Sulphated glycosaminoglycans prevent the neurotoxicity of a human prion protein fragment

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Although a number of features distinguish the disease isoform of the prion protein (PrP^{Sc}) from its normal cellular counterpart (PrP^{C}) in the transmissible spongiform encephalopathies (TSEs), the neuropathogenesis of these diseases remains an enigma. The amyloid fibrils formed by fragments of human PrP have, however, been shown to be directly neurotoxic *in vitro*. We show here that sulphated polysaccharides (heparin, keratan and chondroitin) inhibit the neurotoxicity of these amyloid fibrils and this appears to be mediated via inhibition of the polymerization of the PrP peptide into fibrils. This provides a rationale for the therapeutic effects of sulphated polysaccharides and suggests a rapid *in vitro* functional screen for TSE therapeutics.

INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) are a group of neurodegenerative disorders which are characterized by the appearance of amyloid plaques, vacuolation and neuronal degeneration [1]. The spongiform encephalopathies in humans include sporadic, familial and iatrogenic forms of Creutzfeldt–Jakob disease (CJD), as well as at least three other human TSEs: Kuru, fatal familial insomnia and Gerstmann–Sträussler syndrome [1–4]. Scrapie of sheep and goats is the prototype of the TSEs and shows the characteristic neuropathology with the appearance of amyloid plaques containing amyloid fibrils, vacuolation and astroglial proliferation in the brains of infected animals [1]. The main component of the amyloid fibril is the prion protein (PrP), which is encoded by a highly conserved gene expressed in neurons and some non-neural tissues such as lymphoid cells [5–7]. The gene codes for a 33–35 kDa cell-surface glycoprotein, PrP^{C}, that is anchored to the membrane by an inositol glycophospholipid anchor, and from which the 27–30 kDa prion protein PrP that forms the amyloid fibrils is derived [5–10]. The cellular isoform of PrP^{C} can be converted into the protease-resistant, detergent-insoluble, scrapie isoform PrP^{Sc} [6,11,12]. The molecular events that mediate this conversion, however, remain unknown and current hypotheses include both post-translational modifications, such as glycosylation [2,12], and conformational changes from α-helix to β-sheet [13–17]. Although the latter conformational change is characteristic for the conversion of PrP^{C} to PrP^{Sc} [4], it should be noted that this change is also typical of the formation of all amyloid fibrils [15]. This includes the Aβ-amyloid fibrils and paired helical filaments that form, respectively, the amyloid plaques and neurofibrillary tangles characteristic of Alzheimer's disease (AD) [18–20].

As in the case of scrapie amyloid fibrils, both types of amyloid fibrils seen in the brains of patients with AD are also formed by the conversion of normal cellular proteins to abnormal amyloidogenic forms. The Aβ-amyloid fibrils are composed predominantly of fragments of a larger transmembrane glycoprotein, the β-amyloid precursor protein (βAPP) [20], and the paired helical filaments of a microtubule-associated cytoskeletal protein, tau [21]. Furthermore, similar to the conversion of PrP^{C} to PrP^{Sc}, the molecular modifications in βAPP and tau that give rise to the isoforms found in the Aβ-amyloid fibrils and paired helical filaments remain unknown [20,21]. The demonstration that glycosaminoglycans (GAGs) induced the polymerization of full-length tau under physiological conditions [22,23] follows on from earlier studies reporting similar results on the effects of these sulphated polysaccharides on the polymerization of βAPP fragments [24] and suggests that GAGs may play a pivotal role in the amyloidosis seen in AD [25]. Paradoxically, studies on scrapie models, both *in vitro* [26] and *in vivo* [27], have suggested that sulphated polysaccharides or other anionic compounds, such as dextran sulphate, may have some therapeutic value as they delay progression of the disease [26,28] and appear to inhibit the conversion of PrP^{C} to PrP^{Sc} [24]. We thus examined the effects of GAGs on the polymerization of PrP peptides into amyloid-like fibrils.

Previous work reported that a peptide corresponding to residues 106–126 of the human PrP showed a high intrinsic ability to polymerize *in vitro* to form amyloid fibrils, highly reminiscent of the scrapie amyloid fibrils purified from infected brains [29]. Furthermore, the amyloid fibrils formed by this peptide were shown to be highly toxic to primary rat hippocampal cultures at micromolar concentrations [26]. Additionally, the neurotoxicity of the PrP (106–126) fragment was tested in cortical neurons from mice lacking PrP and a much lower toxic effect than that found in neurons from wild type mice was observed [30]. Other studies have suggested a role for the PrP region comprising the residues 106–126 in the pathogenesis of prion-related encephalopathies [31–33]. On the other hand, the PrP fragment induces astroglial proliferation [34]. The direct neurotoxicity, demonstrated in the assays *in vitro* by adding the PrP

Abbreviations used: PrP, prion protein; PrP^{C}, normal cellular PrP; PrP^{Sc}, disease isoform of PrP; TSEs, transmissible spongiform encephalopathies; CJD, Creutzfeldt–Jakob disease; AD, Alzheimer's disease; βAPP, β-amyloid precursor protein; GAGs, glycosaminoglycans; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; βA, β-amyloid protein.

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fragment, was shown to result from the induction of apoptosis in the hippocampal neurons [26] and suggests a role for PrP fragments in the pathogenesis of TSEs [3,26]. We thus looked at the effects of sulphated and non-sulphated GAGs on the polymerization of a synthetic peptide corresponding to residues 106–126 of the human PrP.

MATERIALS AND METHODS

Materials

GAGs, polylysine and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl Blue) were obtained from Sigma. Dextran sulphate 500 was obtained from Fluka.

The peptide, containing residues 106–126 of PrP, was synthesized in an automatic solid-phase peptide synthesizer (type A430; Applied Biosystems), purified by reverse-phase HPLC on a Nova Pak C_{18} column (Waters) and analysed by mass spectrometry.

Polymer formation

The conditions of Forloni et al. [29] were followed. Alternatively, polymers were obtained by vapour diffusion in hanging drops, as described previously [22]. Peptide (10 μg) was resuspended in 10 μl of buffer containing 0.1 M Mes, pH 6.4, 0.5 mM $MgCl_2$, 2 mM EGTA and 50 mM NaCl in the absence or presence of GAG (0.4 mg/ml) and incubated at 4 °C for four days. Aggregation of the peptide was tested by centrifugation for 15 min at approximately 160000 ***g*** in an Airfuge (Beckman) and the amount of peptide present in the supernatant and pellet was characterized by 16% PAGE (containing Tricine) followed by Coomassie Blue staining. The samples containing the peptide in polymerized form were visualized by electron microscopy after placing them on a carbon-coated grid for 2 min and then staining with 2% (w/v) uranyl acetate for 1 min. The samples were then observed in a JEOL model 1200 EX electron microscope operated at 100 kV.

In some analyses the samples were stained with Congo Red as indicated previously by Fraser et al. [35].

Cell cultures

SH-SY5Y neuroblastoma cell line was maintained in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) foetal calf serum (Gibco BRL), antibiotics (streptomycin and penicillin) and glutamine (2 mM).

For neurotoxicity assays with PrP (106–126), the peptide was resuspended in sterile PBS at 10 mg/ml and incubated for 48 h at room temperature to promote its polymerization. The presence of polymers was determined by electron microscopy. Similarly, the different GAGs (heparan sulphate, chondroitin sulphate, keratan sulphate and hyaluronic acid) were resuspended in sterile PBS at 4 mg/ml. The peptide [PrP-(106–126)], from the previously polymerized stock solution, was mixed with each GAG at a final concentration of 1 mg/ml peptide and 0.4 mg/ml GAG; the mixture was maintained at room temperature for an additional 24 h. A sample of this mixture, in diluted form, in minimal essential medium supplemented with N_2, was added to the cultures.

The cells were plated out onto 24 multi-well plates. When the cells reached 50–60% confluency, the rich medium was changed to minimal essential medium supplemented with N_2, as described by Bottenstein and Sato [36]. Cells were maintained in this medium overnight until the addition of PrP peptide, with or without GAGs, in the same medium. The PrP neurotoxicity was assayed from 0.1 μg/ml to 25 μg/ml, using identical cell culture conditions without peptide or with GAGs alone, as controls. To determine the effect of GAGs on PrP peptide neurotoxicity, peptide and GAGs were mixed in the proportion 1:0.4 (w/w) and increasing amounts (1 to 10 μg/ml) of the different mixtures were added to the culture. The number of cells was determined 5–6 days after treatment. Cell viability was performed using the MTT assay [37].

RESULTS

Polymers of PrP fragment assembled with GAGs

Polymerization was assayed both biochemically and morphologically and the results are shown in Figures 1 and 2. As reported previously [26], the synthetic PrP peptide readily polymerized into amyloid-like fibrils at concentrations of 0.1 to 1 mg/ml (Figure 1, open bars, and Figure 2a), although little polymerization was observed at concentrations as low as 0.01 mg/ml (Figure 1). The amyloid nature of the fibrils formed was further confirmed by Congo Red staining (not shown). Addition of the sulphated GAG, heparan sulphate, significantly inhibited the polymerization of the synthetic PrP peptide (Figure 1, filled bars). Interestingly, the new fibrils observed in the presence of heparan sulphate showed a distinct morphology (Figures 2b and 2c) with a number of twisted fibrils (Figure 2c) and the presence of bound heparan sulphate in these polymers could be detected immunochemically (results not shown). Similar results, with both the inhibition of polymerization and the formation of few morphologically distinct fibrils, were obtained with the other sulphated GAGs tested, keratan sulphate and chondroitin sulphate A, B and C (Figures 2e–2b), but not with the non-sulphated GAG, hyaluronic acid (Figure 2d). A control polycation, polylysine, showed no effect on amyloid fibril formation by the PrP peptide (Figure 2i), excluding a simple charged polymer effect. Additionally, dextran sulphate 500 was also used as a control polyanion since it had been previously used as a potent anti-scrapie agent [26,28]. In this case a morphological change was also observed. Filaments, wider than those assembled in the absence of the compound, were found (Figure 2j). However, a decrease (12 times less) in the amount of aggregated protein

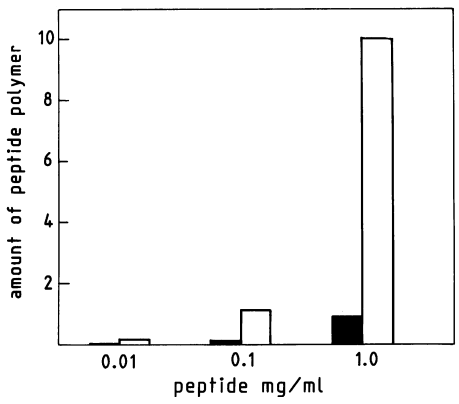

Figure 1 PrP peptide, corresponding to residues 106–126 of the human PrP, was polymerized at increasing concentrations in the absence or presence of a fixed concentration (0.4 mg/ml) of heparan sulphate

The resulting polymers were visualized by electron microscopy and isolated by centrifugation; the amount of polymerized PrP peptide was quantified by densitometry of Coomassie Blue stained SDS/polyacrylamide gels. The amount of polymerized PrP peptide (μg) in the absence (□) or the presence of heparan sulphate (■) is shown.

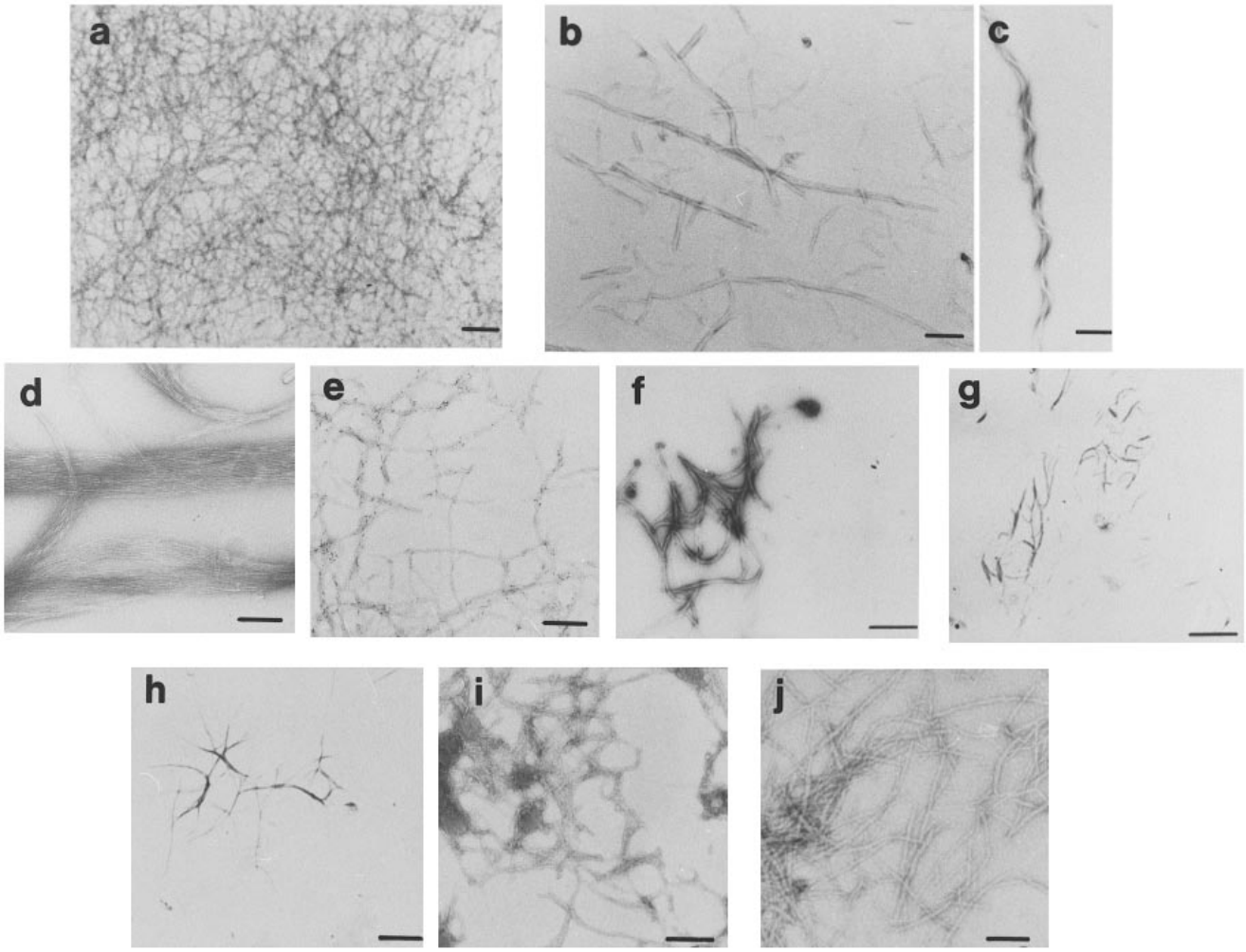

Figure 2 Electron micrographs of the negatively stained polymers assembled from the PrP peptide in the absence or presence of sulphated (heparin, keratan, chondroitin A, B and C) or non-sulphated (hyaluronic acid) GAGs

(**a**) PrP polymers formed by peptide alone. (**b–j**) PrP polymer formed in the presence of heparan sulphate (**b,c**), hyaluronic acid (**d**), keratan sulphate (**e**), chondroitin sulphate A (**f**), chondroitin sulphate B (**g**), chondroitin sulphate C (**h**), polylysine (**i**) and dextran sulphate 500 (**j**). Bars represent 200 nm.

was observed in the presence of dextran sulphate, a decrease similar to that observed in the presence of heparan sulphate (Figure 1). On the other hand, no inhibition of polymerization of the PrP peptide was seen with hyaluronic acid. However, the amyloid-like fibrils formed in the presence of this non-sulphated GAG (Figure 2d) appeared to be distinct from those seen with synthetic peptide alone (Figure 2a), with largely longer, less tangled fibrils observed in the presence of hyaluronic acid. These results demonstrate that sulphated polysaccharides can inhibit the formation of amyloid fibrils by PrP fragments and, given the direct neurotoxicity of these fibrils [29], suggest an alternative explanation for their therapeutic efficacy to that previously proposed [27].

To test whether GAGs, for example, chondroitin sulphate B or keratan sulphate, bind to the PrP peptide polymers, the presence of those GAGs was analysed in PrP peptide polymers isolated by centrifugation on a sucrose cushion, by testing for the reaction of an antibody raised against the GAG with the sedimented polymer. Our results indicate a reaction of the polymer fraction with antibodies raised against chondroitin sulphate B and against keratan sulphate. On the other hand, no sediment was found when GAGs were centrifuged in the absence of PrP peptide (results not shown). These results suggest that GAGs associate with PrP-peptide polymers.

Neurotoxicity of the PrP fragment in the presence or absence of GAGs

To investigate this suggestion further, we examined the effects of both sulphated and non-sulphated GAGs on the direct neurotoxicity of the amyloid fibrils formed by the PrP peptide that had been reported previously in the assays *in vitro*, showing the induction of apoptosis in the hippocampal neurons [26]. In our experiments we looked at the toxicity of the amyloid-like fibrils formed by the synthetic PrP peptide on the cultures of the human neuroblastoma cell line SH-SY5Y. Under the cell culture conditions used, these cells grow predominantly as monolayers, with few cell aggregates, and most cells show a 'flattened' morphology with cytoplasmic extensions (Figure 3C). In agreement with the results reported previously for primary rat hippocampal neuronal

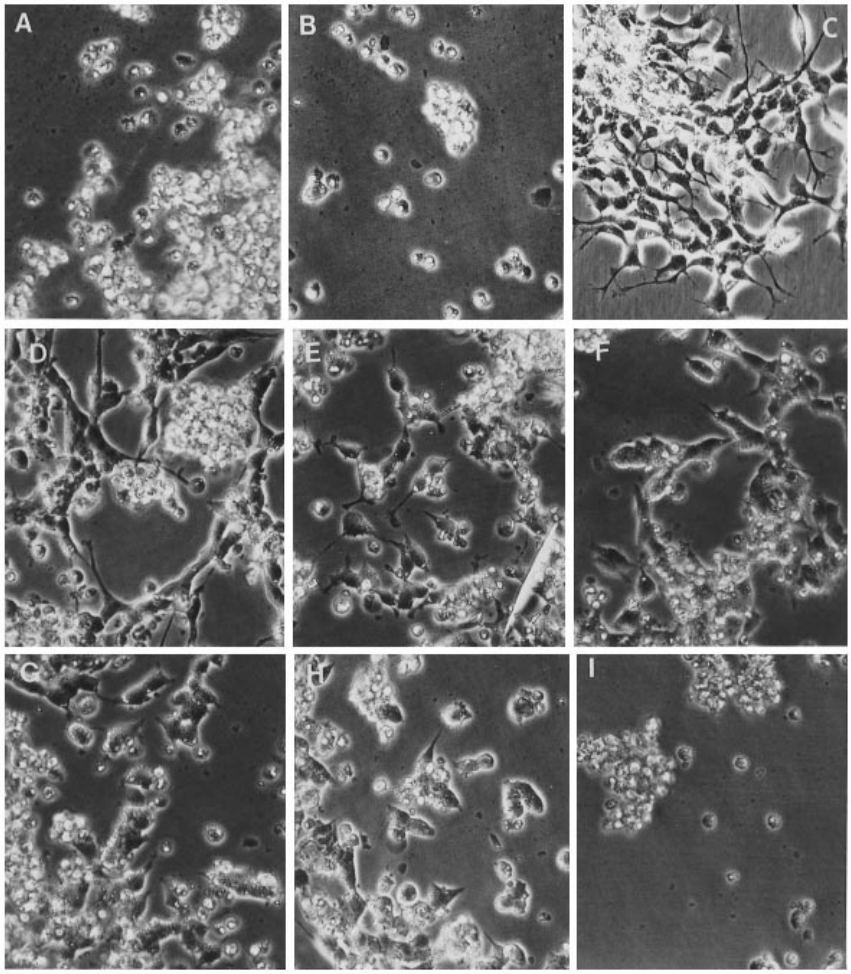

Figure 3 Phase-contrast microscopy of SH-SY5Y human neuroblastoma cells after 5 days of treatment with PrP peptide-(106–126) polymerized in the absence or presence of sulphated (heparin, keratan, chondroitin A, B and C) or non-sulphated (hyaluronic acid) GAGs

(**A,B**) PrP peptide alone, previously polymerized, at final concentrations of 1 μg/ml (**A**) or 10 μg/ml (**B**). (**C**) Untreated control. (**D**) Control treated with heparan sulphate alone. (**E,F**) PrP peptide polymerized in the presence of heparan sulphate (ratio 1:4) at PrP concentrations of 1 μg/ml (**E**) or 10 μg/ml (**F**). (**G–I**) PrP peptide polymerized in the presence of chondroitin sulphate B (**G**), keratan sulphate (**H**) and hyaluronic acid (**I**).

cultures, the amyloid fibrils formed by the synthetic PrP peptide were directly neurotoxic to the SH-SY5Y neuroblastoma cell cultures (Figures 3A and 3B). Cell death again occurs, with the neuroblastoma cells changing their neuron-like morphology, losing their neuritic processes, and becoming rounded before loss of viability (Figures 3A and 3B). As some of the growing cells may sometimes appear rounded morphologically (Figure 3C), cell viability was also quantified by counting cell numbers after vital dye staining using the MTT assay (Figure 4). The neurotoxic effect, clearly detectable at 1 μg/ml, was more evident at 10 μg/ml 5 days after peptide addition (Figure 3). This neurotoxic effect was even more pronounced at higher concentrations, with complete loss of neuroblastoma cell viability 3–4 days after addition of the synthetic PrP peptide at a concentration of 20 μg/ml.

The effect of the addition of GAGs on the neurotoxicity of the PrP peptide was tested at different concentrations of synthetic peptide (1 to 10 μg/ml), in the presence of the different GAGs, and the results obtained are shown in Figures 3 and 4. Paralleling their effects on the inhibition of PrP peptide polymerization, the sulphated GAGs, heparan sulphate (Figures 3E and 3F), keratan sulphate (Figure 3H) and chondroitin sulphate (Figure 3G), all inhibited the neurotoxicity of the synthetic peptide whereas the non-sulphated GAG, hyaluronic acid, again showed no effect (Figures 3I and 4).

To titrate the effect of GAGs on PrP peptide toxicity, PrP peptide (1 mg/ml) was mixed with GAGs (0.4 mg/ml), and increasing concentrations of this mixture (containing from 1 to 10 μg/ml of PrP peptide) were added to the cell cultures. The proportion of viable cells after 5 days of treatment is shown in Figure 4.

Analysis of this neuroprotective effect of the sulphated glycans revealed different efficiencies, with heparan sulphate being the most effective, followed by keratan sulphate and then the chondroitin sulphates (Figures 3, 4A, 4B). Interestingly, the different forms of chondroitin sulphate also showed distinct efficacies, with chondroitin sulphate A being the less effective and chondroitin sulphate C the more effective in cell protection (Figure 4B). Figure 4(A) also indicates the effect of the polyanion, dextran sulphate 500, on neural toxicity. Dextran sulphate 500 prevents peptide toxicity. However, its protection is slightly lower than that of heparan sulphate, which could be due to the

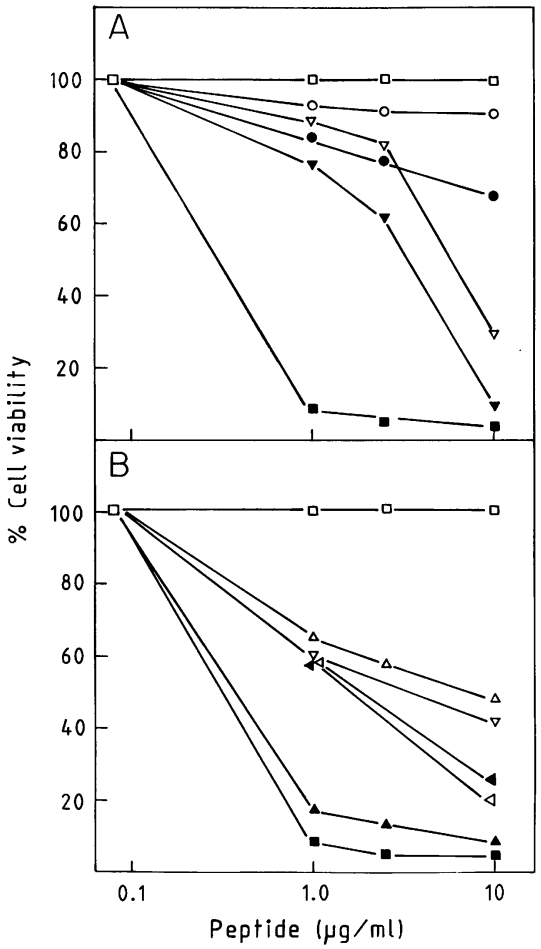

Figure 4 Neuronal cell viability after treatment with polymerized PrP peptide-(106–126)

(**A**) Neuronal cell viability after treatment with PrP peptide 106–126 polymerized in the absence (■) or presence of heparan sulphate (●) or dextran sulphate 500 (▼). The action of heparan sulphate (○) or dextran sulphate 500 (▽), in the absence of PrP peptide, on the cultures is shown. (**B**) As above, but in this case cell viability is shown after treatment with PrP peptide in the absence (■) or presence of keratan sulphate (△), chondroitin sulphate A (◁), B (◀) and C (▽) and hyaluronic acid (▲). Cell viability controls, with only the addition of medium without peptide, are shown (□) in (**A**) and (**B**).

fact that the preparation of dextran sulphate 500 shows by itself, in the absence of PrP peptide, an evident toxicity at 10 μg/ml when the cultures are exposed to the compound for 5 or more days.

DISCUSSION

PrP is located at the cell membrane surface where it can interact with components of the extracellular matrix like GAGs. Extracellular soluble forms have also been identified, not only in the medium of cultured cells, but also in human fluids [38], suggesting that those forms could also interact with components of the extracellular matrix. Some of these components, the GAGs, are associated with the modified toxic form of PrP in amyloid deposits assembled by the aggregation of that protein [39,40].

The functional relevance of GAGs in the nervous system is still unclear. Nonetheless the effects of GAGs have been reported for both neurite formation [41–46] and the survival of neurons [45]. A role for GAGs has also been suggested in neural development or regeneration [47–49]. For example, GAGs can modulate the extracellular localization of wingless protein [50], a factor involved in central nervous system development in *Drosophila*. In neuropathogenesis, it has been proposed that GAGs play a role in the deposition of fibrillar Aβ-amyloid in rat brain [24] and catalyse the formation of paired helical filaments in AD [22,23,51]. It has also been indicated that sulphated glycans may play an important role in the cellular metabolism of PrP [52].

In the present study, a neuroprotective role is suggested for GAGs, since our studies show that the presence of these molecules in sulphated form may decrease the toxic effect of the PrP fragment containing residues 106–126 of the protein.

In this work we have found, using a fragment of the protein that has the capacity for self-assembly and is toxic for cultured neurons, that PrP fragments are able to bind sulphated (such as heparin or keratan) and non-sulphated (such as hyaluronic acid) GAGs with markedly different results. The interaction of sulphated GAGs, such as heparin, with the polymers assembled from the PrP fragment results in the formation of twisted (paired?) filaments with a morphology different to that of the polymers assembled solely from the PrP fragment. Furthermore, the critical concentration required for the assembly of these latter polymers is much lower than that observed for the assembly of GAG–PrP fragment polymers. It is likely that the interaction of the PrP peptide with the GAGs occurs between the basic residues KTNMKH present at the N-terminus of the PrP fragment, and the negatively charged GAGs molecules. However, the effect of the sulphated GAGs is unlikely to be a simple charge effect as the polycation, polylysine, shows no effect on the amyloid fibril formation of the PrP peptide.

Most interestingly, our results show a direct correlation between the extent of amyloid formation and the neurotoxicity of the PrP fragment. Studies on murine scrapie models have shown that sulphated polysaccharides delay progression of the disease [26–28]. The effects *in vivo* were thought to be mediated by the blocking of neuronal cell infection by PrP^{Sc} [50], however, studies *in vitro* have shown that sulphated polyanions may also inhibit the conversion of PrP^{C} to PrP^{Sc} [27,53,54]. The results presented here, on the inhibition of the polymerization of the amyloidogenic PrP peptide by sulphated GAGs, are consistent with the latter hypothesis [27], since the α-helix to β-sheet conformational change seen upon the conversion of PrP^{C} to PrP^{Sc} is accompanied by the formation of amyloid fibrils [4]. However, although it can be suggested that the neuroprotective effect of GAGs may depend on avoiding the conformational change of the PrP peptide, other possibilities for the action of GAGs should not be excluded. It should be mentioned that similar neuroprotective effects of GAGs were obtained against another neurotoxic peptide [the fragment containing residues 25 to 35 of β-amyloid protein (βA)] [55], and it has also been proposed that GAGs may promote fibrillogenic activity in other βA peptides (for example the fragment containing residues 11–28) [56].

Finally, the need for effective therapeutics for the treatment of TSEs has been intensified by the potential epidemic of an increasing number of CJD cases, resulting from the significant public health risk due to the ingestion of bovine spongiform encephalopathy-infected tissues [57,58]. Our study also provides a rapid functional screen for potential CJD therapeutics, by using the direct neurotoxicity of the amyloidogenic peptide comprising residues 106–126 of the human PrP on the human SH-SY5Y neuroblastoma cell cultures as a screening assay *in vitro*.

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