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Scrapie-Associated PrP Accumulation and Agent Replication: Effects of Sulphated Glycosaminoglycan Analogues

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Scrapie-associated PrP accumulation and agent replication: effects of sulphated glycosaminoglycan analogues

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SUMMARY

An abnormally protease-resistant and apparently neuropathogenic form of PrP accumulates in the brains of hosts with scrapie and related transmissible spongiform encephalopathies. Studies with scrapie-infected neuroblastoma cells have highlighted dramatic differences in the metabolism of the normal (protease-sensitive) and scrapie-associated (protease-resistant) isoforms of PrP. Furthermore, this model has been useful in identifying inhibitors of protease-resistant PrP accumulation and scrapie agent replication which are valuable as potential therapeutic agents and as probes of the mechanism of protease-resistant PrP formation. These inhibitors include the amyloid stain Congo red and certain sulphated glycans which are glycosaminoglycans themselves or glycosaminoglycan analogues. The relative potencies of various sulphated glycans correlate with their previously determined anti-scrapie activities *in vivo*, suggesting that the prophylactic effects of sulphated polyanions is due to inhibition of protease-resistant PrP accumulation. These and other observations suggest that an interaction of PrP with endogenous sulphated glycosaminoglycans or proteoglycans is important in protease-resistant PrP accumulation, and raise the possibility that therapies for transmissible spongiform encephalopathies and other amyloidoses could be based on blocking (pre)amyloid-glycosaminoglycan interactions.

1. INTRODUCTION

A defining characteristic of the scrapie and related transmissible spongiform encephalopathies (TSEs) is the accumulation, sometimes in the form of amyloid plaques, of an abnormally protease-resistant isoform of a host protein, PrP (Bolton *et al.* 1982; Diringer *et al.* 1983; Bendheim *et al.* 1984). The fact that the protease-resistant PrP copurifies with infectivity yet does not appear to be associated with any scrapie-specific nucleic acid has led Prusiner (1982) to propose that the protease-resistant PrP is the infectious protein agent of scrapie, as was initially postulated by Griffith (1967) and others. Although this hypothesis is still speculative, it is clear that PrP plays an important role in TSE pathogenesis. Indeed, mice lacking PrP altogether are resistant to scrapie but may be capable of replicating scrapie infectivity at a low level (Bueler *et al.* 1993).

PrP is normally found in a protease-sensitive form in brain and other tissues (Oesch *et al.* 1985; Robakis *et al.* 1986; Rubenstein *et al.* 1986; Hope *et al.* 1986; Meyer *et al.* 1986; Cho 1986; Bendheim *et al.* 1992), and its expression is developmentally regulated (Mobley *et al.* 1988; Manson *et al.* 1992; Lieberburg 1992). Although there is evidence that PrP is involved in lymphocyte activation (Cashman *et al.* 1990), its normal function is otherwise unclear, and mice which lack the protein due to homozygous knockout of its gene appear to develop normally (Bueler *et al.* 1992). During TSE pathogenesis, the abnormal protease-resistant PrP accumulates in the central nervous system and other tissues (Bolton *et al.* 1982; Diringer *et al.* 1983; Rubenstein *et al.* 1986; Shinagawa *et al.* 1986; Rubenstein *et al.* 1991; Race & Ernst 1992). Both PrP isoforms are encoded by the same host gene (Basler *et al.* 1986), and no apparent scrapie-associated differences arise at the level of the mRNA (Chesebro *et al.* 1985; Oesch *et al.* 1985) or primary protein sequence (Hope *et al.* 1986; Stahl *et al.* 1993). Thus the scrapie-specific modification of PrP was thought to arise post-translationally, and this has been borne out by metabolic studies (Borchelt *et al.* 1990; Caughey & Raymond 1991).

The cellular mechanism for the conversion of PrP to the TSE-specific forms is not known. However, substantial progress has been made in understanding the metabolism of both the protease-sensitive and protease-resistant PrP isoforms, and how the accumulation of the protease-resistant PrP can be inhibited. Much of the progress in these areas has come from work with chronically scrapie-infected tissue culture cells such as mouse neuroblastoma (MNB) cells, which can be metabolically labelled and conveniently manipulated *in vitro* (Race *et al.* 1987, 1988). Studies with these cultures have identified dramatic contrasts in the cellular metabolism of the two PrP isoforms, providing insight into TSE pathogenesis.

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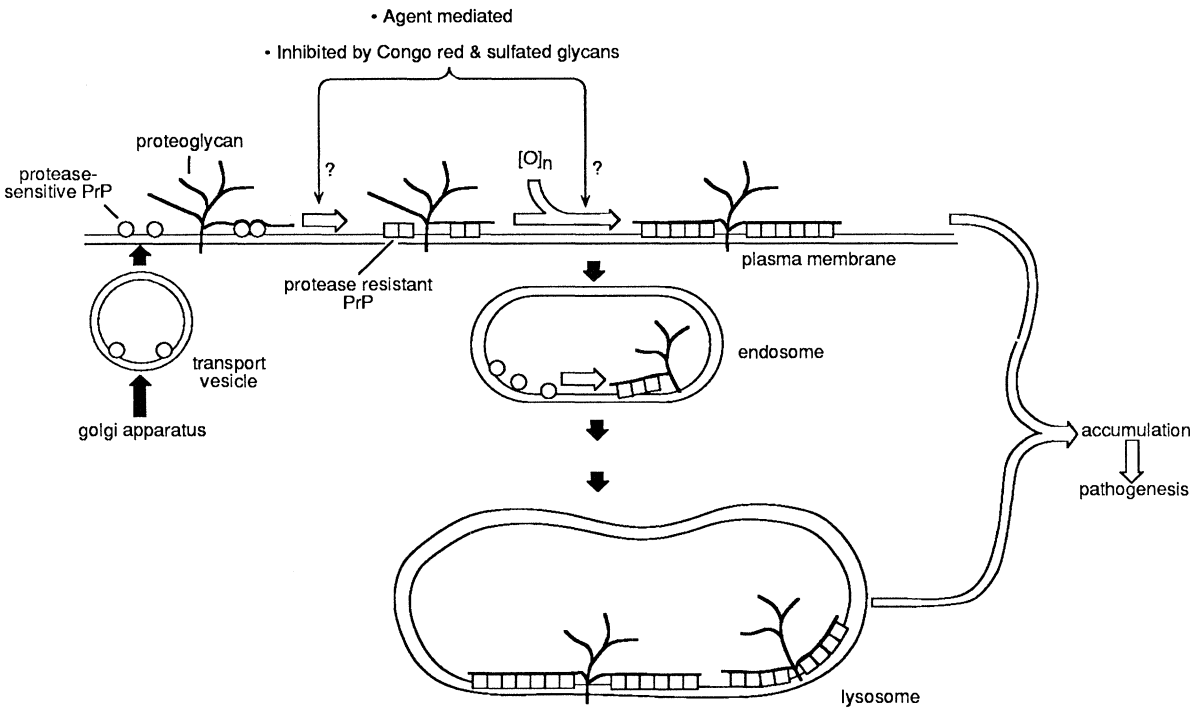

Figure 1. Scrapie-associated formation of protease-resistant PrP-proteoglycan aggregates: a hypothetical model. In Sc^+-MNBs, the conversion of protease-sensitive PrP to the protease-resistant state appears to occur on the cell surface and in endosomes (Caughey & Raymond 1991; Caughey *et al.* 1991*b*; Borchelt *et al.* 1992). Several aspects of the conversion process remain unclear: (i) the structure(s) of the protease-resistant PrP-proteoglycan aggregates; (ii) the order of addition of the components; (iii) the role played by the infectious agent; and (iv) the mechanism of pathogenesis. Congo red and certain sulphated glycans may inhibit protease-resistant PrP accumulation blocking PrP interactions with endogenous sulphated glycosaminoglycans or proteoglycans (Caughey & Raymond 1993).

2. NORMAL PrP METABOLISM

PrP begins its metabolic cycle in the endoplasmic reticulum (ER) where a glycophosphatidylinositol anchor and high-mannose glycans are attached in conjunction with the removal of sequences at the N- and C-termini (Caughey *et al.* 1989; Stahl *et al.* 1990*a*). The high-mannose glycan moieties are converted to complex or hybrid glycans upon passage through the Golgi apparatus (Caughey *et al.* 1989; Endo *et al.* 1989). Most of the mature PrP is anchored to the cell surface by the phosphatidylinositol moiety and can usually be removed with phospholipase or protease treatments (Stahl *et al.* 1987; Caughey *et al.* 1989; Caughey *et al.* 1990). Some intracellular vesicular staining of normal PrP has been observed in MNB cells (Caughey *et al.* 1990) and neurons (Piccardo *et al.* 1990), but this may simply represent the nascent PrP in the ER and Golgi apparatus. Most of the PrP is normally catabolized with a half-life of 3–6 h, but a small proportion can be released into the medium (Caughey *et al.* 1988, 1989; Borchelt *et al.* 1990; Caughey & Raymond, 1991). Soluble forms of PrP have now been identified in the central nervous system as well (Tagliavini *et al.* 1992; Harris *et al.* 1993).

3. SCRAPIE-ASSOCIATED PROTEASE-RESISTANT PrP BIOSYNTHESIS

Pulse-chase metabolic labelling studies have shown that, when MNB cells are infected with scrapie, a small proportion of the total PrP slowly becomes protease- and phospholipase resistant (Borchelt *et al.* 1990; Caughey & Raymond, 1991; Caughey *et al.* 1990; Stahl *et al.* 1990*b*; Safar *et al.* 1991). The conversion to the protease-resistant, metabolically stable state occurs after the apparently normal PrP precursor reaches the cell surface (figure 1) (Caughey & Raymond 1991). Soon after its formation, protease-resistant PrP in MNB cells is exposed to lysosomal or endosomal proteases and truncated at the N-terminus (Caughey *et al.* 1991*b*; Taraboulos *et al.* 1992). Thus formation of the protease-resistant PrP occurs on the plasma membrane or along an endocytic pathway to the lysosomes (Caughey & Raymond 1991; Caughey *et al.* 1991*b*; Borchelt *et al.* 1992). Although the normal protease-sensitive PrP is effectively catabolized, the protease-resistant PrP shows no sign of turnover (Borchelt *et al.* 1990; Caughey & Raymond 1991) and appears to accumulate in lysosomes in scrapie-infected MNB (Sc^+-MNB) cells (Caughey & Raymond 1991; McKinley *et al.* 1991; Caughey *et al.* 1991*b*).

These and other considerations prompted the proposal that the lysosomal accumulation of protease-resistant PrP is important in TSE pathogenesis (Laszlo *et al.* 1992). It is worth noting, however, that much of the protease-resistant PrP in scrapie-infected mouse or hamster brain is not N-terminally truncated (Hope *et al.* 1986; Bolton *et al.* 1987), and has been detected on the plasma membrane (Jeffrey *et al.* 1992), in extracellular amyloid plaques (Bendheim *et al.* 1984) and other apparently non-lysosomal sites (DeArmond *et al.* 1987; Piccardo *et al.* 1990; Diedrich *et al.* 1991). Thus

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it is not clear that translocation to the lysosomes, proteolysis or any other covalent modification of PrP is important in protease-resistant PrP formation or the scrapie disease process. The difference between protease-resistant PrP and normal PrP may be purely conformational or dependent upon an interaction of PrP with another molecule (Hope *et al.* 1986).

4. CONFORMATIONAL STUDIES OF PrP AMYLOID

Although the conformational analysis of protease-resistant PrP by most conventional techniques has been confounded by the insolubility of the material, we used Fourier transform infrared (FTIR) spectroscopy to assess the secondary structure of highly infectious preparations of protease-resistant PrP amyloid fibrils (PrP 27–30) (Caughey *et al.* 1991*a*). These studies revealed that PrP 27–30 has the high β-sheet content characteristic of other amyloids. The β-sheet content (*ca.* 48%) was approximately twice that predicted from the amino acid sequence, but the helix and turn contents were lower than predicted. This suggested that protease-resistant PrP formation might involve a transformation from helix and coil to β-sheet. Recent infrared studies of small synthetic peptide fragments of the PrP sequence are also consistent with this hypothesis (Gasset *et al.* 1992).

5. INHIBITOR STUDIES

The dissection of the mechanism of protease-resistant PrP formation might also be aided by the availability of inhibitors of this process. Furthermore, such inhibitors might lead to therapeutic approaches for the TSEs. We have used Sc^+-MNBs as a screening system for inhibitors of protease-resistant PrP accumulation. In the screening assay, potential inhibitors are added to the culture medium of lightly seeded cells. The cells are grown to confluence and analysed for protease-resistant PrP content by semi-quantitative immunoblotting. The first inhibitor to be identified using this approach was the classic amyloid stain, Congo red, which blocked protease-resistant PrP accumulation in the cells effectively at *ca.* 10 ng ml^{-1} (14 nM) (Caughey & Race 1992). Furthermore, no protease-resistant PrP was detected in the medium of treated cells (figure 2), suggesting that Congo red was not simply causing the release of protease-resistant PrP from the cell surface. Congo red is a sulphonated dye molecule, and we wondered if the sulphated glycans possessing prophylactic anti-scrapie activity in mice and hamsters (Ehlers *et al.* 1984; Ehlers & Diringer 1984; Farquhar & Dickinson 1986; Kimberlin & Walker 1986; Diringer & Ehlers 1991; Ladogana *et al.* 1992) might have anti-protease-resistant PrP activity similar to that of Congo red. Indeed, pentosan polysulphate, iota-carrageenan and dextran sulphate proved to be at least as effective as Congo red on a mass per volume basis (Caughey & Raymond 1993). Other sulphated glycans with no anti-scrapie effect in animals, such as heparin and chondroitin sulphate, were orders of magnitude less effective as inhibitors of protease-resistant PrP accumulation. Thus a correlation was observed between the therapeutic efficacy of these sulphated glycans and their potency as inhibitors of protease-resistant PrP accumulation.

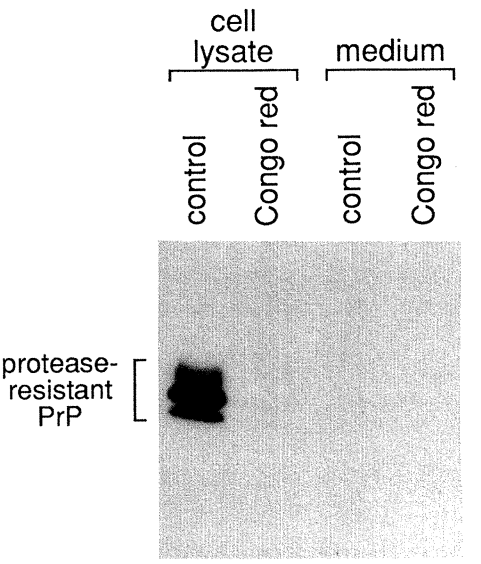

Figure 2. Congo red inhibition of protease-resistant PrP accumulation and lack of release of protease-resistant PrP into the medium of treated cultures. Sc^+-MNB cells (25 cm^2 flasks) were seeded with one tenth of the cells detached from a confluent flask and were grown to confluence in the presence or absence (control) of 100 ng ml^{-1} Congo red (Caughey & Race 1992). Both the cells and the culture medium were tested for the presence of protease-resistant PrP. Protease-resistant PrP was extracted from cell lysates and solubilized in SDS-PAGE sample buffer as described previously (Caughey & Raymond 1993). The media of the cell cultures (10 ml) were centrifuged at 1000 × *g* for 5 min, and the supernatants were then recentrifuged at 184 000 × *g* (ave) for 2 h at 4°C. The 184 000 × *g* pellets were sonicated into 1 ml of the detergent buffer used to lyse the cells, and treated with 20 μg ml^{-1} proteinase K for 30 min at 37°C. After inactivation of the protease with PMSF, the suspensions were centrifuged at 340 000 × *g* (ave) for 40 min at 4°C. The resulting pellets were solubilized in SDS-PAGE sample buffer and, along with the samples from the cell lysates, were analysed for protease-resistant PrP by immunoblotting as described previously (Caughey & Raymond 1993). Equal flask equivalents were loaded into each lane.

The inhibition of protease-resistant PrP accumulation by Congo red and pentosan polysulphate occurred without apparent effects on the metabolism of its apparently normal protease-sensitive precursor or other cellular proteins (Caughey & Race 1992; Caughey & Raymond 1993). The inhibition was primarily due to prevention of new protease-resistant PrP accumulation rather than destabilization of pre-existing protease-resistant PrP. Even after removal of the inhibitors, the accumulation of protease-resistant PrP remained depressed in the cultures, suggesting that the inhibitory effect was not readily reversible. A comparison of the activities of various sulphated glycans, non-sulphated polyanions, dextran and DEAE-dextran provided evidence that the density of sulphation and molecular size are factors influencing anti-protease-resistant PrP activity of these sulphated glycans.

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The potent and selective inhibition exhibited by Congo red and the sulphated glycans suggested that an understanding of their mechanism of action would shed light on the mechanism of protease-resistant PrP accumulation. In this regard, it is interesting to note that all tissue-derived amyloid plaques, including those composed of protease-resistant PrP, contain highly sulphated glycosaminoglycans (GAGs) in the form of heparan sulphate proteoglycan (Snow *et al.* 1989, 1990; Guiroy *et al.* 1991). This observation led to proposals that endogenous sulphated proteoglycans may be involved in the polymerization of proteins into amyloid filaments (Snow *et al.* 1989, 1990; Guiroy & Gajdusek 1989; Guiroy *et al.* 1991). As the inhibitors of protease-resistant PrP accumulation that we have identified are in fact GAGs, or can be viewed as analogues of GAGs, we reasoned that these inhibitors bind to a PrP isoform and competitively inhibit an interaction of PrP with a specific cellular sulphated GAG that is essential for protease-resistant PrP formation or stabilization (Caughey & Raymond 1993; Caughey 1993).

We have obtained evidence that normal protease-sensitive PrP can bind to both immobilization heparin (a highly sulphated GAG) and Congo red, and that either interaction can be blocked with free Congo red or sulphated glycan inhibitors of protease-resistant PrP accumulation (Caughey *et al.* 1994). Thus these inhibitors may bind PrP to prevent the interaction with the appropriate endogenous GAG or proteoglycan, but lack features required to facilitate protease-resistant PrP accumulation within cells by themselves.

How might GAGs influence protease-resistant PrP accumulation? Several mechanisms have been proposed for the role of GAGs in amyloidogenesis generally, including: (i) protecting PrP from proteolysis; (ii) targeting PrP to a particular cellular site that is involved in protease-resistant PrP formation or accumulation; (iii) altering PrP conformational shifts from α-helix to the apparent β-sheet structure that is predominant in amyloid fibrils, including those comprising protease-resistant PrP (Caughey *et al.* 1991*a*); and (iv) acting as a scaffold for the assembly of aggregates (Kisilevsky 1987; Snow & Wight 1989; Snow *et al.* 1989, 1990; Guiroy & Gajdusek 1989; Guiroy *et al.* 1991). Given these possibilities, it is conceivable that differential GAG expression can influence the susceptibility of a given cell to the accumulation of protease-resistant PrP and perhaps, therefore, its susceptibility to TSE infection and pathogenesis. Indeed, very few of the many different tissue culture cells that express PrP are capable of stable scrapie infection, and this may be influenced by their GAG or proteoglycan expression.

6. EFFECT OF CONGO RED ON SCRAPIE AGENT REPLICATION

Given the controversy surrounding the issue of the relation of protease-resistant PrP to scrapie infectivity, we tested the effect of Congo red treatment on scrapie agent replication in Sc^{+}-MNB cells. Congo red treatment sufficient to eliminate protease-resistant PrP from the cultures also eliminated scrapie infectivity as bioassayed in mice (Caughey *et al.* 1993). This observation is consistent with the idea that protease-resistant PrP is a vital component of the scrapie agent, or that efficient agent replication depends on the presence of protease-resistant PrP.

7. A THERAPEUTIC STRATEGY FOR TSEs AND OTHER AMYLOIDOSES

As noted above, several laboratories have shown that certain sulphated glycans and other polyanions have prophylactic value against scrapie in animals, and it appears that the therapeutic mechanism of these compounds may be to block protease-resistant PrP accumulation by interfering with essential PrP–GAG interactions. The frequency of GAG interactions with all types of amyloid deposits raises the possibility that, by a similar mechanism, polyanionic compounds might reduce amyloidogenesis associated with other diseases such as Alzheimer's disease. To the extent that amyloid or preamyloid accumulation is clinically relevant in these various diseases, the potential anti-amyloid effects of these compounds might be of therapeutic value.

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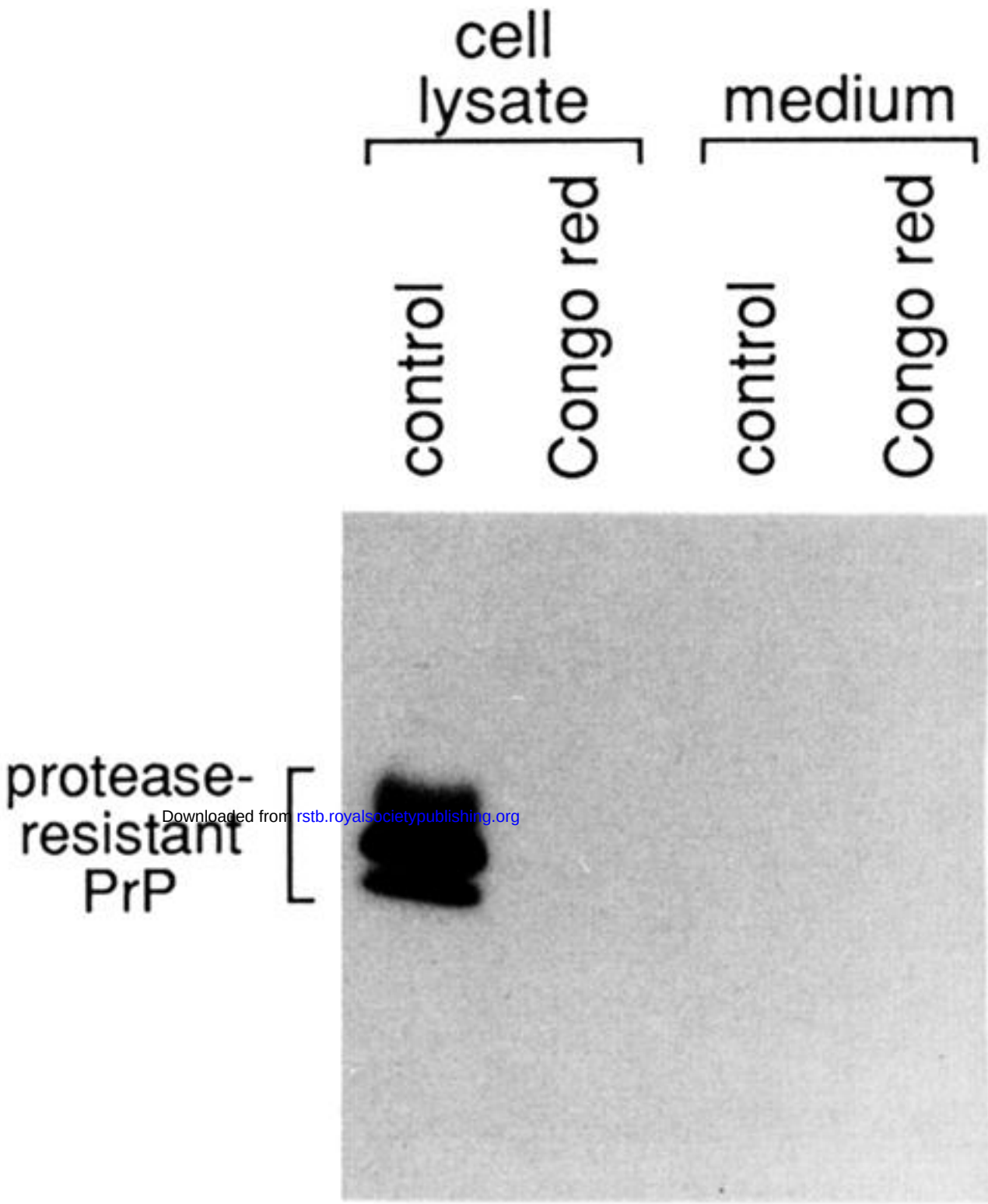
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



gure 2. Congo red inhibition of protease-resistant PrP cumulation and lack of release of protease-resistant PrP to the medium of treated cultures. Sc^+-MNB cells (25 cm^2 asks) were seeded with one tenth of the cells detached from confluent flask and were grown to confluence in the esence or absence (control) of 100 ng ml^{-1} Congo red Caughey & Race 1992). Both the cells and the culture edium were tested for the presence of protease-resistant P. Protease-resistant PrP was extracted from cell lysates d solubilized in SDS-PAGE sample buffer as described eviously (Caughey & Raymond 1993). The media of the ll cultures (10 ml) were centrifuged at 1000 × *g* for 5 min, d the supernatants were then recentrifuged at 184 000 × *g* ve) for 2 h at 4°C. The 184 000 × *g* pellets were sonicated to 1 ml of the detergent buffer used to lyse the cells, and eated with 20 µg ml^{-1} proteinase K for 30 min at 37°C. fter inactivation of the protease with PMSF, the suspen-ons were centrifuged at 340 000 × *g* (ave) for 40 min at 4°C. he resulting pellets were solubilized in SDS-PAGE sample uffer and, along with the samples from the cell lysates, ere analysed for protease-resistant PrP by immunoblotting described previously (Caughey & Raymond 1993). Equal sk equivalents were loaded into each lane.