Molecular Neurobiology

ISSN0893-7648/94/8(2–3): 113–120/$5.60

Inhibition of Scrapie-Associated PrP Accumulation

Probing the Role of Glycosaminoglycans in Amyloidogenesis

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Abstract

Accumulation of an abnormal, protease-resistant form of an endogenous protein, PrP, is a characteristic feature of scrapie and related transmissible spongiform encephalopathies. This abnormal isoform is also present in the amyloid plaques that are often observed in these diseases. In mouse neuroblastoma cells persistently infected with scrapie, the abnormal protease-resistant isoform of PrP is derived from an operationally normal protease-sensitive precursor. Conversion of PrP to the protease-resistant state occurs either on the plasma membrane or along an endocytic pathway by an unknown mechanism. Inhibitors of protease-resistant PrP accumulation have been identified, and these include the amyloid-binding dye Congo red and certain sulfated glycans. The similarity of these compounds to sulfated glycosaminoglycans, which are components of all natural amyloids, has led to the hypothesis that the inhibitors act by competitively blocking an interaction between endogenous glycosaminoglycan(s) and PrP that is critical for amyloidogenic PrP accumulation. The proven prophylactic effect of these sulfated glycans in animal models of scrapie suggests that they represent a group of compounds that might interfere with the pathogenic formation of amyloid in a variety of diseases, such as Alzheimer's disease.

Index Entries: Scrapie; PrP; amyloid; Congo red; glycosaminoglycans; Alzheimer's disease.

Introduction

Scrapie is a transmissible, neurodegenerative disease of unknown etiology that was first recognized in sheep, but can also be transmitted to other mammals. Mouse and hamster models of scrapie have become experimental prototypes for a group of diseases known as the transmissible spongiform encephalopathies (TSEs). Prominent examples of TSEs include kuru, Creutzfeldt-Jakob disease, and Gerstmann-Sträussler Scheinker disease of humans and bovine spongiform encephalopathy of cattle. One of the characteristic features of the TSEs is the accumulation, sometimes in the form of amyloid plaques, of an abnormally proteinase K-resistant isoform of a host protein, PrP *(1–5)*. The fact that the

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protease-resistant PrP isoform (PrP-res) copurifies with infectivity, yet does not appear to be associated with any scrapie-specific nucleic acid, has led to the hypothesis that PrP-res is the infectious scrapie agent *(6)*. Although this hypothesis is still speculative *(7–10)*, it is clear that PrP plays an important role in TSE pathogenesis.

Normally PrP is expressed in a protease-sensitive form (PrP-sen) in brain and other tissues *(5,11–16)*. Although PrP is developmentally regulated *(17–19)* and has been implicated in lymphocyte activation *(20)*, its normal function is unclear. During scrapie pathogenesis, PrP-res accumulates in the central nervous system and other tissues *(1,2,12,21–23)*. PrP-sen and PrP-res are encoded by the same host gene *(24)* with no apparent difference appearing at either the level of the mRNA *(4,5)* or primary protein sequence *(13,25)*. Thus, the scrapie-specific modification of PrP is believed to arise posttranslationally, and this has been borne out by biosynthetic studies *(26,27)*. The mechanism by which PrP is converted to the TSE specific form is not known, but a clear understanding of this process is essential, since it appears to play a crucial role in the pathogenesis and transmission of the TSEs.

Utilizing tissue culture cells persistently infected with the scrapie agent, the biosynthesis of both PrP-sen and PrP-res and their metabolic relationship to each other have been studied *(26–30)*. The recent discovery of a class of compounds that specifically inhibits the accumulation of PrP-res in these cultures has provided insights at a molecular level into a possible mechanism by which PrP becomes protease-resistant and amyloidogenic *(31,32)*. In this article, we will briefly review what is known about the biosynthesis of PrP-sen and PrP-res, and discuss in greater detail studies that define inhibitors of PrP-res accumulation, the effect these inhibitors have in vivo, and insights these inhibitors provide into mechanisms of amyloidogenesis. It is important to note that PrP-res is not always observed in the form of amyloid fibrils, so to equate PrP-res formation with amyloidogenesis is an oversimplification. The process is perhaps more accurately described as an abnormal metabolic stabilization and aggregation of PrP that can ultimately result in the formation of classic amyloid deposits. Nonetheless, the metabolic stabilization of amyloidogenic precursor proteins must occur in all amyloidoses in order for amyloid to accumulate. Therefore, one can regard all the steps of PrP-res formation as potentially relevant to the general understanding of amyloidogenesis.

In Vitro Tissue-Culture Models of Scrapie

Although many diverse tissue-culture cell lines express PrP *(5,33–35)*, few have proven to be susceptible to persistent scrapie infection. The rat pheochromocytoma cell line PC12 can reliably accumulate infectivity to relatively high specific infectivities when differentiated in the presence of nerve growth factor *(36–39)*, but little is known about the biosynthesis of PrP in these cells. Most analyses of PrP biosynthesis in vitro have been performed in scrapie-infected (Sc^+MNB) or uninfected (MNB) murine neuroblastoma cells *(35,40,41)*, or in scrapie-infected hamster brain cells *(42)*. Sc^+MNB cells remain persistently infected, divide, replicate scrapie agent, and accumulate PrP-res, providing an easily manipulated system for studying the biosynthesis of both PrP-sen and PrP-res.

Biosynthesis of Normal and Scrapie-Associated PrP

Pulse-chase experiments using both ^{35}S-methionine-labeled MNB cells and Sc^+MNB cells have led to an understanding of the basic biosynthesis and processing of both PrP-sen and PrP-res and their precursor–product relationship *(26–28,30,43)*. These studies have shown that although the synthesis of PrP-sen in uninfected and scrapie-infected MNBs appears the same, the biosynthesis of PrP-res differs dramatically from that of PrP-sen in Sc^+MNBs.

Biosynthesis of Normal PrP

Nascent PrP is loaded cotranslationally into the endoplasmic reticulum where several events occur: An N-terminal signal peptide is cleaved *(13,44,45)*, a glycophosphatidylinositol (GPI) anchor is attached, and high-mannose glycans are added to one or two potential N-linked glycosylation sites *(28)*. PrP is then translocated into the Golgi apparatus where the high-mannose glycans are converted to complex or hybrid glycans *(28)*. Translocation to the cell surface follows, and PrP is anchored to the plasma membrane via the GPI anchor *(28,46,47)*. The majority of the cell-surface PrP is phospholipase-sensitive, although a small proportion appears to be resistant to phospholipase treatment *(28,29)*. Once on the cell surface, PrP has a half-life of 3–6 h; over time, most PrP appears to be degraded, but some is released into the tissue culture medium *(26–28,34)*. Soluble forms of PrP have also been found in vivo in human cerebral spinal fluid *(48)* and in MNB

cultures expressing the chicken homolog of PrP *(49)*, but the significance of these secreted PrP species is unknown.

Biosynthesis of Scrapie-Associated PrP

Only a small proportion of the available PrP-sen is converted to PrP-res in Sc^+MNB cells *(27,43)*. PrP-res is derived from a proteinase K-sensitive, phospholipase C-sensitive precursor, i.e., PrP-sen, shortly after PrP-sen reaches the cell surface *(27)*. Unlike PrP-sen, PrP-res is resistant to phospholipases and protease treatments *(26,29,47,50)*, and appears to accumulate intracellularly in MNB cells with little, if any, expressed on the cell surface *(26,29,51)*. Shortly after its formation, PrP-res is truncated at its N-terminus by endosomal or lysosomal proteases *(30,43)*. This limited proteolysis of PrP indicates that PrP is already in protease-resistant form on exposure to the proteases, and that the conversion of PrP from the protease-sensitive to protease-resistant form likely occurs either on the plasma membrane or along an endocytic pathway *(27,43,51)*. Furthermore, since the acquisition of protease resistance occurs prior to this truncation *(30)*, it seems that N-terminal cleavage in itself is not necessary for the formation of the bulk of PrP-res. In scrapie-infected hamster brains, only a small proportion of the PrP-res is similarly truncated *(13,45)*, so the importance of this process in vivo is unclear. In Sc^+MNBs, PrP-res eventually accumulates in the lysosome *(27,43,51)*, an organelle that has been postulated to be important in TSE pathogenesis *(52)*. Despite its exposure to endolysosomal hydrolases, PrP-res has a half-life of >48 h *(26,27)*. The metabolic stability of PrP-res can explain its accumulation in vivo, especially in the nondividing cells of the central nervous system.

Inhibition of PrP-res Accumulation

Although studies of the kinetics of PrP-res formation have shown that the conversion of PrP-sen to PrP-res occurs either on the plasma membrane or along an endocytic pathway, the exact nature of the conversion is unclear. The lack of a detectable difference between PrP-sen and PrP-res in terms of primary amino acid sequence or posttranslational modification *(13,25)* has led to the hypothesis that the difference is purely conformational *(13,53)*. According to one model, PrP-res could induce a conformational change in the endogenous PrP-sen, converting it to PrP-res and, thus, effectively "replicating" itself and initiating disease *(13,53,54)*. Interestingly, infrared spectroscopic studies have indicated that PrP-res has a much higher β-sheet content than is predicted theoretically for the PrP polypeptide *(55)*. The induced conformational change model predicts that the close association of similar PrP molecules is a critical step in the conversion of PrP-sen to PrP-res. Experimental support for the importance of interactions between homologous PrP species in PrP-res formation has been obtained in vivo by transgenic animal studies *(56)* and in vitro by studies of the incorporation of heterologous PrP species into the PrP-res produced by scrapie-infected cells *(57)*. Indeed, a single amino acid residue difference can profoundly reduce the efficiency of this conversion *(57a)*. Another hypothesis argues that the association of PrP with another nonprotein component is involved in formation of PrP-res *(13,54,58)*. Compounds that inhibit the formation or accumulation of PrP-res can provide one means of dissecting the conversion process at the molecular level.

Congo Red Inhibits Accumulation of PrP-res

Congo red is a dye that binds to amyloids *(59)* and PrP-res *(60)*, and has long been used as a diagnostic stain of amyloid deposits *(59)*. The binding of Congo red to PrP-res *(60)* suggested that it might affect PrP-res metabolism *(31)*. Indeed, when Sc^+MNB cells are metabolically labeled with ^{35}S-methionine, the presence of Congo red can virtually eliminate the accumulation of radiolabeled PrP-res as defined either by its ability to aggregate or its resistance to proteinase K treatment (Fig. 1) *(31)*. The Congo red treatment does not affect cellular protein metabolism in general nor PrP-sen metabolism in particular, providing evidence that its inhibitory mechanism relates specifically to PrP-res accumulation rather than the biosynthesis of the PrP-sen precursor. Further studies have shown that this effect is irreversible and that long-term accumulation of PrP-res is inhibited in Congo-red-treated cells *(61)*. More recently, it was determined that when Sc^+MNB cells treated with Congo red were injected intracerebrally into susceptible mice, no signs of scrapie were observed, whereas untreated control cells caused disease *(61a)*. Whether this effect was the result of a direct inhibition of PrP-res accumulation or interference with an unidentified scrapie-specific agent is unknown.

Congo red is a small, disulfonated molecule that may stack extensively and mimic a larger sulfated polyanion *(62)*. This postulated behavior of Congo

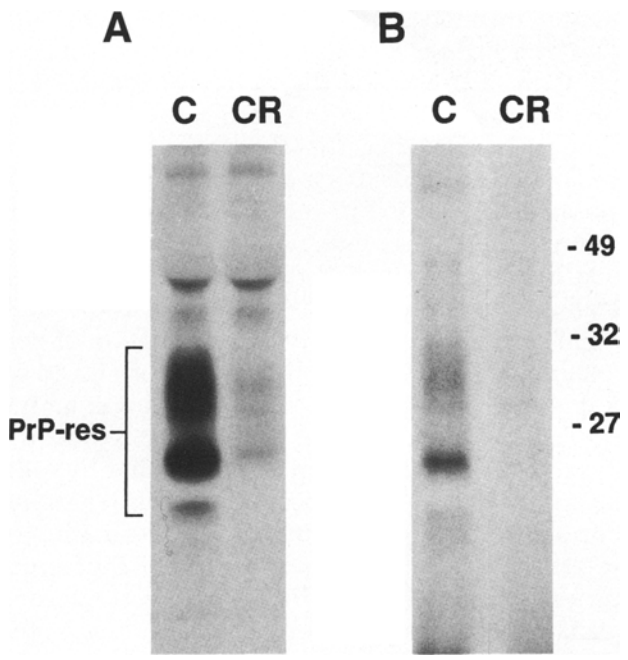

Fig. 1. Effect of Congo red on the labeling of PrP-res defined independently by its aggregation state or proteinase K resistance. Scrapie-infected neuroblastoma cells were metabolically labeled with ^{35}S-methionine in the absence (C) or presence (CR) of an inhibitory concentration of Congo red. The cells were lysed, and PrP-res was separated from PrP-sen by ultracentrifugation **(A)** or proteinase K treatment **(B)** *(31)*. Analysis for PrP-res was by immunoprecipitation and fluorography as described elsewhere *(31)*. The results are from two independent labeling experiments, and the lanes are not directly comparable between the two panels.

red is interesting since certain sulfated polyanions, when injected intraperitoneally into mice either concurrently with or shortly after inoculation with scrapie agent, are known to increase the incubation time of the disease *(63–69)*. In some cases, disease can be prevented entirely *(63,65)*. No PrP-res is detectable in the brains of survivors *(63)*, implying that sulfated polyanions may also inhibit PrP-res formation. Accordingly, the effect of sulfated glycans on PrP-res accumulation in Sc$^+$MNB cells was tested.

Sulfated Glycans Inhibit Accumulation of PrP-res

Sc$^+$MNB cells have been grown in the presence of a variety of potential inhibitors, and the level of PrP-res accumulation compared by immunoblotting *(32)*. Table 1 lists the compounds tested and their relative inhibitory activities. The most potent inhibitors of PrP-res accumulation are pentosan polysulfate, ι-carageenan, and dextran sulfate 500 (DS500, mol wt 500,000), whereas chondroitin sulfate, dextran, and DEAE dextran are among the least potent. In all cases where data are available, the ability of these compounds to inhibit PrP-res accumulation in cell culture parallels their ability to affect scrapie pathogenesis in vivo *(65,67)*, suggesting that their therapeutic effect in vivo may also involve the prevention of PrP-res accumulation.

Certain properties of the sulfated glycans appear to influence their ability to inhibit PrP-res accumulation, including charge density and molecular size *(32,67)*. Several nonsulfated polyanions were ineffective, suggesting that sulfation is important for PrP-res accumulation. However, the mineral heteropolyanion HPA 23 (ammonium-5-tungsto-2-antioniate), an effective prophylactic antiscrapie agent in mice *(68)*, can also block PrP-res accumulation (B. Caughey, K. Brown, and R. Rubenstein, unpublished data). Therefore, sulfate groups are not exclusively required. The level of sulfation may also be important since more densely sulfated compounds (e.g., pentosan polysulfate and iota-carageenan vs chondroitin sulfate and κ-carageenan) appear to be more potent inhibitors (Table 1).

Although chondroitin sulfate and κ-carageenan are similar in sulfation density, chondroitin sulfate has little effect on PrP-res accumulation, whereas κ-carageenan inhibits PrP-res when present at much lower concentrations (Table 1). This observation suggests that other factors may be involved, such as the location of the sulfate groups on the glycan molecule, the interaction of the glycan with other molecules in the cell or the half-life of the compound in culture. Finally, molecular size may also be a contributing factor since dextran sulfate 8 (mol wt 8000) is 10-fold less effective at inhibiting PrP-res accumulation than DS500 (mol wt 500,000) when added at identical concentrations *(32)*.

The Amyloid–Glycosaminoglycan Interaction: The Target of Inhibition?

All of the inhibitors described are sulfated or sulfonated polyanions and, in this respect at least, are similar to the endogenous sulfated glycosaminoglycans (GAGs) of mammalian cells that are often found on the cell surface or in the extracellular

Table 1
Inhibition of PrP-res Accumulation
by Sulfated or Nonsulfated Compounds in Sc+MNB Cells *(61)*

Compound tested	Type of compound, mol wt[a]	IC_{50}[b]
Pentosan polysulfate	Sulfated glycan	1 ng/mL
ι-carrageenan	Sulfated GAG	1 ng/mL
Congo red	Disulfonated dye (696)	8 ng/mL
Dextran sulfate 500	Sulfated GAG (500,000)	9 ng/mL
κ-carrageenan	Sulfated glycan	70 ng/mL
Dextran sulfate 8	Sulfated glycan (8000)	100 ng/mL
Heparin	Sulfated GAG (4000–6000)	100 ng/mL
Chondroitin sulfate[c]	Sulfated GAG	>10 μg/mL[d]
DEAE dextran	Polycation (500,000)	>10 μg/mL[d]
Dextran	Neutral glycan (500,000)	>10 μg/mL[d]
Polygalacturonic acid	Nonsulfated polyanion	>10 μg/mL[d]
Polyglutamic acid	Nonsulfated polyanion (2000–6000)	>10 μg/mL[d]
Salmon sperm DNA	Phosphate polyanion	>10 μg/mL[d]
Yeast tRNA	Phosphate polyanion	>10 μg/mL[d]
Amphotericin B	Polyene antibiotic (924)	>10 μg/mL[d]

[a]Moleular weight in Daltons, where known.
[b]IC = inhibitory concentration; concentration of inhibitor at which the amount of PrP-res decreased by 50%.
[c]Equal mix of types A, B, and C.
[d]Maximum concentration tested.

matrix *(70)*. In addition, it is known that all natural amyloids, including those containing PrP-res, contain such GAGs *(71–73)*. This has led to proposals that endogenous GAGs play a functional role in amyloidogenesis *(71–75)*.

A possible model for the conversion of PrP-sen to PrP-res, and the involvement of sulfated GAGs is illustrated in Fig. 2. Both PrP-sen and endogenous GAGs can be expressed on the cell surface *(28,70)* where the conversion of PrP-sen to PrP-res may occur *(27,43)*. According to the model, the PrP-res precursor (PrP-sen) associates with an endogenous GAG that may already be bound to some PrP-res (Fig. 2). Although PrP-sen is depicted in both monomeric and oligomeric forms, it is not known which form(s) predominates. However, it has been hypothesized that oligomeric forms of PrP may be intermediates in the formation of PrP-res *(13,76,77)*, and the model shown in Fig. 2 reflects this possibility. The association of PrP with the GAG molecule could be mediated by electrostatic interactions of the negatively charged sulfate groups on the GAG with positively charged groups on PrP, but could also be mediated by other unidentified molecules. Binding of PrP-sen to this GAG–PrP-res complex might lead to the close association of PrP-sen with PrP-res and its resultant conversion, by an unknown mechanism, into PrP-res aggregates. Sulfated inhibitors would bind PrP-sen and competitively inhibit its association with the endogenous GAG (Fig. 2B). Presumably, the inhibitors bind PrP-sen but, in contrast to the putative endogenous GAG, lack some function that is required for PrP-res accumulation. The inhibition of PrP-res accumulation in Sc+MNB by polyanions suggests that there is a specific interaction that occurs between cellular GAGs and PrP that is important in the formation or stabilization of amyloid filaments.

It should be emphasized that the model illustrated in Fig. 2 is only one of several possible mechanisms for the potential involvement of GAGs in the formation of PrP-res. For instance, the binding of PrP to GAG may occur after the formation of PrP-res. In this case, the inhibitors might bind to PrP-res and block its interaction with an endogenous GAG required for the long-term metabolic stability of PrP-res within the cell. However, recent studies support the series of events depicted in Fig. 2. For example, PrP-sen binds to immobilized heparin and Congo red, and this binding can be blocked by the addition of free heparin, Congo red, or other sulfated glycan inhibitors *(77a)*.

As has been suggested for GAG–amyloid interactions in general *(71–73,75,78,79)*, the binding of PrP to sulfated GAGs may be involved in the for-

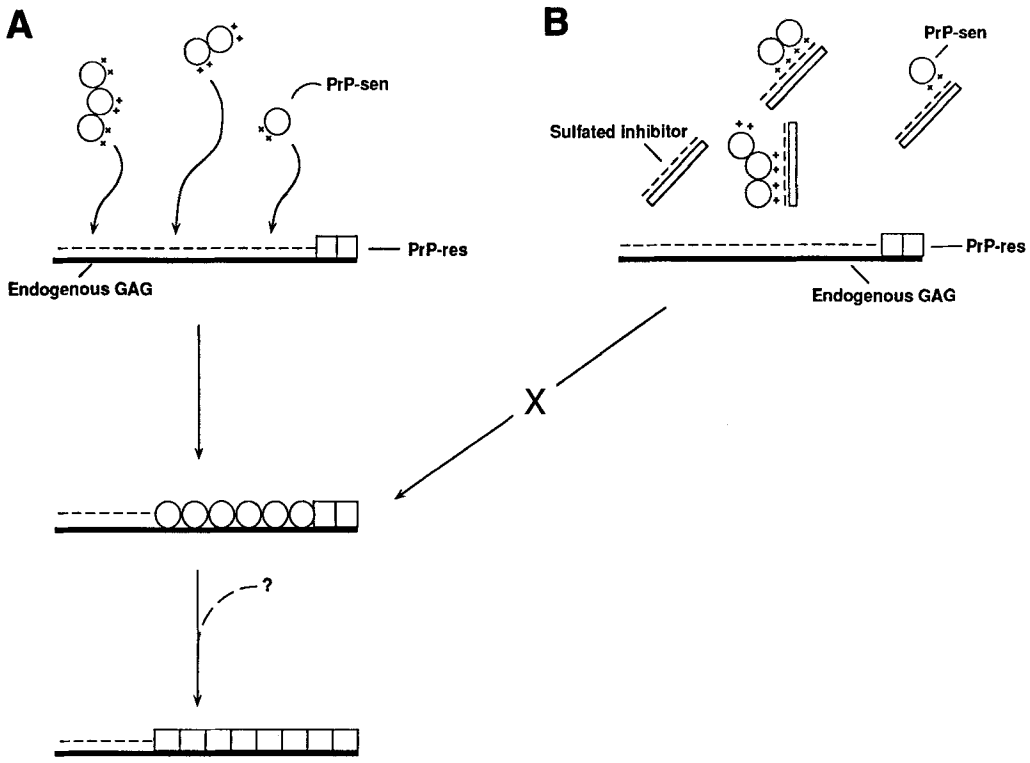

Fig. 2. One possible model for the role of endogenous glycosaminoglycans (GAGs) in PrP-res formation and its inhibition by polyanions. **(A)** PrP-sen monomers or oligomers (open circles) bind to a polyanionic, endogenous sulfated GAG (solid bar) that may already be bound to PrP-res (open squares). The association is assumed to be the result of the direct electrostatic interactions of the negatively charged sulfate groups on the GAG (–) with positively charged residues on PrP-sen (+), but could instead be mediated by other molecules such as multivalent cations bound to negative groups on PrP. PrP-sen is then converted to PrP-res via an unknown mechanism, as depicted by the "?". **(B)** It is hypothesized that a sulfated inhibitor binds to the GAG binding site of PrP-sen, competitively inhibiting the binding of PrP-sen to the endogenous GAG. This would prevent the close association of PrP-sen with PrP-res, and the consequent formation of PrP-res. Only one potential model is illustrated, however; there are other possible orders of addition of the components.

mation or stabilization of PrP-res in several ways. GAGs may act as "scaffolds" for the assembly of PrP-res aggregates or may induce a conformational change of PrP to the predominantly β-sheet structure that is characteristic of amyloid fibrils, including PrP-res *(55)*. In fact, there is evidence that highly sulfated proteoglycans can affect protein folding and induce conformational changes in systemic amyloid precursor proteins *(78,80)*. Sulfated GAGs may also protect PrP from proteolysis. The presence of GAGs in all naturally derived amyloid plaques, coupled with the inhibition of PrP-res accumulation by sulfated glycans and a recent study showing that heparan sulfate proteoglycans can influence the processing of the Alzheimer's amyloid precursor protein in vitro *(81)*, suggests that GAGs are important in a common mechanism of amyloidogenesis.

In Vivo Antiscrapie Activity of Sulfated Polyanion Inhibitors of PrP-res Accumulation

In mice, administration of DS500 prior to scrapie infection, at the time of infection, or for periods up to 3 mo postinfection alters disease incubation times *(63,65,67)*, whereas in hamsters, it must be administered within 2 h of infection to have an effect *(69)*. Apparently, DS500 impairs an early event in scrapie pathogenesis, possibly by interfering with the targeting and penetration of the agent into the central nervous system, replication of the agent, or early accumulation of PrP-res. If endogenous GAGs are a necessary cofactor in the formation or accumulation of PrP-res, the presence of exogenous GAGs could inhibit or delay this process, resulting in the pro-

longed incubation times observed. In this respect, it would be interesting to compare the level of PrP-res accumulation in the spleens of infected mice in the presence or absence of DS500 since the reticuloendothelial system is the earliest site of agent replication in mice *(64)*.

Conclusions

Our increasing understanding of the biosynthesis of PrP-res and the identification of a class of inhibitors that interfere with its accumulation have enabled us to begin to investigate key steps in PrP-res accumulation. New cell-free systems have been developed to test the amyloidogenic capabilities of specific synthetic PrP peptide subunits *(82–84)*. The availability of these cell-free systems as well as the scrapie-infected cell model described here, coupled with our increasing knowledge of the physical properties of inhibitors of the amyloidogenic process in these systems, should facilitate studies of the mechanisms of PrP-res accumulation at the cellular and molecular levels.

The effectiveness of the sulfated polyanions against scrapie infections in animals suggests that these or related compounds could be of therapeutic value in the treatment of human TSEs, such as Creutzfeldt-Jakob disease. Delivery of these molecules directly to the central nervous system would presumably provide the maximum prophylactic effect. Accordingly, a small, partly hydrophobic, Congo red-like molecule might be a more attractive therapeutic agent than a large polyanion because of the greater potential for crossing the blood–brain barrier.

Considering the effects of Congo red and sulfated glycans in the scrapie system and their proposed mechanism of action, it will be important to test whether such compounds might also have beneficial effects against the accumulation of amyloids associated with other diseases, such as Alzheimer's disease. Such studies will help test the generality of the concept that the interaction of endogenous sulfated GAGs with amyloidogenic proteins plays a key role in amyloid accumulation and might therefore be an attractive therapeutic target for the treatment of amyloidoses.

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