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Prion Protein Is Secreted in Soluble Forms in the Epididymal Fluid and Proteolytically Processed and Transported in Seminal Plasma¹

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ABSTRACT

The presence of prion protein in sperm and fluids collected from different parts of the ram genital tract was investigated by immunoblotting with monoclonal antibodies. A slightly immunoreactive 25- to 30-kDa protein was recognized on Western blots of testicular and epididymal sperm extracts. Immunoreactivity increased on ejaculated sperm extracts and 2 other bands at 35 and 43 kDa also reacted. Seminal plasma showed several immunoreactive bands, the main bands being detected at 43 and 35 kDa, whereas less reactive bands were observed at 30, 25, 20, and <14 kDa. All these bands strongly decreased in the seminal plasma after vasectomy, indicating a testicular or an epididymal origin. Testicular fluid showed almost no reactivity, whereas caudal epididymal fluid contained the 2 strong immunoreactive bands at 43 and 35 kDa and in some cases a faint 30-kDa band. The 43-kDa band was also found in the fluid from the proximal caput, whereas the 35-kDa band appeared in the distal caput. Immunoprecipitation of ³⁵S-labeled proteins secreted in the epididymal fluid indicated that the 43-kDa form was synthesized in caput and caudal regions and the 35-kDa form in the distal caput to the distal corpus. Treatment of caudal fluid and seminal plasma by N-glycosidase resulted in the formation of 3 bands: 1 highly reactive at about 25 kDa, a second less reactive at about 28 kDa, and a third at approximately 20 kDa. The pattern of prion protein distribution in epididymal fluids was found to be similar in scrapie-infected rams to that of healthy rams. Cauda epididymal fluid and seminal plasma from infected animals could not be treated directly with proteinase K, because of the presence of protease inhibitors. However, the prion protein immunoprecipitated from these fluids was completely cleaved by proteinase K, whereas in the same conditions this from an infected sheep brain gave the usual resistant band pattern.

epididymis, gamete biology, male reproductive tract, sperm, sperm maturation

INTRODUCTION

The normal cellular form of the prion protein (PrP^c) is a protein inserted in the external face of the plasma membrane via a carboxy-terminal glycosyl-phosphatidylinositol

anchor (GPI). This approximately 230 amino-acid protein possesses 1 disulfide bond, 2 sites of N-glycosylation, and a flexible N-terminal region that contains several octapeptide repeats able to bind metal ions such as copper [1, 2].

Misfolded prion proteins are suspected of being the infectious agent that causes fatal neurodegenerative diseases such as Creutzfeldt-Jakob disease and Gertsmann-Sträussler-Scheinker syndrome in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep (reviewed in [1–3]). This pathological prion isoform, named PrP^{Sc}, has the same sequence as PrP^c but shows a high degree of insolubility in nondenaturing detergent and a relative resistance to proteolysis: whereas PrP^c disappears during limited proteinase K digestion, PrP^{Sc} yields a specific band pattern between 27 and 30 kDa (referred to as PrP^{Res}). These new properties may result from conformational changes induced by a molecular mechanism that is still unclear, but which converts a critical α -helical region into a β -sheet [1, 2]. This molecular transition leads to the formation and accumulation of amyloidogenic aggregates of PrP^{Sc} in the central nervous system that could be responsible for its degeneration [1–3]. PrP^{Sc} also shows different relative levels in the 3 glycosylated isoforms produced by proteinase K treatment, and this “glycoprofile” has been used to differentiate prion strains [4].

The cellular role played by PrP^c is not fully understood: a superoxide dismutase activity, related to its copper binding property, might explain its action in preventing apoptosis by limiting the effect of cell oxidative stress [5–7]. The possible formation of a cation channel following molecular aggregation [8] and roles in cell signaling [9, 10] have also been evoked.

PrP^c is a ubiquitous protein expressed in numerous tissues other than the nervous system [11, 12], and the presence of both the mRNA and the protein has been demonstrated in testicular tissue [11, 13]. A recent study by Shaked et al. [14] showed that the PrP^c is also present on the sperm membrane of different species, including humans. Their results suggested that this sperm membrane protein undergoes a maturational process in the epididymis, the organ that links the testis to the vas deferens in which spermatozoa become fertile [15]. They also reported that the C-terminal part of the protein (and thus the GPI anchor) was removed from the PrP^c and that the protein on the ejaculated sperm membrane may be inserted by its N-terminal end. Finally, they reported that the spermatozoa from PrP^c –/– mice were more susceptible to deleterious effects induced by copper than the spermatozoa from normal mice.

Several studies have indicated that semen is a noninfective tissue [16–18], but some of these findings need to be

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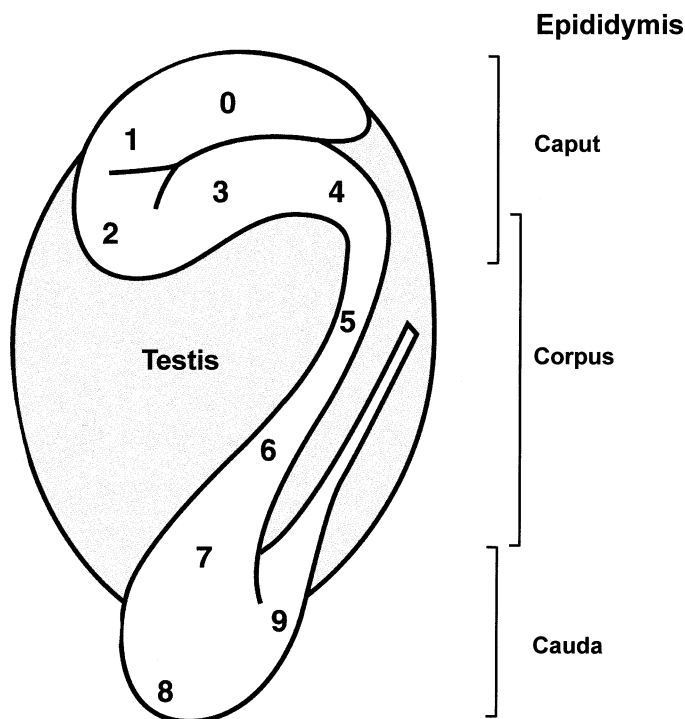


FIG. 1. Schematic drawing of the ram testis and epididymis showing the different zones of fluid collection. Caput epididymis, zones 0–4; corpus epididymis, zones 4–7; cauda epididymis, zones 7–9.

reassessed (see [19] for a recent critical review). For example, to our knowledge there are no results concerning the presence of PrP (PrP will be used throughout the text when the prion protein could either be PrPc or PrPsc) in the male genital secretions from healthy or infected animals.

Our results confirmed the presence of PrPc in the sperm extract and demonstrated that this protein is synthesized in the genital tract and released in large quantities in soluble forms in the epididymal fluid and seminal plasma of the ram where this protein appears to be proteolytically processed. No proteinase K resistant form of PrP could be found in the seminal plasma from scrapie-infected animals.

MATERIALS AND METHODS

Fluid and Sperm Collection

Epididymides and testes were obtained from adult Ile de France or Romanov rams. Testicular fluid and fluids from different epididymal zones (Fig. 1) were collected as previously described [20]. Ejaculated sperm were obtained with an artificial vagina. Spermatozoa were separated from the fluid by centrifugation (10 min, $15\,000 \times g$, 4°C). The fluids were carefully removed and centrifuged again (10 min, $15\,000 \times g$) and used immediately or stored at -20°C . The sperm pellet was suspended in PBS and washed by 2 cycles of centrifugation (10 min, $10\,000 \times g$). For extraction the last pellet was mixed with an equal volume of gel sample buffer then centrifuged and the supernatant boiled.

The epididymis of scrapie-infected Romanov rams (1 VRQ/VRQ and 2 ARQ/VRQ [scrapie susceptible genotypes]) were obtained from animals killed in the terminal stage of illness. Seminal plasma from infected rams were obtained by artificial vagina collection from 8-mo-old to 1-yr-old (A)(V)RQ/VRQ animals, which were confirmed positive for PrPsc by tonsil immunochemistry [21]. Males of the same age and of the ARR/ARR genotype (scrapie "resistant" genotype), maintained in the same flock, were also sampled and used as controls.

Antibodies

Mouse monoclonal antibodies that recognized different epitopes along the sheep PrPc were used in this study (Fig. 2A): SAF32, SAF84, and

8G8 were generously provided by Dr. J. Grassi [22, 23] and P4 by Dr. M. Groschup [24]. The rabbit polyclonal antibodies used for the immunoprecipitation were MH44 and MH48 and were raised against the recombinant VRQ sheep PrPc [12, 25]. All these antibodies reacted on immunoblots with PrPsc extracted from sheep brains (data not shown).

Gel Electrophoresis and Protein Blotting

Gel and sample preparation and methods for isoelectric focusing have previously been described [26]. SDS-PAGE 12% or 6%–16% gradient were used. Semidry transfer of proteins to nitrocellulose was performed over 2 h at $0.8 \text{ mA}/\text{cm}^2$. The blots were blocked with PBS or TBS supplemented with 0.5% (w/v) Tween 20 and 5% (w/v) dry skimmed milk. The second antibody was a goat anti-rabbit or anti-mouse antibody conjugated with peroxidase (dilution 1/2000 to 1/5000; Jackson Lab, West Grove, PA). The peroxidase was revealed with chemiluminescent substrates (West-dura, Pierce, Rockford, IL), and the images recorded on an image analysis station (Fluorchem 8000, Alpha-Innotech, San Leandro, CA) or on film (X-O-mat blue, NEN, Boston, MA). Second antibodies alone gave no reactive bands with the different samples (not shown).

In Vitro ^{35}S Labeling of Epididymal Proteins

^{35}S labeling of the epididymal proteins was performed by in vitro incubation of the epididymal tubule from different regions with a mixture of ^{35}S -methionine and ^{35}S -cysteine (EXPRE ^{35}S , NEN) as previously described [26]. The fluids were collected by microperfusion at the end of the incubation period, separated from the sperm and used directly for immunoprecipitation and SDS-PAGE.

Immunoprecipitation

Immunoprecipitation was performed as described [12] with few modifications. Briefly, epididymal fluids or seminal plasma were diluted 50% with PBS and incubated for 2 h at room temperature with MH44 antibodies (2% [v/v]) or a MH44/MH48 mixture (2%/2% [v/v]) on a rotating wheel. Protein-A Sepharose was then added (10% [v/v]) for another 2 h. The protein-A beads were collected by $15\,000 \text{ g}$ centrifugation (1 min) and washed 4 times with 10 volumes of PBS and a final wash with deionized water. The final protein A pellet was then extracted by boiling with the same volume of reducing sample buffer, centrifuged at $15\,000 \times g$ for 10 min, and the supernatant was used for electrophoresis or stored at -20°C .

Proteinase K Treatment

The PBS-washed protein A-immunoprecipitated complexes were treated with proteinase K (10 $\mu\text{g}/\text{ml}$ final) for 1 h at 37°C . Proteolysis was stopped by addition of sample buffer and heating for 3 min at 95°C . The samples were then separated on gel and Western blotted. The brains from a healthy and a scrapie-infected sheep were homogenized in a lysis solution (10%, w/v [27]) and diluted 50% with PBS. The PrP of these samples was then immunoprecipitated and treated as above.

Protein Deglycosylation

Epididymal fluids and seminal plasma (50 μl) were boiled in the presence of beta-mercaptoethanol (1%, w/v) and SDS (0.2%, w/v), and then 30 μl of a 10% (w/v) Triton X100 solution were added after cooling. A 25- μl aliquot of each sample taken just after boiling was used as a control. The remaining samples were then incubated overnight at 37°C with 3 U of N-glycosidase F (Roche, Meylan, France).

RESULTS

Presence of PrPc in the Sperm Extracts and Fluids from the Genital Tract

Extracts from ejaculated, cauda epididymal and testicular spermatozoa, seminal plasma, cauda epididymal fluid, and testicular fluid collected from the rete testis were separated on 6%–16% SDS-PAGE and transferred to nitrocellulose. The Western blots were then probed with monoclonal antibodies directed against different epitopes along the PrPc polypeptide chain (Fig. 2A).

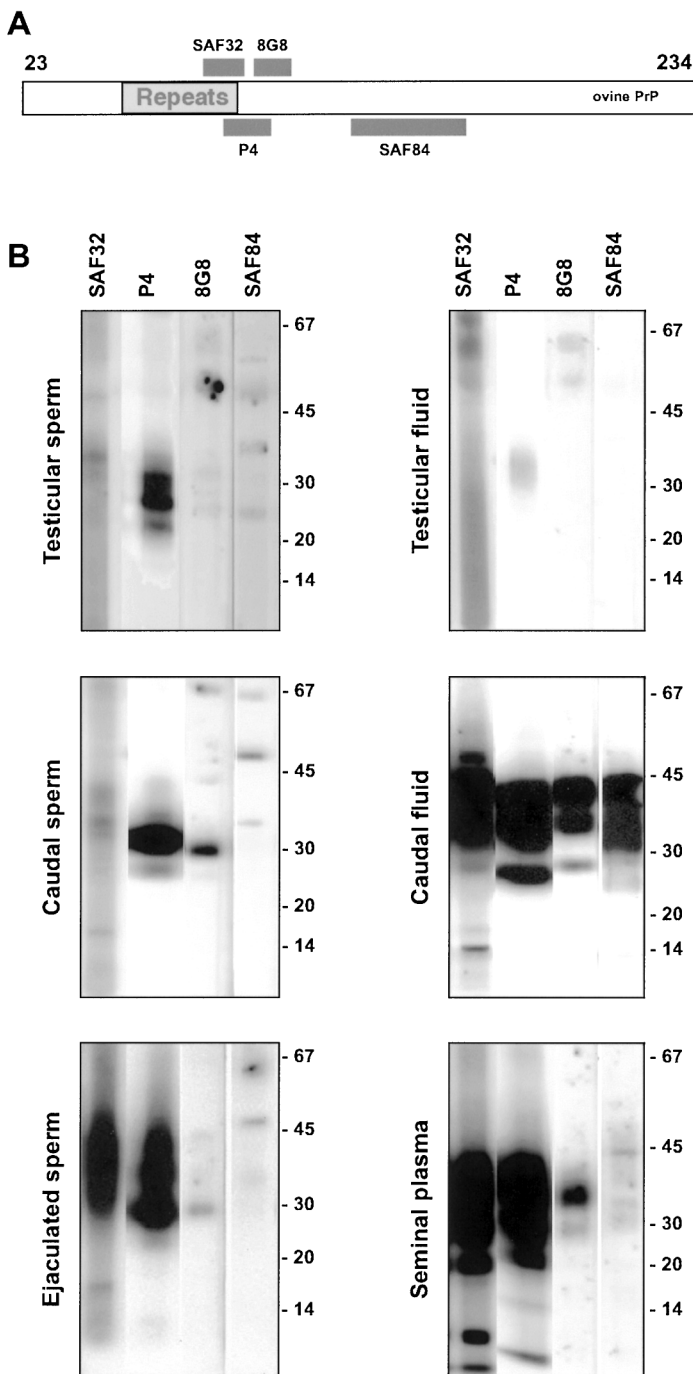


FIG. 2. PrPc in sperm extracts and fluids from different parts of the male genital tract. **A**) schematic representation of the mature ovine PrP with the locations of the 4 polypeptides/epitopes used to generate the antibodies. **B**) On the left-hand side, extract from a 50- μ l pellet of sperm obtained from the testis, the cauda epididymis and the ejaculate were loaded on 6%–16% SDS-PAGE before transfer to nitrocellulose. On the right-hand side, testicular fluid (100 μ l), cauda epididymal fluid, and seminal plasma (50 μ l) were separated. All samples were from the same animal. Western blots were probed with the different monoclonal antibodies as indicated at the following dilutions: SAF32, 1/1000; P4, 1/1000; 8G8, 1/2000; SAF84, 1/2000.

Only the P4 monoclonal antibody clearly recognized bands at around 20, 25, and 30 kDa in the testicular sperm extract (Fig. 2B). A similar response pattern occurred with the extract from cauda epididymal sperm, although the band at 30 kDa reacted more strongly with the P4 and also

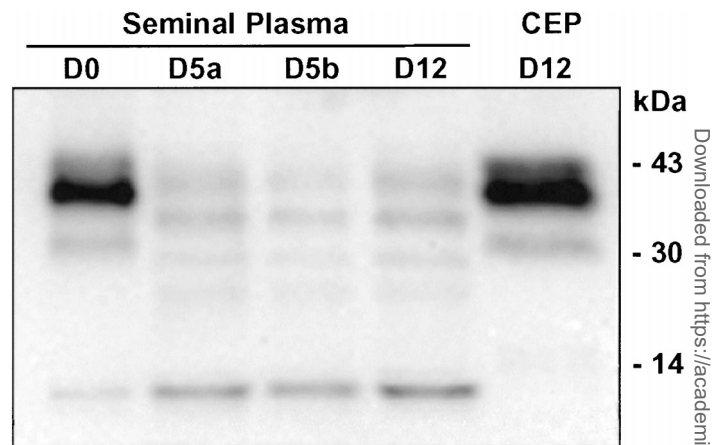


FIG. 3. Effect of vasectomy on the PrPc content of the seminal plasma. Same amounts of proteins (about 100 μ g) were obtained from the seminal plasma before vasectomy (D0) and 5 days (2 ejaculates, D5a and D5b) and 12 days (D12) after surgery, and the cauda epididymal fluid obtained after killing (CEP D12) were loaded on 6%–16% SDS-PAGE and Western blotted. The membrane was probed with the P4 monoclonal antibody described in previous figures (dilution 1/1500).

with the 8G8 monoclonal antibodies. The P4 antibody in ejaculated sperm extract strongly recognized the 30-kDa bands and also 2 other bands at 35 kDa and 43 kDa. It is of note that the SAF32 antibodies also recognized these bands, whereas it gave only a very faint reaction with the previous testicular and epididymal extracts.

The testicular fluid showed only a faint reactive 30- to 35-kDa band with the P4 antibody (Fig. 2). In the cauda epididymal fluid, all the antibodies showed a significant immunoreaction with a 43-kDa band and also with 2 other bands at 35 kDa and less than 30 kDa. In the seminal plasma the P4 and SAF32 antibodies reacted strongly with the 35-, 43-, and 30-kDa bands; the 8G8 antibody only reacted with the 35-kDa band; and the SAF84 antibodies did not react with any of these bands. New compounds at 20 kDa and less than 10 kDa were also recognized by the P4 and SAF32 antibodies.

Although variations in intensity may have occurred between different animals and samples, some due to gel-loading effects, we always observed similar patterns of immunoreactivity with sperm and fluids. The 43-kDa band was in general the major reactive cauda fluid entity: this band decreased in seminal plasma where the 35-kDa band was always predominant (see the following figures). Identical results were also obtained with other monoclonal and polyclonal antibodies that reacted against the same epitopes (not shown).

Seminal Plasma PrPc Originates from the Epididymal Fluid

The results shown in Figure 2 suggested that PrPc in ram seminal plasma originated mainly from the epididymis. One ram was vasectomized after collection of seminal plasma (D0) to ascertain this fact. Vasectomy suppressed the fluid and spermatozoa from the epididymis, and the seminal plasma then comprised only fluid from the accessory glands of the genital tract (seminal vesicle, etc.). Collection was performed 5 (D5) and 12 days (D12) after vasectomy, and thereafter cauda epididymal fluid was obtained from this animal (CEP D12). Western blot of these seminal plasma and cauda fluid samples was probed with the P4 monoclonal antibody (Fig. 3). Before vasectomy (D0) the antibody

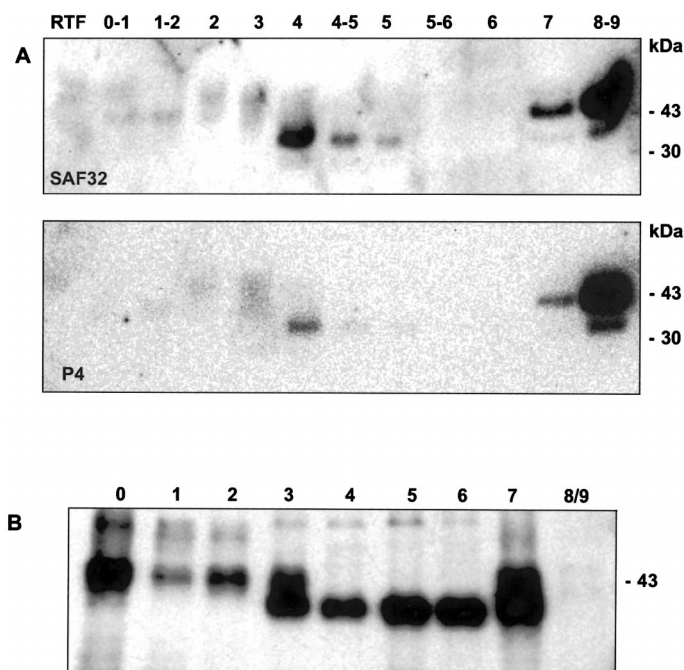


FIG. 4. Distribution and synthesis of PrPc in epididymal and testicular fluids. **A**) Equivalent amounts of proteins (about 100 μ g) from testicular (RTF) and epididymal fluids collected from different zones of the organ were loaded on 6%–16% SDS-PAGE and Western blotted. The Western blots were probed with SAF32 and P4 monoclonal antibodies as indicated. **B**) Autoradiography of immunoprecipitated ³⁵S-labeled PrPc protein from the epididymal fluids collected from different zones of the organ separated on 6%–16% SDS-PAGE (see *Results*).

recognized 1 main 35-kDa band and a faint 43 kDa in the seminal plasma. In the first and the second ejaculates obtained 5 (D5a and D5b) and 12 days (D12) after surgery, almost no reactive bands were observed except for a small 14-kDa compound that might have been a degradation product. Meanwhile the 35- and 43-kDa proteins were still detected in the cauda epididymal fluid obtained from this vasectomized animal (CEP D12).

Distribution of Different PrPc Forms in the Epididymal Fluids

Fluids from different epididymal regions were probed with the SAF32 antibody (Fig. 4A); 2 different bands were observed in the distal caput, 1 clearly reactive at 35 kDa in zone 4 and 1 weak and more fuzzy at about 43 kDa in zones 2 and 3. A strong immunoreaction with the 43-kDa band and a weaker reaction with the 35-kDa band were obtained in the cauda epididymis (zones 8/9). Similar results were obtained using the P4 antibody (Fig. 4A).

PrPc Is Secreted by the Epididymal Epithelium

The ³⁵S-labeled proteins secreted inside the epididymal tubule during *in vitro* incubation were immunoprecipitated with the MH44 polyclonal antibody (this antibody gave the same results on Western blotting as shown in Fig. 4A). The autoradiogram of the immunoprecipitated proteins (Fig. 4B) indicated that the PrPc was synthesized and secreted throughout the epididymis under the different forms observed on Western blotting. In zones 0–3 of the caput region the protein was secreted under the 43-kDa form, then from zones 3 to 7 the 35-kDa protein form was the main labeled form. In the cauda epididymis (zone 7) the 43-kDa

form was again strongly secreted at the same time as the 35-kDa form.

We did not observe any labeled PrPc in the fluid of zones 8/9; This could be due either to a lack of synthesis and secretion or to competition between the high level of unlabeled PrPc accumulated in this fluid and the *de novo* synthesized PrPc. The latter hypothesis was supported by the fact that when a Western blot of the immunoprecipitated biosynthesized proteins was probed with the SAF32 antibody, a very high level of immunoreaction at 43 kDa became clearly apparent in the fluid of zones 8/9, indicating the presence of a large quantity of this protein (not shown).

Biochemical Properties of PrPc from the Genital Tract

The molecular weight of the ram genital tract PrPc was compared on the same gel with the PrP glycoforms present in healthy and scrapie-infected sheep brain extracts (Fig. 5A). The heavier PrPc form from the seminal plasma had a much higher molecular weight than the 35-kDa brain biglycosylated PrPc. This brain form was at about the molecular weight of the lower form observed in the seminal plasma PrPc.

The caudal epididymal fluid, seminal plasma, and brain extract were separated by two-dimensional electrophoresis and the resulting blots probed with the P4 antibody (Fig. 5B). Different immunoreactive spots were observed in the cauda fluid and the seminal plasma for the bands at 43 and 35 kDa. The 43-kDa protein was separated in 2 main spots, one at an isoelectric point (pI) around 6.5 and a second with an acidic pI at about 4. When the same analysis was performed with an overloaded gel, the 35-kDa band formed a fuzzy smear from pIs 7 to 4, indicating a wide charge heterogeneity. The 43-kDa protein in the seminal plasma was found as 1 main spot with a pI of 6.0–6.5 and the 35-kDa form presented a train of small spots also with pIs at around 6.5. The very acidic component from the cauda epididymal fluid was not observed in the seminal plasma. As a comparison the 3 brain PrPc glycoforms gave several series of spots with pIs ranging from 5.8 to 6.5.

In order to remove the N-linked sugar residues of the PrPc, the fluids from zones 2–3, the cauda epididymis, and the seminal plasma were treated by N-glycosidase F (Fig. 6). After treatment the immunoreactive 43-, 35-, and 30-kDa bands present in CEP and seminal plasma generated three similar bands of different intensities: 1 highly reactive at about 25 kDa in the caudal fluid and the seminal plasma, a second less reactive at about 28 kDa, and a third at approximately 20 kDa. Surprisingly the Z2 fluid gave the same result, although the PrPc in this fluid was only a weak fuzzy smear between 30 and 45 kDa. This suggested that in this epididymal zone the protein isoforms were under a wide range of different glycoforms. The relative intensity of the 3 deglycosylated bands varied slightly between experiments, but including protease inhibitors in the deglycosylation media (PMSF 2 mM, EDTA 2 mM, and paraaminobenzamide 2 mM) did not change this final pattern (not shown).

PrPc Distribution in Epididymal Fluids of Scrapie-Infected Animals

Testicular and epididymal fluids were collected from 3 scrapie-infected rams (ARQ/VRQ and VRQ/VRQ genotypes) in the terminal phase of illness and probed with the SAF32 antibody (Fig. 7 shows a typical result on 1 animal). We observed that the distribution of the reactive proteins

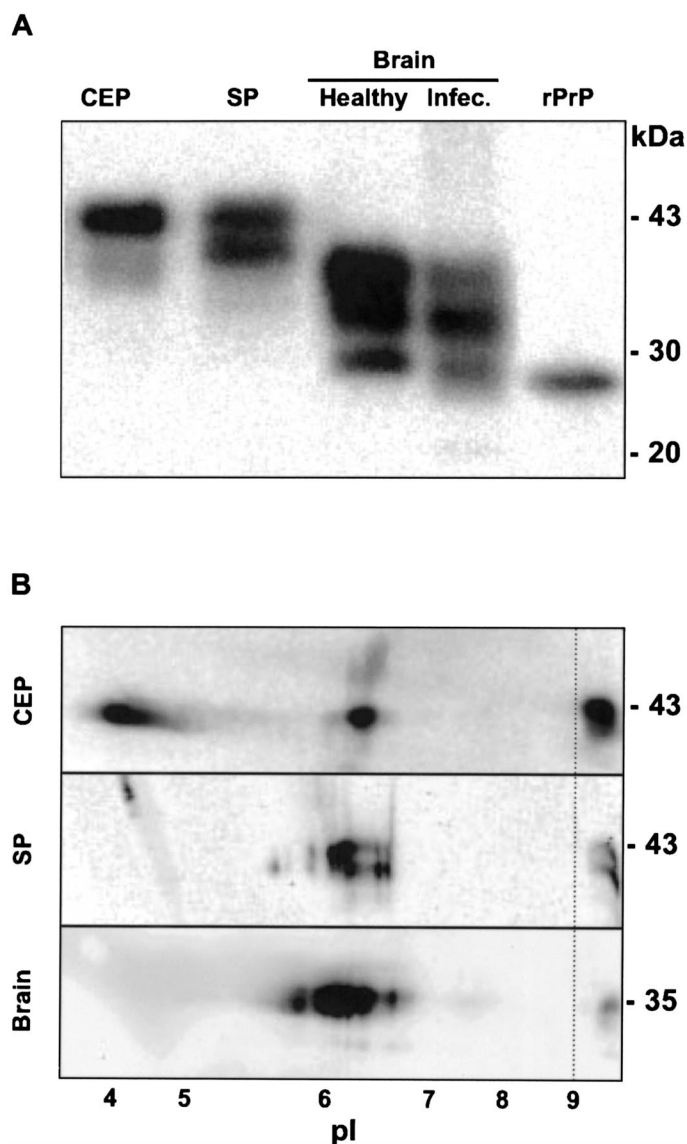


FIG. 5. Electrophoretic comparison of sheep brain, cauda epididymal fluid, and seminal plasma PrP. **A**) Comparison of the molecular weights of sheep brain, cauda epididymal fluid, and seminal plasma PrP. Cauda epididymal fluid (CEP) and seminal plasma (SP) (about 50 μ g of protein) and sheep brain extract from a healthy and a scrapie-infected animal (12 μ l and 2.5 μ l of a 10% (w/v) extract solution, respectively). The 6%–16% SDS-PAGE was transferred to nitrocellulose and probed with the P4 antibody (1/1500). **B**) PrPc comparison by two-dimensional gel electrophoresis of sheep brain, the cauda epididymal fluid, and the seminal plasma. Cauda epididymal fluid, seminal plasma (100 μ g each), and 100 μ l of a 10% (w/v) sheep brain extract were separated by two-dimensional electrophoresis and transferred and probed with the P4 antibody (1/1500). A control lane (right-hand side) was loaded with 20% of each sample.

was almost identical to that obtained in a healthy animal (see Fig. 4): the 43-kDa immunoreactive protein was present in the caput (zones 1–2, 2) and cauda epididymis (zones 7–8 and 9), whereas the 35-kDa form was present in zones 3–4, decreased in zones 4–7, and then reappeared in the caudal fluid. The band heavier than 47.5 kDa in the caput region was obtained only with this animal; this may represent a nonspecific reaction or the formation of a PrPc dimer that can be observed only in certain conditions.

Proteinase K Treatment

Seminal plasma obtained from infected (A)(V)RQ/VRQ Romanov rams (shown positive for scrapie by tonsil im-

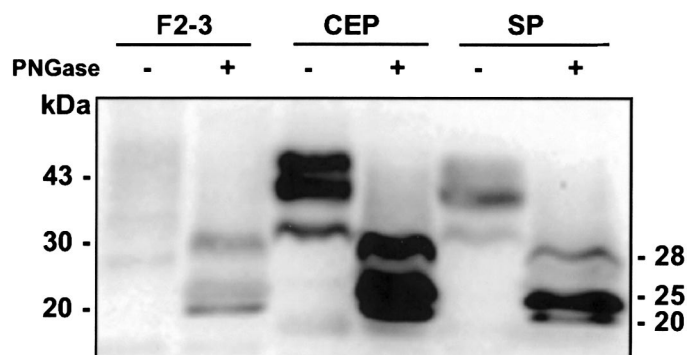


FIG. 6. Effect of N-glycosidase treatment on PrPc from cauda epididymal fluid and seminal plasma. Fluids from zones 2–3, the cauda epididymis (CEP), and the seminal plasma (SP) were treated with N-glycosidase. Treated (+) and untreated (–) samples of each fluid were separated and transferred to nitrocellulose. The blot was probed with the P4 antibody (1/1500).

munochemistry) and scrapie-resistant ARR/ARR rams were treated directly by the addition of 20, 40, and 100 μ g/ml of proteinase K. After 1 h of incubation at 37°C, the immunoreactive PrP pattern of both the infected and the resistant animals remained unchanged (not shown). Similar results were obtained with the cauda epididymal fluid of scrapie-free and infected animals (not shown). We then treated a 10% brain suspension from a scrapie-infected sheep with proteinase K in the presence of cauda epididymal fluid or seminal plasma from different rams. The presence of these fluids abolished the shift in molecular weight (and therefore proteolysis by proteinase K) as compared with standard sucrose conditions (not shown). This result indicated that the presence of proteinase K inhibitors in the cauda fluid and the seminal plasma may have impaired the direct testing for the presence of PrPres in these fluids.

We then used an indirect method in which the PrP present either in the epididymal fluid or the seminal plasma samples was first immunoprecipitated and then treated with proteinase K (10 μ g/ml final concentration for 1 h at 37°C). The PrP immunoprecipitated from brain suspensions of a healthy and a scrapie-infected sheep were treated under the same conditions (Fig. 8).

Western blotting showed that the PrP present in the immunoprecipitates from the seminal plasma of both healthy and scrapie-infected rams was completely cleaved by the protease K treatment. This was also the case for the protein from the epididymal fluid of infected rams. As a control the immunoprecipitated brain PrP from the healthy sheep was completely cleaved by proteinase K, whereas that of

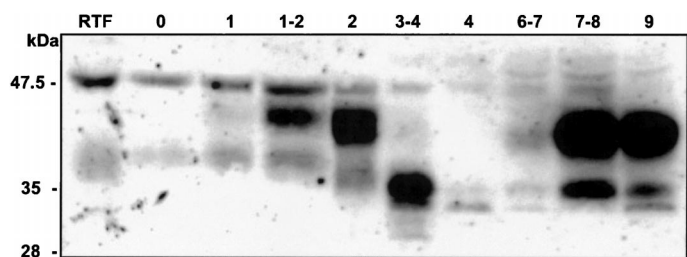


FIG. 7. PrP distribution in the epididymis of a scrapie-infected animal. Rete testis fluid (RTF) and fluids from the different epididymal zones obtained from a scrapie-infected ram (genotype VRQ/VRQ) were separated on 12% SDS-PAGE, transferred to nitrocellulose, and probed with the SAF32 antibody. Equivalent quantities of protein (about 100 μ g) were loaded on each lane.

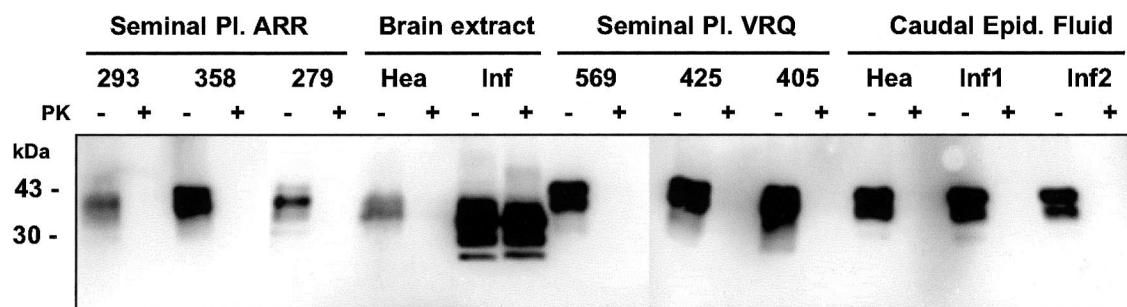


FIG. 8. Proteinase K treatment of seminal plasma, cauda fluid, and sheep brain immunoprecipitated PrP. Equivalent amounts of immunoprecipitated PrP from the seminal plasma of ARR/ARR and (A)(V)RQ/VRQ animals, from the cauda epididymal fluids of a healthy animal (Hea), and infected VRQ/VRQ (inf1) and ARQ/VRQ (inf2) animals, and from the sheep brain extract of a healthy (Hea) and a scrapie-infected (inf) animal were incubated with (+) or without (-) 10 μ g of proteinase K. The samples were separated, transferred, and probed with the P4 antibody (1/1500).

the infected sheep was not, showing that this immunoprecipitation assay could be used to demonstrate the presence of PrPres.

DISCUSSION

Our experimental results indicate that the PrPc is present and transported under a soluble form in the fluids of the male genital tract, and that this protein results mainly from synthesis occurring at different epididymal levels. The 3 main forms observed at 43, 35, and 30 kDa are highly glycosylated. Could these 3 forms represent the equivalent of the mono-, bi-, and nonglycosylated forms observed in the brain [28, 29]? Different glycoform profiles have been demonstrated for this protein in different tissues: e.g., 2 main PrPc forms at 32 and 35 kDa have been found in muscle, whereas lung PrPc ranged from 30 to 40 kDa because of a pattern of multiple glycosylations [12]. However, after deglycosylation these muscle and pulmonary forms gave 1 band at about 28 kDa. In contrast, 3 deglycosylation forms were obtained at about 28, 25, and 20 kDa with the genital tract PrPsc instead of the single \sim 28-kDa nonglycosylated form expected. Because the 25-kDa band was the main deglycosylated form observed in our samples, this suggests that it could be a nonglycosylated PrPc form, but in this case its lower than expected molecular mass suggests it is a truncated PrP. The 28-kDa band was less intense in the seminal plasma where the 43-kDa band was also reduced, suggesting that it originates from the latter. Whether this band is also a nonglycosylated form or a monoglycosylated band remains to be established. The 20-kDa band might be derived from the 35- and 30-kDa proteins, which in this case could also be a nonglycosylated form of proteolytic fragments of PrP.

The 35- and 30-kDa forms seemed predominant in the different seminal plasma we analyzed, whereas the 43-kDa form was generally the main form present in the cauda epididymal fluids. Because all of the PrPc of the seminal plasma is transported within the cauda fluid, this suggests that the 43-kDa form could be converted into lower molecular weight forms at 35 and 30 kDa by proteolytic cleavage that starts in the cauda fluid and increases considerably during the ejaculation process. This ongoing proteolysis also explains the appearance of low molecular weight immunoreactive bands in the seminal plasma. In this case, due to the specificity of the antibodies used in this study, it is most likely that it is the C-terminal portion of the 43-kDa protein that is removed: for example, we noticed that the monoclonal SAF84 (which is our most C-terminal antibody around position 150–160) recognized the PrPc in the cauda

epididymis but not in the seminal plasma, whereas all of the most N-terminal antibodies reacted with the protein. However, the fact that these N-terminal antibodies still recognized the band at 43 kDa raises some concern on this processing, and more work will be needed to understand this discrepancy.

Limited proteolysis that transformed the 43 kDa in low molecular weight forms (35 and 30 kDa) may also occur in the fluid from the anterior regions of the epididymis and could explain the apparent secretion of different isoforms observed. However, it cannot be ruled out that the epididymal epithelium secretes different PrPsc in different regions, since this has already been observed for several other epididymal proteins such as clusterin, epididymal retinoic acid binding protein, and prostaglandin D2 synthase, which are secreted differentially and under different glycosylated isoforms throughout the epididymis [30]. These protein modifications (glycosylation and proteolysis) might also explain the existence of the very acidic form of PrPc in the cauda fluid that disappeared in the seminal plasma.

Another striking and interesting feature is the fact that the PrPc is secreted as a soluble protein in the genital tract fluids. PrPc is normally inserted in the outer surface of the cell membrane via its GPI anchor. There have been very few reports that this protein can be a circulating protein [31]. It has been shown that PrPc can be released from the cell surface *in vitro* using transfected cell lines [32, 33] and neuronal primary cell culture [34]. The authors of these studies concluded that such release results either from a modification of the lipid forming the GPI anchor or that shedding is due to a proteolytic process and that free protein has lost its GPI anchor. We did not specifically investigate whether the epididymal fluid PrPc still retained the GPI tail, nor if GPI modification occurred. If the PrPc retains its GPI anchor after secretion in the epididymis, its behavior could then be similar to that of the Campath-1 antigen or CD52 [35, 36]. This last protein has a GPI tail and is secreted in the epididymal fluid and is inserted in the sperm membrane. The exact mechanism by which this protein and other highly hydrophobic secreted epididymal proteins [37] are transferred from the epididymal epithelium to the sperm membrane remains unknown, but membrane exchange of GPI proteins has been observed in several other cell systems and is known as the "painting" process [38].

Such a mechanism could explain the change in the pattern of PrPc reactivity in the sperm extract between the cauda epididymis and the ejaculate. A small part of the sperm PrPc is already in place when the sperm leave the

gonad, but an increase in the 30-kDa form on caudal sperm occurred, which remained after ejaculation. Variable extents of the 43- and 35-kDa forms could also be found on ejaculated spermatozoa. However, SAF84 and 8G8 antibodies did not react toward the sperm proteins (as observed for the PrPc forms present in the seminal plasma), suggesting that the inserted PrPc could have been cleaved within their C-terminal end. These results are partly in agreement with those of Shaked et al. [14] who suggested that the C-terminal portion of the protein is removed during epididymal passage and that the protein is inserted via its N-terminal in the membrane of ejaculated sperm. However, as in the seminal plasma the N-terminal antibodies still recognized proteins at 43 and 35 kDa on the sperm membrane, and this molecular weight could not fit with the loss of the C-terminal region that comprises the SAF 84 and 8G8 regions. We also observed that the response to some of the antibodies changed during transit, whereas the reactions for other antibodies recognizing a similar epitopic region did not change (data not shown), suggesting that subtle modifications of the PrPc conformation may also occur.

Transmissible neurodegenerative diseases are highly significant for human and cattle health, and also raise important economic problems. The way such diseases propagate is not yet fully understood, and it is important to explore any of the routes that can transmit them. Artificial insemination, which is widely used to improve the genetic value of the animals, could be one of these routes. Several previous studies have suggested that no transmission occurs via semen and artificial insemination [16–18], but some of these findings need to be verified since they were obtained before the discovery of the different sheep *PrP* alleles or using injection in mice, with the problem of the species barrier in transmission and the length in incubation period in the case of scrapie (see [19]). It was therefore important to investigate for the presence of PrPres in the seminal plasma of scrapie-infected animals, since this fluid is very rich in PrPc (by quantification on Western blot with known quantities of recombinant PrPc [25], we estimated the PrPc content to be about 0.1 µg/mg of protein in the seminal plasma and 0.4 µg/mg in the cauda fluid). Unfortunately, it was not possible to challenge the PrPc of these fluids with proteinase K directly, probably due to the presence of protease inhibitors [39, 40]. By immunoprecipitating the PrPc before the action of proteinase K, the indirect method we chose worked perfectly with sheep brain extracts and confirmed the validity of the results, i.e., that large amounts of PrPres were not present in the genital tract fluid of the infected ram even in the late stage of the disease.

Finally, the question concerning the role of the PrPc in the genital tract remains to be answered. As stated above, we observed large amounts of this protein in the cauda epididymis and in the seminal plasma. The PrPc was also present on the sperm membrane. High levels of this protein have also been found in the ewe uterus [12]. Different roles have been suggested for the PrPc, and its antioxidant function has been emphasized. Shaked et al. [14] showed that sperm from wild-type mice were more resistant to the presence of copper than those of PrPc $-/-$ mice. The presence of an antioxidant defense in the sperm-surrounding media is highly important, especially during passage and storage in the epididymis, since this cell lacks the mechanisms to regenerate damaged lipids and proteins.

In conclusion we have demonstrated the synthesis and secretion of soluble PrPc by the epithelial cells lining the epididymis. These cells produce large quantities of a spe-

cific isoform of PrPc that seems to be processed postsecretion in different ways during epididymal transit. Our findings also demonstrate that different forms of PrPc are found in ram spermatozoa, as reported previously for other species [14], and that they are inserted into the sperm membrane mainly during ejaculation. We did not observe the presence of PrPres in the fluid from the genital tract of scrapie-infected rams and, although this result is reassuring, further study will be needed to demonstrate that no infectivity is present in these fluids [41].

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