. It has been suggested previously that the formation of PrP-Cu(II)-glycosaminoglycan assemblies may be crucial entities in the metabolism of PrP (24).

Incubation of Heparin with Prion Inoculum Delays Disease Onset in Hamsters—To test whether heparin can delay prion disease onset by inhibiting binding of PrPSc to its targets in vivo, we incubated normal or PK-digested scrapie brain homogenate in phosphate-buffered saline alone or in phosphate-
buffered saline containing heparin (2 or 4 mg/ml) for 2 h before intraperitoneal inoculation to normal hamsters (Fig. 5).

Administration of normal brain homogenates in the presence or absence of heparin did not produce any adverse effects in any of the animals. Because none of the control animals developed prion disease even after long incubation times, this experiment also shows that incubation of PrPSc with heparin at the present conditions did not convert PrPSc into PrPSc. Preincubation of scrapie brain homogenate with heparin did delay incubation time significantly in both concentrations of heparin used. Together with our in vitro experiments, these results are consistent with the possibility that heparin delayed prion disease onset via the inhibition of PrPSc binding to GAGs on the cell surface. However, because of the complexity of in vivo mechanisms, it is not possible to conclude at this point whether this is the only or major function of heparin as an anti-prion agent.

**DISCUSSION**

Many lines of evidence lead to the conclusion that PrPSc is the major, if not the only, component of the prion. Recently, a new and most awaited result has been provided by the Prusiner laboratory, indicating that an amyloid form of β-sheet recombinant PrP is enough to produce transmissible prion disease when inoculated to mice overexpressing PrPC (31). However, the molecules with which PrPSc approaches its cell targets and subsequently causes conversion of PrPSc and neurodegeneration have yet to be elucidated.

The results presented in this report suggest that GAGs may be the main, if not the only, receptor for PrPSc on the cell surface. The binding of PrPSc to cells was independent from the presence of GAGs only at high concentrations of the prion protein, which were much higher than required to produce minimum incubation time. Interestingly, incubation time for scrapie disease is known to reach a saturation point and cannot be reduced further for a specific disease strain by the inoculation of larger amounts of scrapie homogenates or of purified PrPSc. Consistent with the role of endogenous GAGs in PrPSc binding and internalization is the fact that heparin, an analog of GAGs, can inhibit the binding of PrPSc in cells where GAGs are present.

Interestingly, PrPSc, although it seems not to be required for the primary binding of PrPSc to the cells, has been shown to be closely associated with HS on the cell surface (9, 32). GAGs may constitute the “bridge” between PrPSc on the cell surface and PrPSc in the prion aggregate and cause the internalization of both the normal and scrapie-associated prion proteins. Although HS binds directly to PrPSc, it may have an affinity for the aggregated amyloid-like structure of PrPSc as we have shown previously for Congo Red (34). It is important to note that although the results shown here for CHO cells may not be directly relevant to prion infection, because CHO cells were never shown to sustain prion replication, heparin-like compounds have been shown by us (data not shown) as well as by others to inhibit the binding of PrPSc to neuroblastoma cells (29).

The fact that copper ions also inhibit the binding of PrPSc to cells in the absence of detectable PrPSc further indicates that the direct receptor for PrPSc on the cell surface is another molecule. We hypothesize that copper ions bind to GAGs on the cell surface, causing the internalization of the sugar polymer with the proteins attached to it, including PrPSc. Because the concentration of PrPSc has been shown to determine prion disease incubation time by modulating the concentration of PrPSc formed in the cells (35), our results suggest that the binding of PrPSc to the cell surface is mechanistically separated from the conversion of PrPSc to PrPSc.