Compartmentalization of prion isoforms within the reproductive tract of the ram

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Abstract
Cellular prion protein (Prp(C)) is a glycoprotein usually associated with membranes via its glycosylphosphatidylinositol (GPI) anchor. The trans-conformational form of this protein (Prp(SC)) is the suggested agent responsible for transmissible neurodegenerative spongiform encephalopathies. This protein has been shown on sperm and in the reproductive fluids of males. Antibodies directed against the C-terminal sequence near the GPI-anchor site, an N-terminal sequence, and against the whole protein showed that the Prp isoforms were compartmentalized within the reproductive tract of the ram. Immunoblotting with the three antibodies showed that the complete protein and both N- and C-terminally truncated and glycosylated isoforms are present within cauda epididymal fluid and seminal plasma. Moreover, we demonstrate that in these fluids, the Prp(C) isoforms are both in a soluble state as well as associated with small membranous vesicles (epididymosomes). We also report that only one major glycosylated 25 kDa C-terminally truncated Prp(C) isoform is associated with sperm from the testis, cauda epididymis, and semen, and this form is also present in the sperm cytoplasmic droplets that are released during maturation. In sperm, this C-terminal truncated form was found to be associated with membrane lipid rafts present in the mature sperm, suggesting a role for it in the terminal stages of sperm maturation.

Keywords
within, isoforms, prion, tract, compartmentalization, ram, reproductive, CMMB

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Compartmentalization of Prion Isoforms Within the Reproductive Tract of the Ram

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ABSTRACT

Cellular prion protein (PrpC) is a glycoprotein usually associated with membranes via its glycosylphosphatidylinositol (GPI) anchor. The trans-conformational form of this protein (PrpSc) is the suggested agent responsible for transmissible neurodegenerative spongiform encephalopathies. This protein has been shown on sperm and in the reproductive fluids of males. Antibodies directed against the C-terminal sequence near the GPI-anchor site, an N-terminal sequence, and against the whole protein showed that the Prp isoforms were compartmentalized within the reproductive tract of the ram. Immunoblotting with the three antibodies showed that the complete protein and both N- and C-terminally truncated and glycosylated isoforms are present within cauda epididymal fluid and seminal plasma. Moreover, we demonstrate that in these fluids, the PrpC isoforms are both in a soluble state as well as associated with small membranous vesicles (epididymosomes). We also report that only one major glycosylated 25 kDa C-terminally truncated PrpC isoform is associated with sperm from the testis, cauda epididymis, and semen, and this form is also present in the sperm cytoplasmic droplets that are released during maturation. In sperm, this C-terminal truncated form was found to be associated with membrane lipid rafts present in the mature sperm, suggesting a role for it in the terminal stages of sperm maturation.

INTRODUCTION

The prion protein (Prp) represents a novel mechanism of disease propagation and is based on the conversion of a normal cellular prion protein (PrpC) into a transmissible pathological form (PrpSc) [reviewed in 1, 2]. This trans-conformation is produced by an autocatalytic process induced by PrpSc itself and is the mechanism responsible for the transmissible spongiform encephalopathies such as bovine spongiform encephalopathy (BSE), scrapie in sheep, and the human equivalent, Creutzfeldt-Jakob disease. Infection is mediated, devoid of nucleic acid, by passage of PrpSc from host to host (the protein-only hypothesis), provided they contain a functional Prnp gene [1–3].

PrpC is highly conserved among mammalian species: the similarity at the amino acid level being between 85% and 97% among mammals [4]. The glycoprotein possesses two N-linked glycosylation sites and exists in biglycosylated, monoglycosylated, and nonglycosylated forms [5, 6]. This, as well as proteolytic cleavage within the N-terminal and C-terminal regions of the protein, result in a number of different isoforms being expressed within the various tissues in which the protein is found [7]. Indeed, the expression of PrpC is not restricted to nervous tissues; PrpC is a ubiquitous protein present in almost all tissues throughout the body [8, 9]. PrpC also usually contains a C-terminal glycosyl-phosphatidylinositol (GPI) anchor that enables it to associate with membranes [10, 11]. It has been found to be enriched in sphingolipid- and cholesterol-rich membrane microdomains, known as lipid rafts [12], which are characterized by their relative insolubility at low temperatures in nonionic detergents such as Triton X-100. It has been suggested that this association with lipid rafts is critical for the conformational conversion of PrpC to PrpSc [13, 14]. In vitro, PrpC can be released from the cell surface via the action of exogenous bacterial phosphatidylinositol-specific phospholipase C (PI-PLC), and in vivo soluble forms of the PrpC protein lacking the glycolipid moiety have been reported in serum and human cerebrospinal fluid [15–17].

Despite widespread attention, the cellular function of the Prp remains enigmatic. Previous studies have associated the high levels of expression of PrpC in neuronal cells with a physiological role in synaptic transmission. The protein has been shown to bind metals, particularly copper, via the multiple octa-peptide repeats present in the NH2 terminus region [18–20]. This property may in part account for its antioxidant activity, which has led to reports suggesting a role in inhibiting apoptosis [21]. PrpC has also been implicated in signal transduction because of its position in the external face of the plasma membrane of cells [22, 23], its association with membrane lipid rafts [12], and studies showing its direct interaction with signaling proteins [24, 25].

Our laboratory has previously shown that PrpC is associated with ram sperm [26], a finding consistent with other results indicating that the protein is present in human, bovine, mouse, and hamster sperm [27]. However, at least in human sperm, there is conflicting data as to the isoform(s) present. Although one study has suggested that the protein is C-terminally truncated (being anchored by its N-terminus independent of a GPI-link) and not associated with lipid rafts [27], a more recent study suggested that it is GPI-anchored and N-terminally truncated [28]. Furthermore, we have also shown that different Prp isoforms are present in the ram genital tract fluids and may be processed during epididymal maturation [26]. It remained to be established whether certain isoforms may be transferred from the fluid

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to the sperm. Such a mechanism may be facilitated by hydrophobic membrane vesicles arising from the epididymal epithelium. These vesicles, named "epididymosomes" [29], appear to be similar to seminal plasma prostaticsomes, and they may enable the transport of specific hydrophobic proteins within the reproductive fluid and their transfer to sperm.

In light of these data, we conducted immunological and biochemical studies to characterize the PrpC isoforms present in both the reproductive fluids and sperm from the ram. To do so, we raised antibodies directed against a C-terminal linear peptide sequence (close to the GPI-anchor site) and the recombinant sheep Prp (VRQ allele). With these antibodies and a monoclonal antibody directed against the N-terminal of the protein, we clearly demonstrate that both glycosylated and proteolytic isoforms of PrpC are present in the male reproductive tract. However, the main isoforms differ between sperm and the reproductive fluid, suggesting only a low extent of exchange between these two compartments. We also show that a large part of sperm Prp is associated with the cytoplasmic droplet and that the Prp present in mature sperm is associated with lipid rafts. In the fluid, PrpC isoforms were found in epididymosome vesicles and also in a soluble form, suggesting another type(s) of association to maintain this hydrophobic protein in solution.

MATERIALS AND METHODS

Generation of Antibodies

The mouse monoclonal antibody (P4) directed against an N-terminal peptide was generously donated by Dr. M. Groschup (INEED, Greifswald-Insel Riems, Germany) [30] and is referred to as P4-NT in the text. The rabbit anti-Prp polyclonal antibody 179-CT was raised against the linear peptide 218-ITQYQRESQAYYQRGA-233 corresponding to a C-terminal amino acid sequence of the mature ovine protein, which is situated just before Ser234 bearing the GPI-anchor site. The polyclonal antibody 172-Full was obtained against the complete ovine recombinant Prp protein [31] (VRQ allele; a kind gift from Dr. D. Marc, INRA-Nouzilly, France). Before the first injection, serum from different rabbits were tested on fluids from various epididymal regions by immunoblotting. Animals presenting no reactions were chosen for immunization with either the peptide or recombinant protein. The initial injection included Freund complete adjuvant; 3 weeks later, the first boost, including incomplete adjuvant, was given, followed by two more boosts at intervals of 15 days. The last serum collected was used for the experiments.

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.

Testicular and epididymal fluid and spermatozoa were collected from adult Ile-de-France or Romanov rams as described previously [26, 32]. Ejaculated sperm and seminal plasma were collected with an artificial vagina. Spermatozoa were separated from the fluid by centrifugation (5000 × g, 10 min, 4°C) and then stored at −20°C. The sperm were subsequently washed twice by adding PBS (5000 × g, 10 min, 4°C). Where indicated, the spermatozoa collected from the cauda epididymis or from semen were placed directly on top of a 10%–80% (v/v) linear Percoll gradient and centrifuged (3000 × g, 15 min, 4°C) in order to remove the cytoplasmic droplets. The fraction corresponding to the cytoplasmic droplets was found at approximately 30% Percoll, and the sperm were at the bottom of the gradient. The sperm and cytoplasmic droplets were then washed twice with PBS as described above. Proteins were extracted by addition of an equal volume of nonreducing gel sample buffer to the pellet and centrifuging the sample (15000 × g, 10 min, 4°C). The resulting supernatants were stored at −20°C. Before loading on gels, the samples were mixed with an equal volume of reduced gel sample buffer and heated (95°C, 5 min).

Brains from a healthy sheep and a sheep with scrapie were homogenized in a 10% (w/v) lysis solution [33], diluted 50% with sample buffer, and heated (95°C, 5 min) before gel loading.

Proteolytic Cleavage of Recombinant Ovine Prp

Purified recombinant ovine Prp was cleaved by trypsin (ratio 1/1000 w/w; Roche, Meylan, France) as described previously [31]. After 1 h at 37°C, the reaction was stopped by addition of reducing sample buffer and heating at 95°C for 5 min. The samples were loaded onto gels directly or stored at −20°C.

Preparation of Epididymosomes and Seminosomes

Membrane vesicles from the cauda epididymal plasma and seminal plasma were prepared essentially as previously described [34, 35]. Briefly, cauda epididymal fluid or seminal plasma—freed of spermatozoa and contaminating debris by two successive centrifugations at 15000 × g (10 min, 4°C)—were ultracentrifuged at 45000 × g for 2 h at 4°C (J18; Beckman, Villepinte, France). The high-speed supernatant (HSS) was retained, and the pellet containing the membrane vesicles was suspended in a volume of PBS equal to the initial volume of fluid and centrifuged again under the same conditions. The final pellet was either resuspended in PBS or the proteins extracted directly by addition of reducing sample buffer and heating (95°C, 5 min). The samples were loaded onto gels directly or stored at −20°C.

Triton X-114 Phase Partitioning

Triton X-114 precondensation and phase partitioning was performed as described previously [36]. Briefly, samples were mixed with 1.0% (v/v) Triton X-114 in 10 mM Tris-HCl, 150 mM NaCl, pH 7.4 and left on ice for 20 min. Samples were centrifuged, and a 50 μl aliquot of the supernatant (maximum of 0.5 mg of protein) was layered on top of a 6% (w/v) sucrose cushion in 10 mM Tris-HCl, 150 mM NaCl, 0.06% v/v Triton X-114, pH 7.4, incubated for 3 min at 30°C, and then centrifuged (3000 × g, 30°C, 5 min). The upper aqueous phase was retained, and fresh Triton X-114 was added to a final concentration of 0.5% (v/v). The samples were mixed and left on ice for 5 min before layering on top of the same sucrose cushion and repeating the phase partitioning procedure. After the centrifugation, the aqueous phase was again retained, adjusted to a final concentration of 2.0% (v/v) Triton X-114, mixed and left on ice (5 min), incubated (30°C, 3 min), centrifuged (3000 × g, 30°C, 5 min), and then removed to a fresh tube. The sucrose cushion was removed from the detergent phase, and then it was made up to the same volume as the aqueous phase by adding 10 mM Tris-HCl, 150 mM NaCl, pH 7.4.

Protein Deglycosylation

Fluids and proteins extracted from sperm or epididymosomes were boiled in the presence of 1% (v/v) 2-mercaptoethanol and 0.2% (w/v) SDS for 5 min and then incubated for 2 h at 37°C in the presence of 2% (w/v) CHAPS, 5 mM EDTA, and 5 U of N-glycosidase F (Roche). At the end of the incubation, the reaction was stopped by addition of an equal volume of reduced gel sample buffer and then heating the sample for 5 min at 95°C. In some experiments, the proteins present in reduced sample buffer were deglycosylated by adding 2% (w/v) CHAPS, 5 mM EDTA, and 5 U of N-glycosidase F and incubating for 2 h at 37°C. The reaction was stopped by heating the sample for 5 min at 95°C. Negative controls were conducted by replacing the N-glycosidase F in the reaction mix with an equal volume of H2O.

Preparation of Lipid Raft

All procedures were carried out at 4°C and were based on those published previously [37] with minor modifications. Sperm purified by Percoll gradient centrifugation (as described above) and washed twice with PBS were resuspended in 1% (v/v) Triton X-100 extraction buffer containing 150 mM NaCl, 50 mM HEPES pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, 20 mM NaF, 0.1 mM PMSE, and protease inhibitor cocktail (Sigma, Saint Quentin Fallavier, France). The sperm concentration used was 107 sperm/ml. The homogenate was made 45% (w/v) sucrose by addition of an equal volume of 90% (w/v) sucrose in Buffer A (25 mM HEPES pH 7.4, 150 mM NaCl, 1 mM Na3VO4) and then 1 ml loaded at the bottom of an ultracentrifuge tube. Layers of sucrose were carefully added (2.5 ml of 30% w/v sucrose in Buffer A and then 2 ml of 5% w/v sucrose in Buffer A). The gradients were centrifuged for 14 h at 100000 × g in an SW50.1 rotor (Beckman) at 4°C and subsequently separated...
Different sites of the Prp protein. We first used these antibodies bated with the secondary antibodies alone (data not shown). We replicated for each experiment using material from different sites. Immunoblots shown in the results are representative of the three or more treated samples. The methods of SDS-PAGE have been described earlier (38). SDS-PAGE was conducted on 6%–16% or 10%–20% gradient gels. Gels were first stained with Coomassie blue, and the volume of each sample was concentrated on nitrocellulose over 2 h at 0.8 mA/cm². All membranes were stained with Ponceau-Red to visualize the proteins on the membrane, rinsed in TBS containing 0.05% (v/v) Tween (TBS-T), and then blocked with TBS-T supplemented with 5% (w/v) lyophilized skim milk for 1 h at room temperature. The membranes were rinsed and incubated in TBS-T containing 5% (w/v) milk and primary antibody (179, 1/2000; 172 and P4, 1/4000) overnight at 4°C. The blots were washed 4 times (10 min) with TBS-T, incubated in secondary antibody conjugated to peroxidase (goat anti-rabbit [Sigma] or goat anti-mouse [Jackson Laboratories, West Grove, PA]; I/5000) for 1 h at room temperature, and then washed again in TBS-T. The labeled proteins were detected using a chemiluminescent substrate (West-dura; Pierce, Rockford, IL), and the images were recorded on a digital image analysis station. Immunoblots shown in the results are representative of the three or more replicates carried out for each experiment using material from different animals. No reactive bands were found when the membranes were incubated with the secondary antibodies alone (data not shown).

RESULTS

Characterization of the Anti-Prp Antibodies

We have generated two antibodies directed against different sites of the Prp protein. We first used these antibodies to probe extracts obtained from brains of healthy sheep and sheep with scrapie (Fig. 1A). The P4-NT monoclonal antibody recognized the classical protein-banding pattern of 35, 30–32, and 28 kDa in both brain extracts, corresponding to the biglycosylated, the two monoglycosylated, and the nonglycosylated forms, respectively. The polyclonal antibodies 172-Full and 179-CT recognized these same bands. Furthermore, the same extracts probed with preimmune serum for 172-Full and 179-CT did not reveal any bands (data not shown).

In order to confirm the regional specificity of these antibodies, proteolytic cleavage of the recombinant ovine VRQ protein was performed with trypsin. Conditions were chosen such that the proteolysis was limited to generate only two main fragments: a 15 kDa C-terminal fragment and a 10 kDa N-terminal fragment (31). All antibodies recognized clearly the 25 kDa recombinant protein (Fig. 1B). As expected, the polyclonal antibody 172-Full also recognized the two bands at 10 and 15 kDa obtained after limited proteolysis. The N-terminally directed P4-NT monoclonal antibody recognized only the 10 kDa cleavage product, whereas the polyclonal antibody 179-CT reacted only with the 15 kDa fragment. Similar results were obtained when chymotrypsin was used instead of trypsin to cleave the recombinant protein. The regional specificity of the anti-Prp antibodies are diagrammatically shown in Figure 1C.

Prp in the Reproductive Fluid of the Ram

With all three antibodies, two main immunoreactive bands were mainly found in the fluids from the cauda epididymis (zones 7–9; Fig. 2): a main band at about 43 kDa and another heterodisperse band at 35 kDa. A third band of 28 kDa was also weakly detected by all three antibodies, although it varied between animals (see also Fig. 3). Similarly, the 35 kDa band was found to vary between animals, with some showing strong immunoreactivity and others lower levels. This variation was mainly noted with the polyclonal antibodies. In more proximal regions of the epididymis, only slight reactive bands were observed, but these bands were variable in different animals.

We next addressed the mechanism by which this hydrophobic protein is transported in the fluid. The cauda epididymal fluid was subjected to high-speed centrifugation in order to pellet the membrane vesicles (or epididymosomes) that are present in this fluid (see Materials and Methods).
FIG. 3. Compartmentalization of Prp within the reproductive fluid of the ram. A) Cauda epididymal plasma (CEP) and B) seminal plasma (SP) of the ram were subjected to high-speed centrifugation, and the proteins from the supernatant (HSS) and membrane pellets, corresponding to epididymosomes (EPS) and seminosomes (SMS), were separated via SDS-PAGE and immunoblotted with the three anti-Prp antibodies.

By electron microscopy, this pellet was found to be composed of a homogenous population of membrane vesicles 25–50 nm in diameter [39]. Figure 3A shows that similar patterns of prion reactivity were detected with all three antibodies in the fluid before (cauda epididymal plasma, CEP) and after (HSS) high-speed centrifugation, and in the pellet (epididymosomes, EPS). A similar profile of prion reactivity was found in the supernatant following centrifugation at 100 000 \( g \) (data not shown). Again, the immunoreactivity of the polyclonal antibodies 172-Full and 179-CT to the 35 kDa band was found to vary in the fluid before or after centrifugation; however, they always reacted strongly with this band in the epididymosomes.

A similar experiment was conducted with seminal plasma in order to establish whether there are changes in the types or in the distribution of the prion isoforms upon ejaculation (Fig. 3B). The 43 kDa band was still present in seminal plasma before (SP) and after (HSS) high-speed centrifugation, although at lower quantities such that the 179-CT antibody only faintly detected it. The 35 kDa band was not detected in this fluid before or after centrifugation, and a lower molecular mass band at about 30 kDa became reactive with the P4-NT and 172-Full antibodies. Membrane vesicles, arising from the cauda epididymal fluid and the different accessory glands, were found in the pellet obtained after high-speed centrifugation (seminosomes, SMS). All three antibodies reacted strongly with the 43 and 30 kDa forms, and the 172-Full and 179-CT antibodies also reacted with a lower molecular mass form at about 28 kDa that was not detected in the fluid. Interestingly, this 28 kDa band was not reactive with the P4-NT antibody. These results indicate that in the seminal plasma a proteolytic processing occurs that degrades the prion isoforms both in the fluid and on the membrane vesicles.

Deglycosylation of Prp in Male Reproductive Fluid

We tried to better resolve the individual isoforms present in the cauda epididymal compartments. The high-speed supernatant (HSS) and vesicle pellet (EPS) were treated with N-glycosidase F in order to remove N-linked oligosaccharides from proteins (Fig. 4). Following deglycosylation, all three antibodies detected bands at approximately 35 and 25 kDa in both the HSS and EPS, suggesting that they represent full-length Prp isoforms. The 172-Full antibody also detected minor bands of 20 and 18 kDa in the HSS, and the 20 kDa in the EPS. This 20 kDa band was also detected by the P4-NT antibody, but either was only slightly reactive (HSS) or not detected (EPS) by the 179-CT antibody. Thus, this 20 kDa band could correspond to a C-terminal truncated isoform. Alternatively, although the 179-CT antibody was immunoreactive against the 18 kDa band in the HSS, the P4-NT antibody did not detect it, indicating that it represents an N-terminally truncated isoform. It is most likely that the higher reactive band at 35 kDa has not undergone full deglycosylation.

Prp in the Reproductive Fluid Partitions into the Detergent-Rich Phase after Triton X-114 Extraction

Because full-length Prp is both 'soluble' and associated with epididymosomes, it was of interest to investigate whether the soluble protein retains its GPI-anchor. It has been shown that intact PrpC anchored to the membrane by a GPI anchor 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 [36, 40]. When the HSS and epididymosomes (EPS) were subjected to Triton X-114 phase separation, all of the Prp was detected in the detergent-rich phase after immunoblotting with the 172-Full antibody (Fig. 5). Similar results were obtained with the P4-NT and 179-CT antibodies; no prion reactivity was found in the aqueous phase after the phase separation.

Prp Is Associated with Ram Sperm

Prp associated with ram sperm was investigated using the three antibodies (Fig. 6A). Immunoblotting of testicular
**Compartmentalization of Prion Isoforms**

**Prp in Ram Sperm Is in Lipid Rafts**

Prp can be associated with lipid rafts [12], but a previous study suggested that it is not found in these structures in hamster epididymal sperm [27]. To clarify whether it was associated with these cellular microdomains in cauda epididymal sperm, purified by Percoll gradient centrifugation, proteins were extracted with cold Triton X-100 and the extract subjected to a flotation assay (Fig. 7A). The 24–25 kDa protein was detected by the P4-NT antibody in the buoyant fractions (fractions 4–5, corresponding to the 5%–30% sucrose interface) of the sucrose gradient. Flotillin, a characteristic marker of membrane lipid rafts, was also detected in the same region (fractions 4–6), indicating that it contained lipid rafts. Furthermore, when the same sucrose gradient fractions were immunoblotted with an antibody against the transmembrane protein angiotensin converting enzyme, the majority of the reactivity was restricted to fractions collected from the base of the gradient, indicating that the association of Prp and flotillin with these buoyant fractions is not due to incomplete solubilization of membrane fragments during the extraction process. Moreover, when the main flotillin reactive fraction from the sucrose gradient (fraction 4 [F4]) was centrifuged in order to pellet the raft, both Prp and flotillin were found associated with the pellet (Fig. 7B). Deglycosylation of the raft pellets resulted in a shift in the molecular mass of the Prp to 17 kDa (Fig. 7C), indicating that it is the same isoform as that present in the cytoplasmic droplets (see Fig. 6C).

**Discussion**

In this study, we have addressed the biochemical characteristics of Prp isoforms present in the male reproductive tract. The results demonstrate that full-length, as well as truncated N-terminal and C-terminal, isoforms exist within the reproductive fluid, and a C-terminally truncated isoform is associated with ram sperm. Significantly, we found that the prion isoforms are compartmentalized within the male reproductive tract. The majority of the Prp was found in the fluid where it is transported both bound to membrane vesicles (epididymosomes) and as a soluble species. Furthermore, our results show that distinct cellular lipid microdomains known as lipid rafts are present in the mature male gamete, and that a C-terminally truncated prion isoform can be associated with these cellular microdomains in cauda epididymal sperm.

**Figure 5**

Triton X-114 Prp phase partitioning of Prp in the male reproductive fluid. The HSS and epididymosome extracts (EPS) were subjected to Triton X-114 phase separation. The detergent-rich phase (DT) and aqueous phase (AQ) were collected, separated by SDS-PAGE, and immunoblotted with the anti-Prp antibody 172-Full.

**Figure 6**

Prp is associated with ram sperm. (A) Testicular (T), cauda epididymal (Cd), and ejaculated (Ej) sperm extracts were separated by SDS-PAGE and immunoblotted with the three anti-Prp antibodies. (B) Extracts from cauda epididymal sperm before (NP) and after Percoll-gradient separation (P) and the isolated cytoplasmic droplets (CD) were probed with the 172-Full and P4-NT antibodies. (C) Extracts from cauda epididymal sperm (Cauda Sp) and the isolated cytoplasmic droplets (Cyt Drops) were treated with N-glycosidase F.
form associates with these rafts. These findings are summarized in Figure 8.

We have previously shown that different Prp isoforms are secreted by the epididymal epithelium [26]. By RT-PCR and Northern blotting we have observed that Prp mRNA is expressed in the tests and all regions of the epididymis (unpublished results). Since Prp is encoded by a single exon [41], differences in the prion isoforms must arise from post-translational or postsecretory modifications. Thus, we employed three antibodies in order to establish the proteolytic isoforms of prion present in the male reproductive tract: a well-characterized monoclonal antibody directed against an N-terminal peptide sequence [30] and two new antibodies raised in our laboratory. The demonstrated specificity of these antibodies has enabled us to identify both C-termi-nally and N-terminally truncated sheep prion isoforms. This was important because conflicting results exist on the types of truncated protein associated with sperm [27, 28]. Shaked et al. [27] have described in human, bull, hamster, and mouse that a C-terminal truncated prion was associated with sperm, a finding consistent with these results and our previous data in the ram. Meanwhile, Peoc’h et al. [28] reported in mature human sperm the presence of an N-terminal truncated protein that, following treatment of ejaculated sperm with PI-PLC, resulted in a reduction in Prp staining (as measured by flow cytometry), thus leading to their suggestion that the protein is GPI-anchored.

The results obtained here with the new antibodies confirmed and emphasized our previous work [26] and show that the main Prp isoform associated with ram sperm is C-terminally truncated. This, along with the failure of Triton X-114 to extract the protein from sperm, indicates it does not retain its GPI anchor in these cells. Moreover, we demonstrate that this Prp isoform is present in sperm as they leave the testis and that there is no significant increase in the amount of this isoform associated with cauda or ejaculated sperm (see Fig. 6A). Thus, it is concluded that this isoform does not arise as a result of proteolytic cleavage during epididymal maturation or upon ejaculation, or during protein transfer from the epididymal fluid. As reported for humans [27], we show that the unglycosylated form of this sperm-associated Prp is approximately 17 kDa. The failure of both the 179-CT antibody and an antiserum that preferentially recognizes the glutamate at residue 200 [27, 42] (corresponding to Glu203 in the ovine Prp sequence) to detect this band in sperm extracts suggests that it is generated by cleavage at a site N-terminal to residue 203 (see Fig. 8). However, it retains at least one N-glycosylation site (Asn184), and the kDa change in its apparent mass upon deglycosylation suggests it is a monoglycosylated isoform. As well, it implies the disruption of the disulfide bond between Cys182 and Cys217, which may be significant as this intermolecular bonding has been reported to provide stability to the C-terminal domain and play an important role in the conversion of Prp C to Prp SC [43].

Previous work using mouse and guinea pig sperm have localized caveolin-1, a protein known to associate with lipid rafts, to both the sperm acrosome and flagellum [44]. We have shown that despite being C-terminally truncated, some Prp of mature ram sperm is found in these rafts. Thus, prion may associate with these rafts through a GPI-independent mechanism. The failure of Shaked et al. [27] to detect prion in lipid rafts may be due to the majority of the protein being localized to the cytoplasmic droplets of sperm [45]; however, these were also found to be rich in caveolin-1 in mouse sperm [44]. We have overcome this problem by using sperm devoid of their cytoplasmic droplets in the flotation assay so that the results are not confounded by proteins arising from these droplets. Although the GPI-anchor is an exoplasmic determinant of raft association, a number of recent cell-free studies have clearly demonstrated the interaction of Prp with model membranes, including sphingolipid-cholesterol-rich raftlike liposomes, can occur in a GPI-independent manner [46–48]. These findings have been confirmed in a recent study using a neuroblastoma cell line, which reports that Prp contains a determinate within the 23–90 N-terminal ectodomain that can mediate lipid raft association independently of the GPI-anchor [49].

Our data suggests that there is a partitioning of the different biochemical isoforms of the protein between the fluid phase and cellular phase within the male reproductive tract with little transfer between the two. Significantly, this might be achieved by posttranslational modifications of the protein (such as proteolytic cleavage and glycosylation) in order to impart a specific localization and functionality on particular Prp isoforms. In this respect, because mature sperm represent cells with no capacity for gene transcription, studies utilizing sperm from Prp-knockout and wild-type mice, as well as mice overexpressing the Prnp gene, should provide a good model in which to study Prp isoform function.

Interestingly, although the Prnp gene is transcribed throughout the epididymis, and Prp is secreted under dif-

**FIG. 7.** Prp in ram sperm is associated with lipid rafts. Proteins from cauda epididymal sperm, purified by Percoll gradient centrifugation, were extracted with ice-cold Triton X-100 and subjected to a flotation assay through a sucrose gradient. Following centrifugation, nine fractions (1–9) were collected (9, bottom fraction). A) An equivalent volume from each fraction was separated by SDS-PAGE and immunoblotted with the anti-Prp P4-NT antibody, an anti-flotillin, and an anti-angiotensin-converting enzyme (ACE) antibody. B) Fraction 4 (F.4) was centrifuged and the supernatant (Sup) and pellet (PT) were probed with the anti-Prp P4-NT antibody and the anti-flotillin antibody. C) Extracts from the F.4 pellet were deglycosylated by N-glycosidase F and probed with the P4-NT antibody.
ferent forms by the epididymal epithelium [26], recent results from our laboratory suggest that glycosylation occurs intracellularly and proteolysis most likely takes place in the luminal fluid (unpublished data). Since Prp was mainly detected in large quantities in the fluid from the distal (cauda) epididymis, this could indicate that proteolytic degradation of Prp occurs in more proximal regions of the epididymis. Proteolytic processing of the isoforms was also found to occur upon ejaculation, although Prp was still found in both soluble and membrane vesicle-associated forms. The majority of the Prp associated with seminosomes most likely arises from epididymosomes, because the latter represents the main source of vesicles present in this fluid [39].

The major immunoreactive bands in the cauda epididymal fluid were the heterodisperse 43 and 35 kDa forms and a faint 28 kDa band. Upon deglycosylation, the main immunoreactive band in the fluid was at 25 kDa and corresponded to full-length Prp. The 43 kDa band most likely represents the biglycosylated product of this full-length protein; biglycosylation therefore accounts for about 15–18 kDa of the apparent molecular mass of the mature protein in the fluid. Our results indicate that glycosylated products of this full-length prion isoform may retain its GPI-anchor because the prion isoforms partitioned into the detergent-rich phase following phase separation with Triton X-114. Alternatively, cleaved Prp may exist in a hydrophobic complex with other proteins. This could explain why we failed to detect the C-terminally truncated isoform in the aqueous phase following the phase separation. We have attempted to release Prp from epididymosomes by incubating them with PI-PLC, but incubation at 37°C had a deleterious effect on the integrity of the membrane vesicles and could not be used to assess Prp release into the surrounding medium. Furthermore, treatment of cauda epididymal fluid with PI-PLC failed to result in a shift of the Prp isoforms from the detergent-rich phase to the aqueous phase or a change in the relative mobility of the protein on an SDS-PAGE gel [10, 50]. In contrast, when brain extracts from scrapie-infected mice were treated with PI-PLC Prp partitioned into the aqueous phase following Triton X-114 phase separation, the characteristic mobility shift in the GPI-anchorless form could be clearly detected following separation on an SDS-PAGE gel (data not shown). This may indicate that soluble Prp in the cauda epididymal fluid is resistant to cleavage by PI-PLC in a similar manner to that reported for another GPI-containing protein present in epididymal fluid, PH20 [51]. Definitive evidence for the presence of a GPI-anchor on the Prp present in the reproductive fluid will require studies involving the purification and direct structural determination of the anchor using techniques such as mass spectroscopy and/or metabolic labeling experiments using epididymal tissue.

The presence of the heterodisperse band at 35 kDa probably represents glycosylated products of the C-terminally and N-terminally truncated unglycosylated isoforms of 20 and 18 kDa, respectively (see Fig. 8). The 32–35 kDa band may also be due to small levels of monoglycosylated full-length product. The faint 28 kDa band could represent monoglycosylated products of the terminally truncated isoforms as well as unglycosylated full-length protein. The 18 kDa C-terminal fragment detected in the fluid is probably analogous to the C1 terminal fragment reported in brain extracts that is cleaved at His-111 or Met-112 of the human sequence [52], because this facilitates the generation of a form that is not detected by the P4 antibody. This cleavage is thought to be part of the normal metabolic processing of the protein [52]. The 20 kDa N-terminal fragment detected in the fluid is distinct from that found in sperm. It presumably arises from a different proteolytic cleavage mechanism at a site downstream from that which generates the sperm-associated isoform. Cleavage most likely occurs in the region 197–218 because this would generate a 20 kDa unglycosylated isoform not detected by the 179-CT antibody, and our present results suggest that it retains both N-linked

![FIG. 8. A schematic drawing of the Prp isoforms present in the male reproductive tract. Full-length, as well as C-terminally truncated and N-terminally truncated, isoforms of Prp are compartmentalized between the sperm, reproductive fluid, and membrane vesicles (epididymosomes).](image-url)
glycosylation sites. In the male genital tract, whether these proteolytic cleavage products represent the degradation of the full-length protein or functional isoforms of the protein has not been established. At least some of the same isoforms were found to be associated with epididymosomes and soluble in the fluid, raising questions as to the mechanism by which these two pools of protein are compartmentalized.

In conclusion, the findings of this study show definitively that both glycolytic and proteolytic isofoms of Prp are present within the reproductive fluid and gamete of the male. We provide the first evidence for a C-terminally truncated prion isoform associated with lipid rafts in sperm and retains at least one N-glycosylation site. Our results also show that in the male reproductive fluid, Prp exists as a full-length protein and in C-terminally truncated and N-terminally truncated isoforms. Part of the fluid protein is associated with epididymosomes, but the mechanism by which this "membrane-associated" protein is also able to exist in a "soluble circulating" state within this fluid warrants further investigation.

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