Characterization of Proteins in Membrane Vesicles from Scrapie-infected Hamster Brain

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SUMMARY

Previous studies have shown that the scrapie agent is highly membrane-associated. We examined the protein composition of gradient fractions enriched for large membrane vesicles prepared from scrapie-infected and uninfected hamster brain using various methods to extract membrane proteins. We also examined proteins in detergent-extracted membrane vesicles fractionated on CsCl gradients. No qualitative differences in protein composition were seen comparing scrapie-infected and uninfected samples by one-dimensional gel electrophoresis. Extraction of proteins from membrane vesicles by phenol, pyridine, perchloric acid or lithium diiodosalicylate also failed to reveal any unique proteins in scrapie-infected hamster brain. Attempts to solubilize hydrophobic proteins (proteolipids) from CsCl gradient fractions into organic solvents were unsuccessful. These findings indicate that any hydrophobic protein associated with the scrapie agent is not a proteolipid, and that the ability of solvents to reduce scrapie infectivity is not a result of extraction of a proteolipid.

INTRODUCTION

Scrapie is a naturally occurring disease of sheep that is similar to kuru and Creutzfeldt-Jakob disease in man. Very little is known about the physicochemical nature of the aetiological agent of scrapie except that the agent has an essential protein (Prusiner et al., 1981b; Cho, 1983; Lax et al., 1983). Previous studies have shown that the scrapie agent is primarily associated with cell membrane (Millson et al., 1971; Semancik et al., 1976), and that treatment with organic solvents or high concentrations of ionic detergents reduces infectivity (Millson et al., 1976). These results suggest that the scrapie agent may require lipid–protein interactions for infectivity (Marsh et al., 1984a), and that membrane components may protect the scrapie agent from inactivation by treatments that damage nucleic acids (Latarjet, 1979; Kimberlin, 1982; Dees et al., 1985a). Recent studies have reported that a unique 26000 to 30000 mol. wt. protein can be detected in scrapie-infected hamster brain after protease treatment (Bolton et al., 1982; Diringer et al., 1983). It has also been claimed that this protein by itself is the aetiological agent of scrapie (Prusiner, 1982; Prusiner et al., 1983). We report here studies characterizing proteins in gradient fractions of hamster brain enriched for large membrane vesicles. No proteins unique to scrapie were detected.

METHODS

Agent and bioassay. The scrapie agent used in these studies was serially passaged in outbred hamsters after adaptation from the Chandler strain of mouse scrapie as previously described (Kimberlin & Marsh, 1975). Infectivity was determined by the method of incubation interval assay (Prusiner et al., 1981a) after intracerebral inoculation into weanling male hamsters purchased from Harlan Sprague Dawley (Indianapolis, Ind., U.S.A.).

Membrane vesicle and CsCl density gradient fractions. Membrane vesicles from scrapie-infected and uninfected hamster brain were prepared on an iodinated density gradient medium as described previously (Marsh et al., 1984a). Briefly, plasma membrane-enriched homogenates from scrapie-infected or age-matched 12-week-old healthy hamsters were sonicated, then separated on Nycodenz® using rate zonal centrifugation. Fractions

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enriched for large membrane vesicles, containing \(10^8\) LD50/ml and 180 \(\mu g/ml\) of protein, were used for protein characterization studies. In some instances, vesicle fractions were extracted with 0.5% Triton X-100 and re-fraccionated by equilibrium density centrifugation in CsCl. A CsCl fraction having a density of 1.28 g/ml and containing an infectivity titre of \(10^{8.4}\) LD50/ml was used for further studies.

**Protein analysis and electrophoretic techniques.** Protein concentrations were determined by a colorimetric dye binding assay (Bradford, 1976). Proteins from membrane vesicles or CsCl density gradient fractions were examined by one-dimensional gel electrophoresis in 12% or 16% discontinuous polyacrylamide gels (Laemmli, 1970). Proteins in gels were visualized by silver staining (Wray et al., 1981) or by autoradiography after radiolabelling with \(^{125}I\) (Greenwood et al., 1963). All autoradiographs were prepared using a Cronex Lightning-Plus Enhancing Screen at \(-80^\circ C\).

**Membrane glycoprotein extraction techniques.** Phenol extraction of membrane glycoproteins was performed according to procedures described by Kathan et al. (1976). A 10 ml suspension of membrane vesicles in 50 mM-Tris-HCl, 100 mM-NaCl, pH 7.8, were extracted by the addition of hot (65 °C) 50% phenol. Vesicles and phenol were stirred for 10 min, then cooled to room temperature. Phenol and water phases were separated by centrifugation at 3000 g for 15 min. The upper water phase containing extracted proteins was removed and dialysed for 3 days against distilled water. After dialysis, phenol-extracted proteins were concentrated by a Savant Speed Vac Concentrator, then tested for infectivity and examined by gel electrophoresis.

Pyridine extraction of membrane proteins was performed using the method of Blumenfeld et al. (1970). A 10 ml suspension of membrane vesicles in distilled water was mixed with 0.5 vol. ice-cold redistilled pyridine containing 0.3 ml 2-mercaptoethanol per 100 ml. The clear solution was dialysed overnight against distilled water at 4 °C. The turbid dialysate was clarified by centrifugation at 3000 g for 15 min at 4 °C. Clarified supernatants containing pyridine-solubilized proteins were removed from the pellets, dialysed for an additional 72 h at 4 °C, then concentrated as described. Pyridine pellets and concentrated supernatants were tested for infectivity, and the supernatants examined by gel electrophoresis.

Membrane proteins were solubilized with 0.3 M-lithium diiodosalicylate (LIS) following the procedure of Marchesi & Andrews (1979). A 10 ml suspension of membrane vesicles in LIS was stirred at room temperature for 2 h and two vol. distilled water added. Ten min after the addition of distilled water, the LIS-membrane protein mixture was centrifuged at 4 °C at 45 000 g for 30 min. The supernatant was dialysed and concentrated. LIS pellets and concentrated supernatants were tested for scrapie infectivity, and examined for protein composition by gel electrophoresis.

Glycoproteins were also extracted by adding perchloric acid (60 \(\mu l/ml\) of a 70% solution) to 10 ml suspensions of membrane vesicles followed immediately by the addition of an equal volume 0.6 M-perchloric acid. Acid-soluble proteins were separated by centrifugation at 4 °C for 1 h at 100 000 g. Supernatants were dialysed against distilled water for 72 h at 4 °C followed by concentration as described. Concentrated acid-soluble proteins and pellets were examined for infectivity and protein composition.

**Solvent solubilization and proteolipid extraction techniques.** Membrane proteins solubilized by butanol or pentanol were prepared according to Maddy (1966). Equal volumes of butanol or pentanol were added to 10 ml suspensions of membrane vesicles in cold distilled water, vigorously mixed, then held on ice for 20 min. Butanol- or pentanol-water phases were separated by low-speed centrifugation at 4 °C for 15 min at 3000 g. The water phase was removed and the interface and solvent phases washed twice with 0.5 vol. of distilled water. The aqueous phase washings were pooled and centrifuged at 4 °C for 30 min at 5000 g. Supernatants were removed from pelleted material and concentrated.

CsCl fractions were treated by two methods designed to extract hydrophobic membrane proteins (proteolipid). Ten ml samples were extracted by the basic Folch procedure, which uses a chloroform:methanol solvent system, and by Cabello's butanol technique (Boyan & Clement-Cormier, 1984). Pellets and concentrated supernatants were tested for infectivity, and the protein composition of the supernatants was examined by gel electrophoresis.

**RESULTS**

**Membrane vesicle proteins**

Fig. 1 shows the proteins associated with membrane vesicles prepared from scrapie-infected and uninfected hamster brain. No discernible qualitative differences could be seen.

**Glycoprotein extracts from membrane vesicles**

Proteins separated by gel electrophoresis of LIS supernatants and pellets are shown in Fig. 2. LIS solubilization enriches for proteins with molecular weights ranging from approximately 23 000 to 33 000. No differences in the protein profiles of scrapie-infected or uninfected hamster brain were seen either in the pellets or the supernatants.
Fig. 1. Silver-stained proteins from membrane vesicles separated on a 16% discontinuous gel. (a) Proteins from uninfected hamster brain; (b) proteins from scrapie-infected brain; (c) standard protein markers with the molecular weights (× 10^{-3}) indicated. No proteins unique to scrapie-infected tissue can be seen.

Fig. 3 shows 125I-radiolabelled membrane proteins extracted using phenol. Several proteins were extracted into the aqueous phase having molecular weights ranging from 12000 to 60000. No proteins were found that were unique to scrapie-infected tissue.

The perchloric acid-soluble 125I-labelled proteins separated by gel electrophoresis are shown in Fig. 4. The major protein solubilized by perchloric acid as indicated by tyrosine labelling had a molecular weight of approximately 21000. Two other minor proteins were seen at 66000 and at 10000. No qualitative difference was apparent; however, the 21000 mol. wt. protein from infected tissue appeared to be increased in amount when compared to uninfected hamster brain.

Proteins with molecular weights of 66000 and 45000 were found in pyridine-solubilized extracts (Fig. 5). Fig. 5 also shows that butanol and pentanol solubilized many proteins from membrane vesicles. Again, no qualitative differences were evident in the protein profiles of solvent-solubilized membrane proteins from infected compared to uninfected tissue.

**Proteins in CsCl fractions**

No differences were detected in proteins associated with CsCl fractions from scrapie-infected and uninfected hamster brain, and extraction of CsCl gradient fractions using techniques designed to extract proteolipids were unsuccessful. Autoradiography of 125I-labelled materials from Folch or butanol proteolipid extracts separated by gel electrophoresis revealed only
Fig. 2. Electrophoretic separation of LIS-solubilized proteins (d and e) and LIS-insoluble pellets (b and c). (b and d) Proteins from uninfected hamster brain; (e and c) proteins from infected brain. (a) Molecular weight \(( \times 10^3 \) ) standards. No differences in protein profiles of pellets or supernatants are apparent.

radioiodinated lipids (Fig. 6). Radioiodinated materials shown in Fig. 6 were resistant to proteinase K digestion and migrated in both neutral and polar lipid solvent systems (Dees et al., 1985b).

Effects of protein extraction procedures on infectivity

The effects of glycoprotein extraction procedures on scrapie infectivity are shown in Table 1. All techniques were found to reduce infectivity to various degrees. Only a small percentage of infectivity remained in any fraction after extraction. The majority of infectivity remaining after treatment with pyridine, perchloric acid, or LIS was in pelleted materials. Solvent extraction of membrane vesicles resulted in an equal division of infectivity between pelleted materials and solubilized supernatants.

DISCUSSION

The physicochemical nature of the scrapie agent has not yet been determined. It has been demonstrated that the agent has an essential protein or proteins required for infectivity (Prusiner et al., 1981b; Cho, 1983; Lax et al., 1983). Some investigators have suggested that the agent is entirely proteinaceous in nature and contains no nucleic acid (Lewin, 1982; Prusiner, 1982). A 26000 to 30000 mol. wt. protein has been identified in protease-treated preparations which appears to be unique to scrapie-infected tissue and co-purifies with scrapie infectivity (Bolton et al., 1982; Diringer et al., 1983). Studies on subcellular fractionation have shown that the majority of scrapie infectivity is associated with the cell membrane (Millson et al., 1971; Semancik et al., 1976). Previous studies
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Fig. 3. Membrane proteins solubilized by phenol extraction and separated on a 16%, polyacrylamide gel after radiolabelling with 125I. The autoradiograph and diagrammatic representation show that proteins with molecular weights ranging from 12000 to 66000 are solubilized from membrane vesicles by phenol. Small arrows indicate individual bands which could be visualized in the autoradiograph but may not be apparent in the photograph. No unique proteins can be found in phenol-solubilized proteins from scrapie-infected tissue (b) compared to proteins from uninfected hamster brain (a).

on membrane proteins from our laboratory have failed to identify unique proteins in scrapie-infected tissues (Marsh et al., 1984a, b). In this study, we performed a more extensive examination of membrane vesicle proteins by using techniques that extract membrane glycoproteins. No unique proteins were seen in samples containing greater than 10⁹ LD₅₀ of scrapie infectivity.

Since it has been hypothesized that cell lipids may protect the scrapie agent (Latarjet, 1979), and that a hydrophobic protein is associated with scrapie infectivity (Prusiner et al., 1981b), we used techniques that solubilize hydrophobic membrane proteins. However, solubilization of membrane vesicles using organic solvent systems failed to reveal any evidence of a unique hydrophobic protein associated with scrapie-infected tissues. Furthermore, proteolipid extraction techniques failed to recover a proteolipid from CsCl density gradient fractions containing 10⁹ LD₅₀ of scrapie infectivity. Proteolipid extraction techniques were found to remove only lipids that were still associated with the membrane residue after detergent extraction and CsCl fractionation. It is possible that the lipid remaining in membrane residues after detergent extraction is in part responsible for the hydrophobicity of the agent. Any membrane protein associated with the agent must have hydrophobic regions with which the
lipid is associated. However, since the proteins do not appear to partition into organic solvents, they are either highly aggregated or contain hydrophilic regions that cause them to remain with the water phase.

The association of the scrapie agent with cell membrane suggests that some essential membrane feature, such as lipid–protein interaction or lipid bilayer structure, might be required for scrapie infectivity. Many cell membrane enzyme complexes require essential lipids and lipid–protein interactions for proper function (Jorgenson, 1982). In this study, we provide further evidence that lipid–protein associations may be necessary for scrapie infectivity. Treatments that disrupt membrane vesicles by extracting membrane proteins were found to have reduced infectivity in the solubilized supernatants compared to pelleted residues. These findings are similar to other studies which have failed to solubilize more than 1% of scrapie infectivity present in cell membrane using various combinations of anionic and non-ionic detergents (Marsh et al., 1984b). These results suggest that the scrapie agent is either an insoluble membrane or submembranous complex, or that the soluble form of the agent is less infectious than insoluble aggregates.

Fig. 4. Few proteins were solubilized from healthy (a) or scrapie-infected (b) hamster brain membrane vesicles by perchloric acid. A protein with a molecular weight of 21000 can be seen in both lanes, but appears to label more intensely in the infected sample. The polyacrylamide concentration in the gel is 16% and the $^{125}$I-radiolabelled proteins were visualized by autoradiography with an enhancing screen. Molecular weight (×10$^{-3}$) markers are on the left.
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Fig. 5. Extraction of membrane vesicles with pyridine (b and c), butanol (d and e) or pentanol (f and g) failed to reveal any proteins unique to scrapie-infected brain (c, e and g) when compared to proteins from uninfected brain (b, d and f). (a and h) Standard molecular weight ($\times 10^{-3}$) markers. The polyacrylamide concentration is 16%, and proteins are stained with silver.

Table 1. Effects of various methods of protein extraction of membrane vesicles on scrapie infectivity

<table>
<thead>
<tr>
<th>Treatment and fraction</th>
<th>Titre*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>$10^7$</td>
</tr>
<tr>
<td>Phenol–water supernatant</td>
<td>$&lt;10^1$</td>
</tr>
<tr>
<td>Pentanol supernatant</td>
<td>$10^{5.5}$</td>
</tr>
<tr>
<td>Pentanol pellet</td>
<td>$10^{5.5}$</td>
</tr>
<tr>
<td>Butanol supernatant</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Butanol pellet</td>
<td>$10^{6.5}$</td>
</tr>
<tr>
<td>Perchloric acid supernatant</td>
<td>$10^4$</td>
</tr>
<tr>
<td>Perchloric acid pellet</td>
<td>$10^6$</td>
</tr>
<tr>
<td>Pyridine supernatant</td>
<td>$10^2$</td>
</tr>
<tr>
<td>Pyridine pellet</td>
<td>$10^6$</td>
</tr>
<tr>
<td>LIS</td>
<td></td>
</tr>
<tr>
<td>Control (no treatment)</td>
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</tr>
<tr>
<td>0.3 M-LIS supernatant</td>
<td>$10^7$</td>
</tr>
<tr>
<td>0.3 M-LIS pellet</td>
<td>$10^{6.2}$</td>
</tr>
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</table>

* Infectivity was determined by the method of incubation interval assay (Prusiner et al., 1981a). Titres are expressed as LD$_{50}$ per ml with volumes adjusted to original starting preparations.
Fig. 6. No radioiodinated proteins were found when CsCl density gradient fractions were extracted using the Folch technique (a and b) or butanol (c and d). (a, c and e) Proteins from uninfected tissue; (b, d and f) proteins from scrapie-infected tissue. Lanes (e) and (f) have been treated with 100 μg/ml proteinase K for 1 h at 37°C. The material that is radioiodinated is proteinase-resistant. The radioiodinated material was found to migrate in neutral and polar lipid solvent systems, indicating that it is iodinated lipid.

We have previously shown that large aggregates of the scrapie agent appear to have a higher titre when infectivity is measured by the method of incubation interval assay compared to endpoint dilution (Marsh et al., 1984b). Further studies are necessary to determine what treatments may give artefactually high titres due to aggregation when infectivity is measured by incubation interval assay. For example, previous reports of a unique protein associated with scrapie-infected tissue (Bolton et al., 1982; Diringer et al., 1983; Hilmert & Diringer, 1984) have used purification schemes favouring aggregation of the agent. Since the demonstration of a unique scrapie protein requires proteinase K treatment, the effect of aggregation on differential protection to proteolytic digestion needs to be assessed.

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REFERENCES


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