The epididymal soluble prion protein forms a high-molecular-mass complex in association with hydrophobic proteins

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Publication Details

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Abstract
We have shown previously that a 'soluble' form of PrP (prion protein), not associated with membranous vesicles, exists in the male reproductive fluid [Ecroyd, Sarradin, Dacheux and Gatti (2004) Biol. Reprod. 71, 993-1001]. Attempts to purify this 'soluble' PrP indicated that it behaves like a high-molecular-mass complex of more than 350 kDa and always co-purified with the same set of proteins. The main associated proteins were sequenced by MS and were found to match to clusterin (apolipoprotein J), BPI (bacterial permeability-increasing protein), carboxylesterase-like urinary excreted protein (cauxin), beta-mannosidase and beta-galactosidase. Immunoblotting and enzymatic assay confirmed the presence of clusterin and a cauxin-like protein and showed that a 17 kDa hydrophobic epididymal protein was also associated with this complex. These associated proteins were not separated by a high ionic strength treatment but were by 2-mercaptoethanol, probably due to its action on reducing disulphide bonds that maintain the interaction of components of the complex. Our results suggest that the associated PrP retains its GPI (glycosylphosphatidylinositol) anchor, in contrast with brain-derived PrP, and that it is resistant to cleavage by phosphatidylinositol-specific phospholipase C. Based on these results, the identity of the associated proteins and the overall biochemical properties of this protein ensemble, we suggest that 'soluble' PrP can form protein complexes that are maintained by hydrophobic interactions, in a similar manner to lipoprotein vesicles or micellar complexes.

Keywords
mass, molecular, forms, protein, hydrophobic, soluble, association, complex, high, proteins, prion, epididymal, CMMB

Disciplines
Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details
The epididymal soluble prion protein forms a high-molecular-mass complex in association with hydrophobic proteins

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INTRODUCTION

PrPC (cellular PrP, where PrP stands for prion protein) is a glycoprotein of unknown function, highly conserved among mammalian species [1]. This ubiquitous protein is found in almost all tissues, where it is usually attached to the external face of cell membranes by a GPI (glycosylphosphatidylinositol) anchor [2]. The conformational conversion of PrPC into its pathological isoform (PrPSC transconformational form of PrP) is thought to be responsible for the progression of TSEs (transmissible spongiform encephalopathies), such as BSE (bovine spongiform encephalopathy), scrapie in sheep and Creutzfeldt–Jakob disease in humans [3,4]. The conformational conversion of PrPC into PrPSC is thought to be an autocatalytic process; however, its interaction with an unidentified factor (named protein ‘X’) has also been proposed [7].

PrP has been found in multiple circulating isoforms in different body fluids [8–12], and a proportion of this PrP has been shown to be associated with exosome-like membranous vesicles [13,14]. In vitro, PrP can be released from the cell surface via the action of exogenous bacterial PI-PLC (phosphatidylinositol-specific phospholipase C). However, we have shown that a non-membranous ‘soluble’ form of PrP is present in the male reproductive fluid, which partitions into the detergent-rich phase following treatment with Triton X-114, suggesting that it may retain its GPI anchor [13]. It is of importance to understand the mechanism by which these membrane-associated proteins are able to exist in a ‘soluble’ state in this fluid, since the transport of PrP in body fluids may be a key event in the transmission and progression of TSEs.

MATERIALS AND METHODS

Reagents and antibodies

Unless otherwise specified, all chemicals were purchased from Sigma–Aldrich (St Quentin Fallavier, France). Purified recombinant ovine PrP was a gift from Dr D. Marc (INRA, Nouzilly, France). The mouse monoclonal antibody (P4) was donated by Dr M. Groschup (Institute for Novel and Infectious Emerging Diseases, Greifswald-Insel Riems, Germany) [15]. The anti-PrP polyclonal antibody, 172 antibody, was a gift from Dr T. Yamashita (Department of Agrobioscience, Iwate University, Morioka, Japan) [18]. The anti-17 kDa polyclonal antibody has already been characterized [13]. The anti-CRD antibody (where CRD stands for cross-reacting determinant) and its positive control [phospholipase C-solubilized porcine MDP (where MDP stands for membrane dipeptidase)] were gifts from Professor N. Hooper (School of Biochemistry and Microbiology, University of Leeds, Leeds, U.K.) [16,17]. The full-length polyclonal anti-cauxin antibody was a gift from Dr T. Yamashita (Department of Agro-bioscience, Iwate University, Morioka, Japan) [18]. The anti-17 kDa polyclonal antibody has already been described [19].
Biological samples

Experiments on animals were conducted according to the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction. Cauda epididymal fluid was collected from adult Ile de France or Romanov rams as described previously [8,20]. Spermatozoa were separated from the fluid by centrifugation (5000 g, 10 min and 4 °C), the fluid was collected, centrifuged again (15,000 g, 10 min and 4 °C) and then ultracentrifuged at 45,000 g for 2 h at 4 °C in order to remove the exosome-like vesicles (epididymosomes) present in this fluid [13]. This fluid sample is referred to as CEP-HSS (cauda epididymal plasma high-speed supernatant) throughout the text. In some experiments, the supernatant after this ultracentrifugation was ultracentrifuged at 100,000 g for 60 min to isolate the high-speed supernatant (HSS) [21]. Cauda epididymal fluid was collected from adult Ile de France or Romanov rams as described previously [8,20]. Spermatozoa were separated from the fluid by centrifugation (5000 g, 10 min and 4 °C), the fluid was collected, centrifuged again (15,000 g, 10 min and 4 °C) and then ultracentrifuged at 45,000 g for 2 h at 4 °C in order to remove the exosome-like vesicles (epididymosomes) present in this fluid [13]. This fluid sample is referred to as CEP-HSS (cauda epididymal plasma high-speed supernatant) throughout the text. In some experiments, the supernatant after this ultracentrifugation was ultracentrifuged again at 120,000 g for 2 h at 4 °C. The fluids were then stored at 4 °C. Samples were mixed with an equal volume of reducing sample buffer [62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 0.05% Bromophenol Blue, 10% (v/v) glycerol and 2.5% (v/v) 2-mercaptoethanol], such that the final concentration of 2-mercaptoethanol was 2.5%, and then heated (95 °C, 5 min) before loading on to gels.

Purification

The CEP-HSS was purified by two successive rounds of column chromatography using an HPLC system (Prosys; Biosepra, Villeneuve-la-Garenne, France). CEP-HSS (5 ml) was concentrated 2-fold with a 10 kDa cut-off membrane before loading on to a gel-filtration column (Hiload 16/60, Superdex 200; Pharmacia, St Quentin en Yvelines, France). Elution was carried out at a flow rate of 0.7 ml/min with 300 mM NaCl and 50 mM Tris/HCl (pH 7.5) buffered solution. PrP-positive fractions (determined by immunoblotting) were pooled, dialysed against 300 mM NaCl and 50 mM Tris/HCl linear gradient (1 ml/min) applied for 60 min. PrP-positive fractions were again determined by immunoblotting, pooled, dialysed and then concentrated before being used in the sucrose-density gradients and centrifugation experiments (see below). Calibration of the gel-filtration Superdex 200 column was performed with a calibration kit (gel filtration standards; 670, 158, 52, 21, 12 and 7 kDa) (Bio-Rad Laboratories, Marnes la Coquette, France).

Sucrose-density-gradient ultracentrifugation

Aliquots of CEP-HSS and the pooled and concentrated PrP-positive sample following the two-step HPLC purification were applied to the top of a 5–20% (w/v) linear sucrose gradient (prepared in PBS with protease inhibitors) (protease inhibitor cocktail; Sigma–Aldrich). The gradients were centrifuged at 160,000 g for approx. 16 h at 4 °C in a Beckman SW41 swing-out rotor and separated into 17 fractions (0.7 ml/fraction). An aliquot of each fraction was mixed with reducing sample buffer, heated (95 °C, 5 min) and loaded on to the gels. Equivalent PrP-positive fractions (collected from the sucrose gradients overlaid with the HPLC-purified samples) were pooled and used in subsequent experiments.

Gel electrophoresis and immunoblotting

The methods of isoelectric focusing and SDS/PAGE were the same as those described previously [21]. Before loading on to gels, samples were mixed with non-reducing sample buffer [62.5 mM Tris/HCl, pH 6.8, 2% (w/v) SDS, 0.025% Bromophenol Blue and 10% (v/v) glycerol] or reducing sample buffer. In other experiments, the calcium-binding properties of proteins in the complex were examined by including 2 mM EGTA or 1 mM CaCl2 in the sample buffer. Samples mixed with non-reducing sample buffer were not boiled before loading on to the gels. SDS/PAGE was performed on 6–16% gradient gels and proteins were semi-dry transferred to nitrocellulose for 2 h at 0.8 mA/cm2. All membranes were stained with Ponceau Red to visualize the proteins on the membrane, rinsed in TBS (Tris-buffered saline; 50 mM Tris/HCl and 150 mM NaCl, pH 7.6) containing 0.05% Tween 20 (TBS-T) and then blocked with TBS-T supplemented with either 5% (w/v) skimmed milk powder (anti-PrP and anti-clusterin antibodies) or 3% (w/v) BSA (anti-cauxin antibody). The membranes were rinsed and incubated in TBS-T containing either 5% skimmed milk powder or 1% BSA and primary antibodies (172, P4 and anti-17 kDa: 1:4000; anti-cauxin: 1:5000; and anti-clusterin: 1:10,000) overnight at 4 °C. The peroxidase-conjugated secondary antibodies used were goat anti-rabbit (1:5000; Sigma–Aldrich) or goat anti-mouse (1:5000; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.). Immunoblotting with the anti-CRD antibody was performed as described previously [16,22]. The immunoreactive proteins were detected using a chemiluminescent substrate according to the manufacturer’s instructions (West-dura; Pierce, Rockford, IL, U.S.A.) and the images were recorded on an image analysis station, or by exposing the membranes to a film. No reactive bands were found when the membranes were incubated with the secondary antibodies alone (results not shown). In some cases, after probing with the anti-CRD antibody, membranes were stripped [2% SDS, 5% (w/v) 2-mercaptoethanol and 62.5 mM Tris, pH 6.8] (1 h, 60 °C), washed with TBS and then probed with an anti-PrP antibody (172 antibody).

Non-reducing/reducing PAGE

In some experiments, an aliquot of the purified complex was mixed with an equal volume of non-reduced sample buffer and non-reducing/reducing PAGE was performed essentially as described previously [23]. Briefly, after subjecting the non-reduced fraction to electrophoresis in the first dimension, the gel lane containing the separated proteins was cut and immersed in SDS sample buffer containing 2.5% (w/v) 2-mercaptoethanol for 20 min at room temperature (22 °C) with constant agitation. Each gel lane was then applied horizontally on top of another 6–16% gel, covered with reduced sample buffer and electrophoresis was performed in the second dimension. The gel was then silver-stained.

MS amino acid sequence analysis

The identification of the major protein spots from one- and two-dimensional gels was obtained by MS. The Coomassie Blue-stained spots were cut with a sterile scalpel blade into small blocks. The blocks were rinsed, then reduced and alkylated with iodoacetamide and incubated overnight at 37 °C in a microtube with 12.5 ng/μl of trypsin (sequencing grade; Roche, Meylan, France) in 25 mM NH4HCO3, as described previously [24]. The tryptic fragments were extracted, dried, reconstituted with 0.1% formic acid and sonicated for 10 min.

Tryptic peptides were analysed either directly by MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS; M@LDI-L/R; Waters Micromass, Manchester, U.K.) or sequenced by nano-LC-MS/MS (Q-TOF-Global equipped with a nano-electrospray ionization source; Waters Micromass) in automatic mode. The peptides were loaded on to a C18 column (AtlantisTM dC18; 3 μm, 75 μm × 150 mm NanoEaseTM, Waters Micromass) and eluted with a 5–60% linear gradient in 30 min at a flow rate of 180 nl/min [buffer A, water/acetonitrile (98/2, v/v), 0.1% formic acid; buffer B, water/acetonitrile
(20/80, v/v), 0.1% formic acid). The peptide masses and sequences obtained were either matched automatically to proteins in a non-redundant database (NCBI) using the Mascot program (http://www.matrixscience.com) or de novo sequenced using the ProteinLynx Global Server program (Waters Micromass) and blasted manually against the current databases (http://npsa-phlb.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_blast.html).

Enzyme assays

Aliquots (20 µl) of each fraction from the sucrose gradients overlaid with the HPLC-purified fraction were assayed for esterase activity against p-NPA (p-nitrophenylacetate) and SOD (superoxide dismutase) activity using the xanthine (5 mM) and xanthine oxidase (1.0 unit)-based system for production of superoxide anion. Esterase activity assays were conducted in 50 mM Tris/HCl (pH 7.5) containing 2% (v/v) 2-mercaptoethanol, the sample carefully mixed and centrifuged again. This procedure was repeated using Tris buffer containing Tris/HCl (pH 7.5) and the sample carefully mixed and centrifuged for the last time and is not induced by the conditions used during the purification steps also sedimented to the same fractions of the sucrose-gradient. Approximately 11 main protein bands were detected by silver staining, ranging in mass from approx. 10 to 150 kDa. The protein peak was found in fractions 16–23; a major peak at approx. 120–170 mM NaCl (fractions 16–23; Figure 1C). Immunoblotting with the anti-PrP antibody showed reactivity in fractions 18–23, corresponding to the main protein peak. A similar pattern of protein was obtained when we reversed the order of the HPLC purification steps (results not shown), suggesting that the co-eluting proteins may be associated with PrP as part of a high-molecular-mass complex.

In order to confirm the existence of this complex, we subjected the final HPLC-purified PrP-containing fractions to sucrose-gradient ultracentrifugation. Figure 2(A) shows that almost all of the proteins found to co-elute with PrP following the HPLC purification steps also sedimented to the same fractions of the sucrose-gradient. Approximately 11 main protein bands were detected by silver staining, ranging in mass from approx. 10 to 150 kDa. The protein peak was found in fractions 7 and 8: PrP reactivity reflected this protein distribution with a peak of reactivity in these same fractions. These fractions correspond to approx. 11–13% (w/v) sucrose (d 1.042–1.051). A sucrose-gradient sedimentation using CEP-HSS showed an identical profile of PrP reactivity (Figure 2B) with a peak in fraction 7 corresponding to the same density range, indicating that the complex is present in vivo and is not induced by the conditions used during the purification steps. Furthermore, when purified recombinant PrP was applied to the top of a sucrose gradient and subjected to the same centrifugation procedure, it was found in the top fraction recovered from the gradient (results not shown).

Identification of proteins that are part of the high-molecular-mass complex

The purified protein complex recovered from the sucrose gradient was separated by two-dimensional SDS/PAGE (Figure 3). The main proteins were found to have acidic pl values, ranging from 4.0 to 6.8, and were found to be composed of trains of several spots corresponding to isoforms of differing molecular mass and/or pl. Coomassie Blue staining of the two-dimensional polyacrylamide gels revealed at least six main protein spots and/or trains (labelled 1–6 in Figure 3). Immunoblotting of transfected proteins with the anti-PrP antibody showed PrP as a smear ing spot with an acidic pl of approx. 4.5 and mass of 40–43 kDa. Protein spots corresponding to PrP could not be visualized even after silver staining.

The six main protein trains were excised from the gel, digested with trypsin and MS-based protein identification was performed...
on the peptide fragments (Table 1). From this analysis, we have identified proteins matching to clusterin (apolipoprotein J) (covering trains 1 and 2), BPI (bacterial permeability increasing-protein), β-mannosidase, β-galactosidase and carboxylesterase-like urinary excreted protein (cauxin) as the main proteins co-purifying with PrP reactivity. Table 1 shows the peptide sequences obtained that were matched to each protein. Similar results were obtained when we used the excised bands from a one-dimensional SDS/polyacrylamide gel to identify these proteins. No supplementary bands could be identified.

Clusterin and cauxin are associated with PrP in male reproductive fluid

We confirmed the presence of both clusterin and a cauxin-like protein in the complex by immunoblotting with specific antibodies. First, clusterin reactivity was found to be present in the same fractions as PrP following gel-filtration HPLC (fractions 15–23; Figure 4A), ion-exchange HPLC (fractions 18–23; Figure 4B) and sucrose-gradient ultracentrifugation of the HPLC-purified sample (fractions 5–10; Figure 4C). Clusterin reactivity was also detected in later fractions following the gel-filtration HPLC (Figure 4A), suggesting that not all the clusterin interacts with PrP. Reactivity against the anti-cauxin antibody was also found in fractions 5–10 recovered from the sucrose gradient overlaid with the HPLC-purified sample (Figure 5A). We then tested whether the esterase activity of the cauxin-like protein was retained within the PrP-associated complex. Esterase activity was detected in fractions 5–11 with a peak of activity in fractions 6–8 (Figure 5B). These fractions corresponded to those in which PrP was found (see Figure 2A).

As some studies have reported SOD activity of PrP [26,27], we also tested whether the PrP-associated complex had such activity. Using a xanthine–xanthine oxidase-based assay system, we found no evidence for SOD-type activity in any of the fractions isolated from the sucrose gradient, including those containing PrP (results not shown).

A hydrophobic 17 kDa fluid antigen is also part of the protein complex

During the purification of this protein complex, we observed that two proteins of 23 and 17 kDa (see Figure 1C) had similar properties (highly hydrophobic and similar molecular masses).
Soluble prion protein is part of a high-molecular-mass structure.  

Figure 2 Sucrose-gradient sedimentation analysis of soluble PrP

(A) The pooled and concentrated HPLC-purified sample (1 ml) and (B) initial CEP-HSS (1 ml) were separated by centrifugation through a 5–20% (w/v) linear sucrose gradient. The gradients were collected into 17 fractions (fraction 17, bottom). An aliquot of the first 13 fractions was separated by SDS/PAGE and the gel was silver-stained (A) or stained with Coomassie Blue (B). Equivalent gels for each gradient were transferred on to nitrocellulose and immunoblotted with the P4 anti-PrP monoclonal antibody.

Figure 3 Two-dimensional PAGE analysis of proteins associated with soluble PrP

Proteins found to co-purify with PrP were subjected to two-dimensional-PAGE as described in the Materials and methods section. (A) The major proteins visible following Coomassie Blue staining of the gel are shown. These proteins are numbered according to their subsequent analysis by MALDI–TOF-MS and nano-LC-MS/MS. (B) An equivalent two-dimensional gel was transferred on to nitrocellulose and immunoblotted with the P4 anti-PrP monoclonal antibody.

to epididymal fluid proteins that we have already characterized but did not match to any protein present in the current databases [19]. Using an antibody directed against the 17 kDa protein, we confirmed its presence in the purified complex (Figure 6).

Table 1 Summary of proteins identified as being part of the soluble PrP-associated high-molecular-mass complex in male reproductive fluid

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Protein</th>
<th>NCBI accession no.</th>
<th>Peptides matching protein (n)</th>
<th>Peptide sequences</th>
</tr>
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<tbody>
<tr>
<td>1 and 2</td>
<td>Clusterin</td>
<td>P17697</td>
<td>9</td>
<td>TPYHPTMFEFTNNDR LYYQLLSYDOK QIKTQIEQTNEER</td>
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<td></td>
<td></td>
<td></td>
<td>GSLFFNPK KLLSSLQEAK RQDITQYSPFSPPPR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LYYQLSYDOK ASINDELFQDORFLR DKEQEMSTEGRG</td>
</tr>
<tr>
<td>3</td>
<td>BPI</td>
<td>P17453</td>
<td>6</td>
<td>ICEVNSTVSSX LOPYFTFLPPTR TNPQYAR</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>GLYACOGVTLRQD KDWAGDYLYAPPR GEFFSARH</td>
</tr>
<tr>
<td>4</td>
<td>β-Mannosidase precursor</td>
<td>Q29444</td>
<td>7</td>
<td>ICEVVTSTVNM LQPYFQTPVTTK BNPGIVNVR</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>GLDYACQQGVLTLK LDKVAGVDYLYAPPR</td>
</tr>
<tr>
<td>5</td>
<td>Carboxylesterase-like urinary protein precursor</td>
<td>BAC22577</td>
<td>3</td>
<td>YWANFAR FVGGAFKFL GQFGFDTYDEHAR QYFVLVR</td>
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<td>GLDYACQQGVLTLK LDKVAGVDYLYAPPR</td>
</tr>
</tbody>
</table>

Figure 4 Co-purification of clusterin with soluble PrP

The fractions collected following (A) gel-filtration HPLC, (B) cation-exchange HPLC and (C) sucrose-gradient ultracentrifugation were immunoblotted with an anti-clusterin antibody (1:10000) as described in the Materials and methods section. Below each panel, the fractions containing PrP reactivity are indicated (+) (same fractions as in Figures 1A, 1B and 2A respectively).

Further characterization of the PrP-associated high-molecular-mass complex

We conducted studies to determine the manner by which members of the complex interacted. We firstly looked at the role of ionic interactions. Figure 7 shows that up to 0.75 M NaCl failed to cause the dissociation of PrP and clusterin, and their subsequent detection in the filtrate through a 100 kDa cut-off membrane. A high concentration of NaCl (1 M) resulted in only a small amount of PrP being detected in the filtrate. Addition of the reducing agent...
Figure 5 Association of the auxin-like protein with the PrP-associated high-molecular-mass complex

(A) Fractions 1–14 recovered from the sucrose gradient overlaid with the HPLC-purified product were immunoblotted with an anti-auxin antibody (1:10,000) as described in the Materials and methods section. The fractions containing PrP reactivity are marked with ‘+’ (same fractions as in Figure 2A). (B) Fractions 1–17 from this same sucrose gradient were assayed for esterase activity against p-NPA. Esterase activity was measured as the increase in A_{405} per min and is reported in arbitrary units. The positive control (+ve Ctrl) consisted of 0.02 unit of porcine liver esterase.

Figure 6 A 17 kDa hydrophobic protein is part of the high-molecular-mass complex

Aliquots of the CEP, CEP-HSS and purified PrP-associated complex (Cmplx) were separated by SDS/PAGE, transferred on to nitrocellulose and probed with an antibody directed against the 17 kDa protein.

(2-mercaptoethanol) was able to disrupt the complex and resulted in PrP and clusterin both passing through the membrane.

We further examined the effect of reducing disulphide bonds of proteins in the complex by successive non-reducing/reducing SDS gels. When no disulphide bonds are involved in maintaining the structure or multimeric form of a protein, it migrates through an SDS/polyacrylamide gel at the same molecular mass under non-reduced and reduced conditions; it is therefore found on the diagonal after staining (Figure 8A). However, some proteins were displaced from this diagonal; these included the following.

(i) A major protein of approx. 60–70 kDa (reduced) that migrated in multiple forms of approx. 150 and 80 kDa under non-reducing conditions. This protein was found to correspond to the auxin-like protein (Figure 8B).

(ii) A protein that migrated at approx. 100 kDa under non-reducing conditions and at 75 kDa under reducing conditions. This protein which was not identified.

(iii) A 40–35 kDa reduced protein, which migrated at approx. 100 kDa under non-reducing conditions and corresponded to clusterin (Figure 8B). Immunoblotting with the anti-PrP antibody showed...
no change in its molecular mass under non-reducing and reducing conditions (Figure 9B).

Since the ‘soluble’ PrP present in the male reproductive fluid is reactive with an antibody directed against the C-terminal sequence that bears the GPI-anchor site [13], it was of interest to investigate whether it also retained the GPI anchor. It has been shown that GPI-anchored PrP is found in the detergent-rich phase after Triton X-114 phase partitioning, but remains in the aqueous phase when the GPI anchor is absent [22,27]. After treatment with PI-PLC, we subjected the CEP-HSS to phase separation by Triton X-114 (Figure 9A). In the absence of PI-PLC, the ‘soluble’ PrP present in the CEP-HSS partitioned into the detergent-rich phase and remained in this phase following treatment with PI-PLC. This was not due to its association with other complex members, as treatment with Triton X-114 resulted in the disruption of the complex and clusterin being partitioned into the aqueous phase. Similar results were obtained when the purified complex was used in the place of CEP-HSS (results not shown).

These results suggested that the GPI anchor of the ‘soluble’ PrP was resistant to cleavage by PI-PLC. This was supported by results obtained using the anti-CRD antibody that detects the phospholipase-cleaved form of GPI-bearing proteins [17,22]. The anti-CRD antibody was able to detect the PrP from scrapie-infected sheep when the extract was treated with PI-PLC, and PI-PLC-solubilized porcine MDP used as a positive control (Figure 9B). However, it failed to detect PrP from CEP-HSS after PI-PLC treatment. This was not due to a lack of protein, as PrP was readily detected by an anti-PrP antibody in these samples. Again, similar results to the CEP-HSS were obtained when the purified complex was used in this experiment (results not shown). Also, no apparent change in the relative SDS/PAGE mobility of PrP from CEP-HSS or the purified complex was observed following treatment with PI-PLC. In contrast, when brain extracts from scrapie-infected sheep were treated with PI-PLC and run on the same gel, the characteristic upshift in the GPI-anchorless form could be detected [2,28], in particular in the non-glycosylated 25 kDa form (Figure 9B). Also, when we conducted experiments aimed at removing any lipid from the inositol ring prior to PI-PLC treatment, the PrP remained unreactive with the anti-CRD antibody (results not shown).

DISCUSSION

PrP is a 28 kDa protein known to exist in a number of different glycoforms and, in the male reproductive fluid, sugar moieties can add up to 15–18 kDa to its apparent molecular mass [13]. In previous studies, we have demonstrated that a part of the PrP present in this fluid was linked to exosome-like vesicles and that a large part remained in a ‘soluble’ state in the bulk phase [13]. In the present study, using both HPLC and sucrose-gradient sedimentation (two purification methods based on different physical principles), we show that this ‘soluble’ PrP behaves like a high-molecular-mass protein of more than 350–400 kDa. We also observed that the PrP always co-purifies with the same proteins, which were identified by both MS and immunoblotting, suggesting that they were associated in a supramolecular structure.

A number of studies have been conducted aimed at identifying PrP-interacting proteins, since they should help to provide insights into the physiological functions of PrP and/or explain how PrP can be transformed into PrPSc. However, a number of these studies utilized molecular biology approaches (such as yeast two-hybrid systems and expression library screening) or in vitro assays, and thus some of these interactions may not be significant in vivo. One protein shown to interact with PrP in a body fluid is plasma and, which binds PrP and PrPSc from different species [29–31]. Here, a ‘soluble’ PrP form that is naturally found in the epididymal fluid was found to co-purify with a number of hydrophobic proteins, as well as with proteins thought to interact with hydrophobic domains and/or lipids. This ‘soluble’ PrP exists in a different form than that present in spermatozoa or their cytoplasmic droplets [13,32], and arises from its secretion into the cauda epididymis [8].

Some of the proteins found to co-purify with ‘soluble’ PrP are already known to exist in high-molecular-mass complexes in other bodily fluids. For example, clusterin, which can act as an extracellular chaperone [33,34], is a well-characterized apolipoprotein [35–37] and is found in relatively high concentrations in male reproductive fluid and in the brain [38]. Interestingly, in blood, this protein is typically associated with lipoprotein vesicles [35]. Many of the reported biological ligands of clusterin are significantly hydrophobic, and clusterin has been shown to bind to hydrophobic regions of exposed proteins to solubilize them and protect them from precipitation [34,39]. A link between clusterin expression and PrP has been suggested previously by studies in which clusterin has been co-localized to fibril-forming deposits [40,41], and the aggregation of the prion peptide 106–126 has been shown to be prevented by clusterin [42]. Recently, it has been shown that the clusterin knockout mouse has a shorter incubation time and different PrPSc deposition than wild-type mice when infected with BSE, suggesting a role for clusterin in the compartmentalization of PrPSc [43].

BPI plays a role in immune protection from Gram-negative bacteria and, recently, the mRNA for this protein was found to be expressed in the testis and epididymis in mice [44]. It is a member of the lipid transfer/lipopoly saccharide-binding protein gene family and is related to two mammalian lipid transport proteins, CETP (cholesteryl ester transfer protein) and PLTP (phospholipid
transfer protein) [45]. It has been shown to bind phospholipids and may itself have lipid transfer activity, enabling phospholipid transfer from membranes to high-density lipoprotein particles [45]. β-Galactosidase and mannosidase have been shown to associate with membrane vesicles in the male reproductive fluid [46,47], and to be enzyme-active within these vesicles [46,48]. Cauxin, a carboxylesterase-like enzyme, is present in the kidneys and urine of domestic cats [18]. Cauxin’s biological role is yet to be determined, but, since the protein identified here retains its enzymatic activity within this complex, it may be involved in lipid and cholesterol processing such as cholesterol esterification. This is the first report that a cauxin-like protein is present in the male reproductive fluid and associates with PrP. We also found that a 17 kDa hydrophobic protein, which we have previously characterized [19], also associates with this protein complex. This protein shows no homology with proteins from current databases and its biochemical properties showed that it is a very hydrophobic compound.

We have partially characterized some features of this supra-molecular association. First, it involves disulphide-bond-containing proteins, including clusterin and the cauxin-like protein, and addition of 2-mercaptoethanol disrupts the complex, indicating a structural role for these proteins. Secondly, the most abundant proteins do not bind calcium, as evidenced by the lack of change in their electrophoretic mobility [46,47].

The existence of lipoprotein vesicles may enable the association of PrP with the other components due to their hydrophobic properties and not through direct protein–protein interactions, in a similar manner to proteins associated in lipoprotein vesicles found in blood plasma and cerebrospinal fluid. For example, the fractions following gel-filtration HPLC, in which the PrP complex was found, are similar to those in which low-density lipoprotein vesicles elute from cerebrospinal fluid [37]. These lipoprotein vesicles have specific lipid and protein compositions and different combinations of lipid and protein produce lipoprotein particles of different density and size. Although we have not specifically addressed its lipid content, we noted the evidence of large amounts of lipid in the PrP-associated complex when it was run on SDS/polyacrylamide gels and subsequently silver-stained to visualize the proteins. However, even when a hydroxylamine treatment, aimed at removing any lipid directly attached to the inositol ring [49], was used before PI-PLC treatment, we found that ‘soluble’ PrP was still unreactive with the anti-CRD antibody (results not shown). Together, our results suggest that ‘soluble’ PrP retains its GPI anchor, which is resistant to PI-PLC cleavage in a similar manner to that reported for another GPI-containing protein present in epididymal fluid, PH20 [50]. Definitive evidence towards the presence of a GPI anchor on this ‘soluble’ form of PrP will require experiments involving the direct structural determination of the anchor, using techniques such as MS and/or metabolic labelling.

The existence of lipoprotein vesicles may enable the association of PrP with this complex through its GPI anchor and also facilitate the resistance of this anchor to PI-PLC cleavage. Also, its incorporation into these vesicles may not be dependent on direct protein–protein interactions with other members. Evidence for this latter proposal is supported by our finding that immunoprecipitation with antibodies fails to bring down other complex members (H. Ecroyd and J.-L. Gatti, unpublished work). We suggest that this is probably due to the proteins having a much higher affinity for the antibodies than other proteins in the lipoprotein complex. Immunoprecipitation is therefore likely to cause the protein to be simply displaced from the vesicle and even vesicle disassembly. Further investigations will be necessary to detail the nature of the lipid vesicle that maintains these proteins together.

In conclusion, here we report the identification and the biochemical characterization of a ‘soluble’ PrP-associated high-molecular-mass complex from male reproductive fluid that resembles micelles or lipoprotein vesicles. We have identified a number of proteins present in this structure, which are in agreement with this hypothesis. To date, the presence of such lipoprotein vesicles in the epididymal fluid has not been described, but their existence could explain how numerous hydrophobic proteins found in this fluid remain in a soluble state and how some are transferred between cells [51]. They may also enable clearance of hydrophobic proteins in a state that prevents their precipitation and aggregation, a process particularly relevant to PrP transport in terms of disease propagation. As such, this purified PrP-associated complex may provide a valuable model to study a mechanism by which PrP could be transferred between cells.

REFERENCES
