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The Human “Prion-like” Protein Doppel Is Expressed in Both Sertoli Cells and Spermatozoa*

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The prion-like Doppel protein (Dpl) has many biochemical and structural properties in common with the cellular prion protein (PrP^c), and the physiological role of neither protein is known. Experimental data suggest either direct or indirect interaction between the two proteins. In this study, we investigated the expression pattern and biochemical characteristics of Dpl in human tissues and in Chinese hamster ovary cells transfected with wild-type or variant human Dpl gene constructs. Human Dpl appears to be a glycosylphosphatidylinositol-anchored glycoprotein with N- and O-linked sugars. It was found on Sertoli cells in the testis, on the flagella of epididymal and mature spermatozoa, and in seminal plasma. Dpl coexists only with N-terminally truncated isoforms of PrP^c on mature spermatozoa. The localization of human Dpl on both Sertoli cells (somatic cells) and spermatozoa (germinal cells) strongly suggests that this protein may play a major role in human male fertility. Finally, our data indicate that spermatozoa are thus an interesting model for studies of the potential interaction between Dpl and PrP^c.

gene, which encodes Dpl (2, 3). This relationship was confirmed by constructing *Prnd*-overexpressing transgenic mice, in which the age at onset of ataxia was inversely correlated with Dpl levels (4, 5).

Prnd is located 16 kb downstream from *Prnp*, the gene encoding the cellular prion protein (PrP^c), and is thought to result from the ancestral duplication of this gene. For this reason, the corresponding protein was named Dpl, an acronym derived from downstream prion protein-like gene. Despite this relationship, PrP^c and Dpl present numerous differences and have specific features. PrP^c is a ubiquitous glycoprotein produced in large amounts in neurons (6). The mature protein is ~210 amino acids long, binds copper with high affinity via its N-terminal octapeptides, and may be involved in the regulation of oxidative stress (7). It has also been shown to prevent neuronal death *in vitro* (8), suggesting that it may play a role in cell death and/or apoptosis and that it may also be involved in cell signaling (9, 10). More importantly, a change in the conformation of PrP^c to generate an abnormal isoform, named PrP^{Sc}, is the molecular basis of the prion diseases, a group of fatal transmissible neurodegenerative diseases, including Creutzfeldt-Jakob disease in humans.

The occurrence of ataxia with cerebellar Purkinje cell degeneration in two aging prion protein (PrP)¹ knockout (*Prnp*^{0/0}) mouse lines named *Ngsk* and *Rmco*, together with a large-scale sequencing program, led to the discovery of the first prion-like protein, Doppel (Dpl), in mice (1, 2). The ataxia observed in these mouse lines was linked to overexpression of the *Prnd*

The mature Dpl protein is ~125 amino acids residues long and presents no region with a sequence similar to the octapeptide region of PrP^c. In contrast to what has been shown for PrP^c, Dpl does not seem to be required for prion disease progression or for the generation of PrP^{Sc} (11, 12). Whereas PrP^c is ubiquitous (13), Dpl seems to be tissue-specific in mice, sheep, and cattle (2, 14). Unlike PrP^c, Dpl gene transcripts are present only in very small amounts in the brains of wild-type adult mice and are principally found in the heart and testis (2). Although these differences indicate that the two proteins have different fates, Dpl and PrP^c have retained a number of important features in common, probably due to their common origin. Both are cell-surface proteins and present a 25% sequence homology, mainly in the C-terminal domain, resulting in similar patterns of folding (2, 15).

The most intriguing observation to date remains the correction of the ataxic phenotype following the introduction of a wild-type PrP transgene in some *Prnp*^{0/0} mice overexpressing *Prnd* in the central nervous system (4, 5, 16). This correction suggests that there is a direct or indirect interaction between Dpl and PrP^c. No precise physiological role has yet been attributed to either PrP^c or Dpl.

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¹ The abbreviations used are: PrP, prion protein; PrP^c, cellular prion protein; PrP^{Sc}, scrapie prion protein; HuPrP^c, human cellular prion protein; Dpl, Doppel; HuDpl, human Doppel; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PI-PLC, phosphatidylinositol-specific phospholipase C; PNGase F, peptide N-glycosidase F; FITC, fluorescein isothiocyanate; CHO, Chinese hamster ovary; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; GPI, glycosylphosphatidylinositol.

Most recent studies have focused on murine Dpl, and few data are available concerning human Dpl (HuDpl). Lu *et al.* (17) produced a recombinant putative mature HuDpl (residues 24–152) and showed that HuDpl contains two disulfide bonds between residues 94 and 145 and between residues 108 and 140 and has a high α -helix content (40%). The apparent molecular mass of the mature protein is 14.9 kDa, corresponding to the predicted molecular mass.

Three polymorphisms affecting residues 26 (T26M), 56 (P56L), and 174 (T174M) of the coding sequence of the human Dpl gene, *PRND*, have been described. No association has been established between these polymorphisms and the development of human prion diseases (18, 19). However, the specific positions of these polymorphisms raise questions concerning their possible effects on the processing of the protein (18).

We therefore investigated the cellular distribution and biochemical properties of HuDpl in various tissues, focusing on the male reproductive system and on cells transiently transfected with various HuDpl DNA constructs. We also studied the structure of PrP^c and its distribution in the male reproductive system, with the aim of investigating possible PrP^c/Dpl interactions under physiological conditions. We found that HuDpl was present on both Sertoli cells and mature spermatozoa. These findings concerning the distribution of the protein and further characterization of the protein may provide new insight into the function of Dpl in human reproduction.

EXPERIMENTAL PROCEDURES

Reagents—McCoy's medium, trypsin, fetal calf serum, antibiotics, glutamine, bovine serum albumin (BSA), diamidobenzidine, Tween 20, Dulbecco's phosphate-buffered saline (PBS), phosphatidylinositol-specific phospholipase C (PI-PLC), and normal goat serum were obtained from Sigma (Saint-Quentin Fallavier, France). FuGENE 6 reagent and Mini-Complete protease inhibitor tablets were purchased from Roche Molecular Biochemicals (Meylan, France). Peptide *N*-glycosidase F (PNGase F) and restriction enzymes *Bam*HI and *Hind*III were purchased from New England Biolabs Inc. (Hitchin, United Kingdom). The glycoprotein deglycosylation kit containing PNGase F, endo- α -*N*-acetylglucosaminidase, α 2–3,6,8,9-neuraminidase, β 1,4-galactosidase, and β -*N*-acetylglucosaminidase was supplied by Calbiochem. Antifading solution was obtained from Dako Corp. (Trappes, France). We obtained pcDNA3 from Invitrogen (Cergy Pontoise, France).

Antibodies—DDC39 is a polyclonal rabbit antiserum directed against a synthetic peptide encompassing amino acids 68–88 of HuDpl (Neosystem SA, Strasbourg, France). Dop151 is a monoclonal antibody obtained by immunizing mice with recombinant HuDpl (amino acids 28–152) produced in *Escherichia coli*. Recombinant HuDpl was generously provided by Dr. Sorgato (Dipartimento di Chimica Biologica, University of Padova). Dop151 specificity was verified by Western blotting on the recombinant HuDpl protein. SAF32, SAF70, and Pri917 are monoclonal antibodies directed against the PrP^c protein. SAF32 and SAF70 were elicited in knockout mice immunized with denatured scrapie-associated fibrils from the brain of an infected hamster. SAF32 recognizes the N-terminal octarepeat region of HuPrP^c (amino acids 59–89), and SAF70 recognizes the region encompassing residues 156–162 (18). Pri917 was raised in Biozzi mice by immunization with a synthetic peptide (20) and binds to the C-terminal part of HuPrP^c (amino acids 216–221). Monoclonal antibody 3F4 recognizes residues 109–112 of HuPrP^c (Senetek, Napa, CA). Secondary antibodies were obtained from Southern Biotechnologies (peroxidase-conjugated anti-rabbit antibody; Montrouge, France), Dako Corp. (peroxidase- and FITC-conjugated anti-mouse antibodies), and Sigma (FITC-conjugated antibody).

Plasmid Construction—The *PRND* sequence encoding Thr²⁶/Pro⁵⁶/Thr¹⁷⁴ HuDpl (considered the wild-type form) or its variants (T26M, P56L, and T174M) was inserted into the expression vector pcDNA3 as follows. The previously described *PRND* coding sequences from four control individuals (18) were amplified under standard conditions with the following primers, introducing restriction sites for *Hind*III (forward) and *Bam*HI (reverse): forward, 5'-GATCAGAGCTTAATGAG-GAAGCACCTGAGCTGG3'-; and reverse, 5'-CTGATCGATCCTCAT-TATTTACCGTGAGCCAGAT3'-. The PCR products were cleaved with

the appropriate restriction enzyme and inserted into pcDNA3. Each DNA construct was verified by sequencing.

Cell Cultures and Their Treatment—Chinese hamster ovary (CHO) cells were cultured in McCoy's medium supplemented with 5% fetal calf serum, 1% glutamine, and 1% penicillin/streptomycin in an atmosphere of 5% CO₂ and 95% air and transiently transfected using FuGENE 6 reagent as recommended by the manufacturer. Cell viability was assessed by the trypan blue exclusion test 24 h after transfection. To obtain cell lysates, cells were washed three times with PBS, suspended in lysis buffer (150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 20 mM Tris-HCl, pH 7.5) at 4 °C to which a protease inhibitor tablet had been added, and incubated for 30 min on ice. The resulting cell lysates were centrifuged at 4000 × *g* for 10 min, and the supernatant was stored at –80 °C until used.

Tissue Preparations—Human tissues were removed post-mortem from two adult individuals (one male and one female) free of human prion diseases. Human organs were homogenized at 4 °C in lysis buffer in an automated crusher (Ultraturax, Staufen, Germany). The homogenates were centrifuged, and the supernatants were frozen at –80 °C until used. Protein concentrations were determined by the BCA technique (Bio-Rad, Marne-la-Coquette, France). Human testicular tissue were obtained from infertile patients in whom a surgical recovery of testicular or epididymal spermatozoa was attempted for assisted reproduction. Testicular samples with normal spermatogenesis and normal structure of interstitial tissue were obtained from three men with obstructive azoospermia. Three testicular samples from men with secretory azoospermia and high follicle-stimulating hormone levels were also analyzed. Two had Sertoli-cell-only syndrome, characterized by a lack of germinal cells in all seminiferous tubule sections analyzed; and in one sample, a complete spermatogenesis could be observed only in some tubule sections (hypospermatogenesis). Blood was obtained from healthy voluntary donors.

Seminal Plasma and Sperm Preparation—Human spermatozoa were obtained from healthy voluntary donors with normal sperm characteristics according to the World Health Organization criteria (21). After collection, semen samples were incubated for 30 min at 37 °C for liquefaction and layered onto a two-phase Percoll gradient (47.5 and 95%). The Percoll gradient was centrifuged for 20 min at 300 × *g*, and the overlying seminal plasma was recovered and centrifuged at 10,000 × *g* for 10 min. Protease inhibitors were added to the supernatant depleted of spermatozoa, which was then frozen at –80 °C until used. In the lower fraction (95% Percoll), the pellet of motile spermatozoa was washed twice and resuspended in human tubule fluid supplemented with 1% BSA. The cells were either used immediately or incubated for 18 h in human tubule fluid supplemented with 1% BSA under 5% CO₂ in air to allow the completion of capacitation. Testicular spermatozoa were obtained by dissecting fresh human testicular biopsy samples in Ferticult medium to extrude germ cells. In one patient with excretory azoospermia, spermatozoa were collected from the epididymis for diagnostic assessment and smeared onto a slide. All samples were obtained after the patients had given written informed consent.

Deglycosylation Experiments—Where indicated, cell lysates or tissue homogenates were treated with 10,000 IU/ml PNGase F for 3 h at 37 °C to generate *N*-deglycosylated HuDpl and HuPrP^c proteins. In some experiments, samples were incubated for 0, 15, 30, 45, 60, and 120 min at 37 °C with 100 IU/ml PNGase F. Lysates were also treated with PNGase F and *O*-deglycosidase enzymes for 3 h at 37 °C to generate *N*- and *O*-deglycosylated HuDpl, respectively. Alternatively, lysates were treated with 0.2 IU/ml neuraminidase together with PNGase F.

Western Blotting—Cell lysates or tissue homogenates were loaded onto 12 or 15% SDS/glycine-polyacrylamide gel. Proteins were electroblotted onto Immobilon P membranes (Millipore, Saint-Quentin en Yvelines, France), and HuDpl or HuPrP^c was detected with the antibodies indicated and a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody. The blots were developed using an enhanced chemiluminescence protocol (Pierce).

***N*-terminal Sequencing of HuDpl**—For microsequencing, HuDpl was immunoprecipitated from a testicular homogenate using Dop151 conjugated to Sepharose beads. Briefly, the antibody was incubated with protein A-conjugated Sepharose beads (Amersham Biosciences, Saclay, Paris) in 150 mM HEPES and 5% BSA for 2 h at 4 °C. Precleared crude testicular lysates were then incubated overnight with the beads. After three washings in PBS the beads were heated for 10 min at 100 °C in Laemmli buffer, and the supernatant was subjected to two-dimensional electrophoresis. Protein spots were electroeluted and sequenced by Edman degradation. MALDI-TOF mass spectrometric analysis of digested peptides was performed on a Biflex mass spectrometer equipped with a gridless delayed extraction operating in the reflector mode. A list

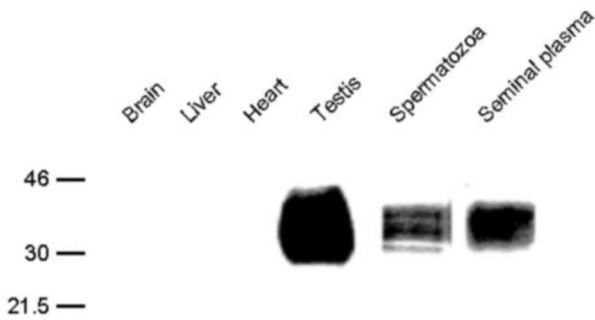


FIG. 1. **Dpl production in human tissues and fluids.** Organ homogenates or cell lysates were run on a 12% SDS-glycine gel. HuDpl was detected with the DDC39 antibody (1:10,000 dilution). The pattern observed was obtained with 8 μ g of total protein from human testis, 60 μ g of total protein from other tissues, and 4 μ l of seminal fluid (100 μ g of total protein). 50 μ g of total protein equivalent to 25×10^6 spermatozoa were loaded onto the gel. Molecular masses are indicated in kilodaltons.

of the masses of peptides was obtained for the protein digests. This peptide mass fingerprint was then submitted to the Swiss Protein Database² to identify the protein. To identify the sugars, the mass strategy developed by Carr *et al.* (22) was used. N-terminal sequencing and MALDI-TOF mass spectrometric analysis were performed in the Roche Pharma Research Department (Basel, Switzerland).

Detergent Insolubility—Detergent insolubility was assayed as previously described (23), with slight modifications. We incubated 500 μ l of cell-free seminal plasma at 4 °C for 20 min with 1% Triton X-114 in PBS supplemented with protease inhibitors. This mixture was centrifuged at $3000 \times g$ for 10 min. The supernatant was incubated for 10 min at 37 °C and centrifuged at $10,000 \times g$ for 60 s. The aqueous phase was then collected and submitted to methanol precipitation (A1), and the detergent phase was removed and placed on ice. We added 500 μ l of PBS and split the resulting mixture into two aliquots. One aliquot was incubated with 5 IU/ml PI-PLC for 2 h at 4 °C. Both aliquots were then incubated for 10 min at 37 °C and centrifuged at $10,000 \times g$ for 60 s. Both the aqueous (A2) and detergent phases were collected and subjected to methanol precipitation, followed by Western blotting.

Immunohistochemistry—Human testicular biopsy samples were fixed in 4% formaldehyde and embedded in paraffin. Sections (4 μ m) were cut, cleared by incubation in alcohol, and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ for 30 min. Sections were incubated with 1.5% normal goat serum in 1% BSA and 0.05% Tween in PBS for 1 h and then with primary antibodies for an additional hour at room temperature. Sections were washed with PBS, incubated with horseradish peroxidase-conjugated anti-mouse antibody for 1 h, and washed three times. The sections were finally treated with diaminobenzidine for 10 min in the dark. Washed sections were rapidly counterstained with Hemalum. For PrP^c detection, the sections were first dipped in boiling citrate buffer (0.1 M sodium citrate and 0.1 M citric acid, pH 6.0) for 10 min and then allowed to cool before peroxidase inhibition. Negative controls were performed by incubating sections with the secondary antibody alone.

Immunofluorescence—Capacitated spermatozoa (2×10^6 cells/ml) were washed twice with PBS, deposited in the wells of a microscope slide, and air-dried. For PrP^c detection, slides were dipped in boiling citrate buffer for 10 min and cooled. Slides were incubated for 30 min at room temperature with 3% BSA in PBS and then treated for 1 h with primary antibodies in 1% BSA in PBS. Spermatozoa were washed three times and incubated for 1 h with FITC-conjugated anti-mouse antibody in 1% BSA in PBS. The slides were washed, mounted into anti-fading solution, and examined by fluorescence microscopy. Negative controls were performed using either a mouse IgG isotype control or PBS instead of the primary antibody. The smears of testicular and epididymal spermatozoa were treated for immunofluorescence as described above for ejaculated spermatozoa. For transfected cells, Dop151 was incubated with living cells on coverslips for 1 h at 4 °C, fixed by incubation with cold methanol for 30 min, and detected with FITC-conjugated anti-mouse antibody. The coverslips were mounted in anti-fading solution, and the cells were observed by fluorescence microscopy.

Cytofluorometry—Spermatozoa were washed twice with PBS, incubated in 10% normal goat serum in PBS to avoid nonspecific binding,

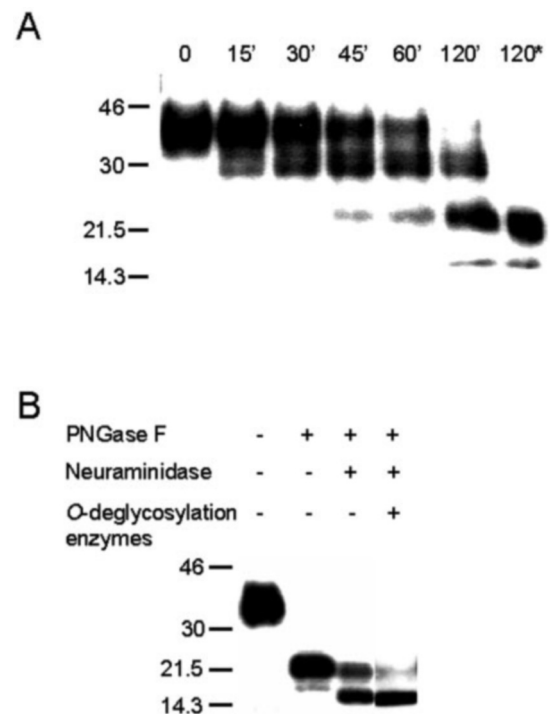


FIG. 2. **HuDpl is N- and O-glycosylated.** *A*, time course of PNGase F digestion of HuDpl. Aliquots of human testicular homogenates (8 μ g) were digested with 100 IU/ml PNGase F for 0, 15, 30, 45, 60, and 120 min as indicated. 120* indicates a 100-fold excess of PNGase F, which was used to ensure the complete deglycosylation of the molecule during a 2-h incubation period. HuDpl was detected with the DDC39 antibody (1:10,000 dilution). Molecular masses are indicated in kilodaltons. *B*, deglycosylation experiments in which testicular HuDpl was digested with both N- and O-deglycosidases. HuDpl was detected with the DDC39 antibody (1:10,000 dilution). We loaded 4 μ g of total protein from human testis onto the gel. Molecular masses are indicated in kilodaltons.

and then incubated for 1 h with either Dop151 or SAF70 in 3% normal goat serum in PBS. They were rinsed and incubated for 1 h with FITC-conjugated anti-mouse antibody. The cells were washed, fixed in 3% paraformaldehyde in PBS, and analyzed by flow cytometry with CELL-Quest software (BD Biosciences). In some experiments, cells were incubated for 1 h at 37 °C with PI-PLC (5 IU/ml), rinsed, and stained as described above.

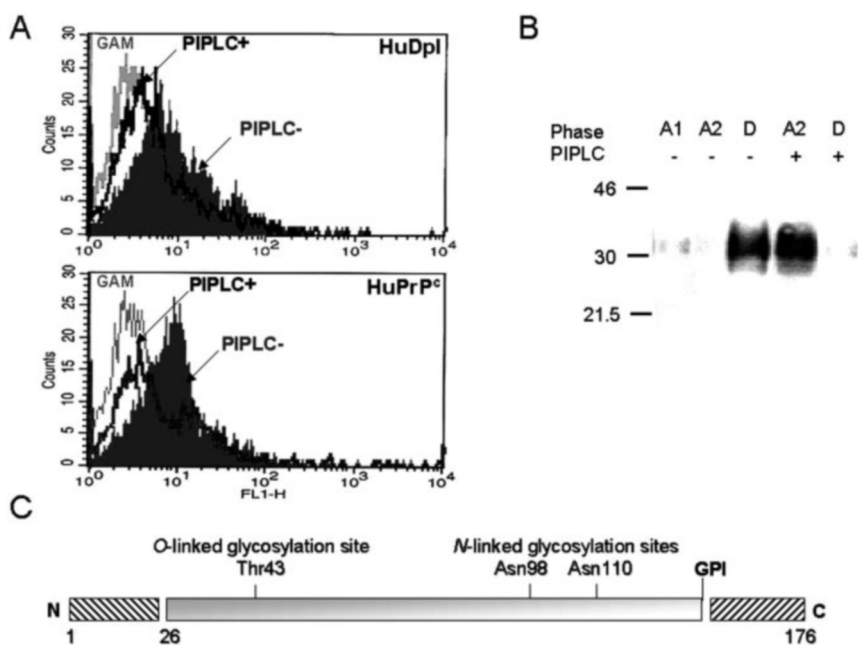
RESULTS

Biochemical Detection and Characterization of HuDpl—Various human organs and tissues were subjected to immunoblotting to test for the presence of HuDpl. We used a polyclonal antibody directed against the N-terminal part of HuDpl, which recognized a large heterodisperse band in human testis with an apparent molecular mass of 29–40 kDa (Fig. 1). No HuDpl was detected in the brain, heart, or liver (Fig. 1) or in the spleen, lung, lymph nodes, ovary, leukocytes, erythrocytes, or serum (data not shown). Edman degradation and MALDI-TOF mass spectrometric analysis of the protein immunoprecipitated from the testis confirmed that this protein was indeed HuDpl, with the N-terminal sequence TRGIKHKRIKWN, indicating that the mature HuDpl protein begins at the polymorphic residue 26.

Two potential N-glycosylation sites were identified in the HuDpl sequence at Asn⁹⁸ (NVT) and Asn¹¹⁰ (NAT); we therefore analyzed testicular lysates after deglycosylation with PNGase F (Fig. 2A). The kinetics of deglycosylation indicated that both sites were occupied. We detected one intermediate deglycosylation step giving rise to a band migrating at 27–28 kDa (Fig. 2A). Prolonged incubation with an excess of PNGase F resulted in the detection of two bands, a minor band migrating at 14–15 kDa (probably corresponding to unglycosylated

² Available at www.expasy.org/tools/findmod.

FIG. 3. HuDpl appears to be GPI-anchored. *A*, surface expression and GPI anchoring of HuDpl and HuPrP^c. Flow cytometric experiments were conducted with human mature ejaculated spermatozoa. The sensitivity to PI-PLC of HuDpl and HuPrP^c on mature spermatozoa was investigated by FACS analysis with Dop151 (1:25 dilution) and SAF70 (1:50 dilution), respectively. The results presented are representative of two independent experiments. A negative control was performed using the goat anti-mouse (*GAM*) secondary antibody alone. *B*, Triton X-114 partitioning of HuDpl from cell-free seminal plasma before and after addition of 5 IU/ml PI-PLC. Analysis was performed by Western blotting with the DDC39 antibody (1:10,000 dilution). *A1* and *A2*, first and second aqueous phases, respectively; *D*, detergent phase, as specified under "Experimental Procedures." Molecular masses are indicated in kilodaltons. *C*, schematic representation of HuDpl. The human mature protein after cleavage of the N- and C-terminal peptides is shown in gray. The N- and O-linked glycosylation sites identified by mass spectrometry are indicated above the diagram.



HuDpl) and a second band of 16–22 kDa, suggesting the existence of additional post- or co-translational modifications insensitive to PNGase F. Indeed, assays combining N- and O-deglycosylation resulted in the detection of a single band of ~14–15 kDa, probably corresponding to the mature unglycosylated full-length protein (Fig. 2B). Digestion of the N-deglycosylated extract of HuDpl with neuraminidase alone led to a detectable shift in the apparent molecular mass of the protein, indicating the presence of sialic acid in the oligosaccharide residues in the side chains of the glycoprotein (Fig. 2B). MALDI-TOF analysis of HuDpl confirmed that a mucin-type O-glycosylation occurred at Thr⁴³, with the carbohydrate moiety GalNAc branching from the first residue (data not shown).

HuDpl was also detected in both ejaculated sperm extracts and seminal plasma as a large heterodisperse band of 29–35 kDa (Fig. 1). After PNGase F treatment, HuDpl from spermatozoa and seminal plasma displayed the same pattern of migration as seen for the testis (data not shown), indicating N- and O-glycosylation. Flow cytometric analyses of HuDpl staining were performed on mature spermatozoa with or without preliminary incubation with PI-PLC. PI-PLC treatment led to a marked decrease in the fluorescence intensity resulting from HuDpl staining (Fig. 3A), indicating GPI anchoring of the protein.

HuDpl from seminal plasma was shown to partition into the Triton X-114 hydrophobic fraction (Fig. 3B). PI-PLC treatment resulted in the partitioning of HuDpl into the aqueous phase, demonstrating that HuDpl has an intact PI-PLC-sensitive GPI anchor in seminal plasma.

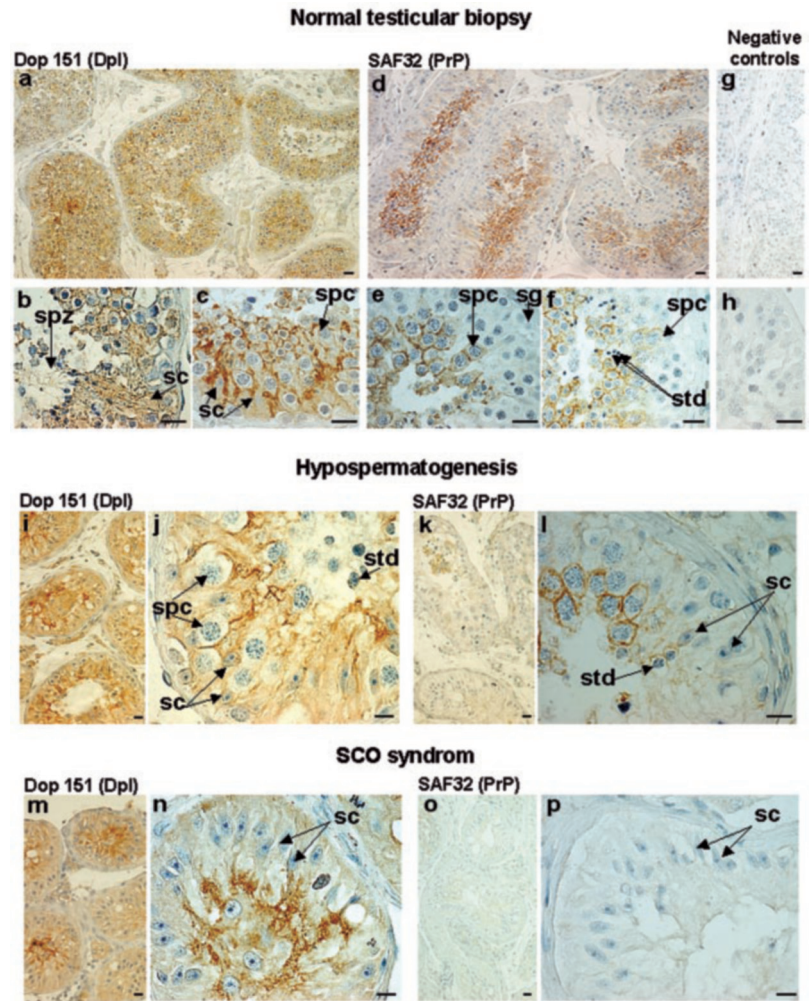
HuDpl Is Found Mainly on Sertoli Cells and Spermatozoa—As Dpl seemed to be present only in the male reproductive system in humans, we characterized in more detail its distribution in the testis and at the surface of spermatozoa. Immunohistochemistry experiments in which Dop151 was incubated with normal biopsy sections showed that HuDpl was present mostly in the seminiferous tubules, at the location of the Sertoli cells (Fig. 4, a–c). In testicular biopsy sections from patients suffering from hypospermatogenesis or Sertoli-cell-only syndrome, in which Sertoli cells are more easily visible due to the partial or total absence of germinal cells, this reactivity was confirmed; a markedly higher staining was observed on the adluminal side of the cells (Fig. 4, i, j, m, and n).

To determine the location of HuDpl on spermatozoa, we carried out indirect immunofluorescence experiments with Dop151 and spermatozoa collected at three different points in their maturation: from the testis, epididymis, and semen. No fluorescence was observed on testicular spermatozoa, whereas faint staining was observed on the flagella of epididymal spermatozoa (Fig. 5c), and more intense staining was observed on the flagella of mature ejaculated spermatozoa (Fig. 5, e and f).

PrP^c Is Present as a Full-length Protein and Truncated Isoforms in the Male Reproductive System—HuPrP^c was detected by Western blotting in testicular extracts and seminal plasma as a heterodisperse band with an apparent molecular mass of 27–35 kDa, similar to that obtained with brain extracts (Fig. 6, A and B). PNGase F treatment of testicular homogenates generated three isoforms with apparent molecular masses of 27–30, 21–22, and 18 kDa (Fig. 6A). These isoforms, also present in the brain (24, 25), corresponded to the unglycosylated full-length protein and two N-terminally truncated products (C1 and C2), respectively. HuPrP^c from seminal plasma partitioned into the Triton X-114 detergent phase, suggesting that the GPI anchor is conserved. On human testicular sections, HuPrP^c was detected mostly on spermatocytes and spermatids, but not on spermatogonia (Fig. 4, d–f, k, and l). A weak staining of Sertoli cells was observed on biopsy sections from patients with Sertoli-cell-only syndrome (Fig. 4, o and p), but was indistinguishable from the background.

HuPrP^c detected in mature sperm extracts had an apparent molecular mass of 27–30 kDa, lower than that observed in the brain, testis, or seminal plasma. Two isoforms of HuPrP^c with apparent molecular masses of 21–22 and 18 kDa after deglycosylation were detected, indicating that the smaller size of sperm HuPrP^c probably resulted from truncation of the protein. The truncated region was mapped with antibodies specific for different epitopes along the length of the protein (Fig. 6C). On spermatozoa, HuPrP^c was recognized by antibodies binding to the C terminus, such as Pri917 (residues 216–221) and SAF70 (residues 156–162), but not by SAF32, an antibody that binds to the N terminus of PrP (residues 59–89). Monoclonal antibody 3F4 (residues 109–112) recognized HuPrP^c on spermatozoa only weakly and detected only the 21–22-kDa deglycosylated isoform. This suggested that HuPrP^c is present on spermatozoa only as N-terminally truncated fragments, simi-

FIG. 4. Location of HuDpl and HuPrP^c in the testis. HuDpl is located on Sertoli cells, whereas HuPrP^c is found on germ cells. Peroxidase staining of testicular biopsy samples from morphologically normal testis were carried out using Dop151 (1:100 dilution; *a–c*) or SAF32 (1:50 dilution; *d–f*). A negative control was performed by incubating sections with the secondary antibody alone (*g* and *h*). *spz*, spermatozoon; *sg*, spermatogonium; *sc*, Sertoli cell; *spc*, spermatocyte; *std*, spermatid. Immunohistochemical staining of testicular biopsy samples from patients suffering from either hypospermatogenesis (Dop151 (*i* and *j*) and SAF32 (*k* and *l*) or Sertoli-cell-only syndrome (Dop151 (*m* and *n*) and SAF32 (*o* and *p*)) was carried out for comparison. Bars = 10 μ m.



lar to those present in the brain (C1 and C2). The proportion of the two fragments differed in the spermatozoa and testis, with the 18-kDa fragment accounting for a larger proportion of the fragments than the 21–22-kDa fragment in the spermatozoa. Consistent with the results obtained by Western blotting, SAF32, which binds to the octarepeat region of PrP^c, stained only weakly rare spermatozoa (Fig. 5*i*), whereas SAF70 gave punctate staining of the sperm head region (Fig. 5, *j* and *k*). No immunoreactive signal was detected on the flagella. In flow cytometric experiments, PI-PLC treatment reduced the staining of HuPrP^c by SAF70, suggesting the PI-PLC-sensitive GPI anchoring of the protein to the spermatozoa (Fig. 3A).

Transient Production of HuDpl and Its Variants in CHO Cells—With the aim of further characterizing HuDpl and its variants, we transiently transfected CHO cells that did not produce endogenous Dpl with an expression vector carrying the Thr²⁶/Pro⁵⁶/Thr¹⁷⁴ PRND coding sequence, which was considered to represent the wild-type form. The results of immunoblotting experiments with transfected cell lysates, before and after deglycosylation, confirmed the data obtained for human testis. HuDpl was produced as a highly glycosylated and heterogeneous form of 29–37 kDa, and an unglycosylated full-length form was obtained after combined *N*- and *O*-deglycosylation. No cleaved forms were detected with the antibodies used (Fig. 7A). Indirect immunofluorescence experiments demonstrated that HuDpl was expressed at the cell surface, giving a punctate pattern of membrane fluorescence associated with cytoplasmic fluorescence (Fig. 7B).

We investigated the expression of various DNA constructs

with the previously described polymorphisms at codons 26, 56, and 174 of PRND to explore the possible impact of these variants on HuDpl processing. For instance, the T26M variation is likely to affect signal peptide cleavage according to predictions made by the method of von Heijne (26). The P56L variation is located in a stretch of 25 residues highly conserved in mammals (2, 18). The T174M polymorphism occurs in the C-terminal hydrophobic sequence, which is thought to be removed before addition of the GPI anchor at Gly¹⁵². Expression of the HuDpl DNA constructs had no toxic effect on the transfected cells, as almost 100% viability was observed 24 h after transfection. All proteins were present at the cell surface, as shown by indirect immunofluorescence; and their apparent molecular masses before and after PNGase F treatment were similar to that of Thr²⁶/Pro⁵⁶/Thr¹⁷⁴ HuDpl (Fig. 7, A and B). These data suggest that the PRND variants have little effect, if any, on HuDpl cellular trafficking.

DISCUSSION

In this study, we first investigated the distribution of Dpl in humans and then focused on the male reproductive system, the only location in which HuDpl was found. The expression pattern of the Dpl gene (*Prnd*) in adult tissues has been studied in mouse, sheep, and cattle. *Prnd* transcripts are abundant in adult mouse testis and heart and are detected at low levels in the brain (2). Northern blotting has demonstrated the presence of *Prnd* mRNA in the brains of newborn mice, raising questions as to the role of Dpl during development (27). *Prnd* is strongly expressed in the testes of sheep and cattle, whereas only low

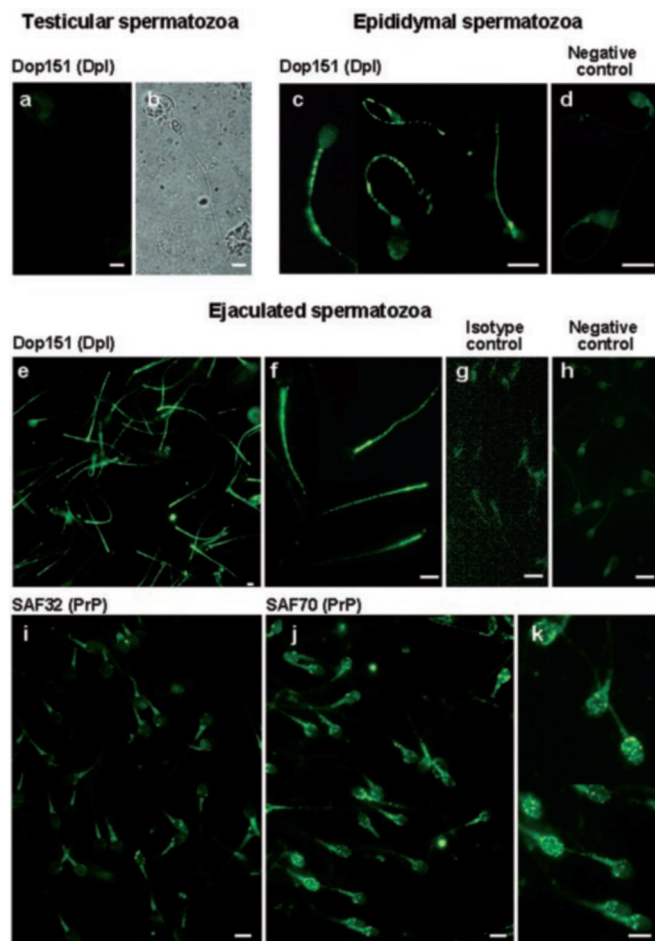


FIG. 5. Location of HuDpl and HuPrP^c on spermatozoa. HuDpl is present on the flagella of both epididymal and mature ejaculated spermatozoa. Immunofluorescence staining of testicular (a), epididymal (c) and mature ejaculated spermatozoa (e–f) was carried out using Dop151 (1:25 dilution). For comparison, testicular spermatozoa obtained after dissection of the biopsy sample are shown in bright-field microscopy (b). Immunofluorescence staining of mature ejaculated spermatozoa was carried out using SAF32 (1:50 dilution; i) and SAF70 (1:50 dilution; j and k). Negative controls were carried out using PBS instead of primary antibody (d and h) or a mouse IgG isotype control (g). Bars = 5 μ m.

levels of expression are detected in the epididymis (cattle), ovary, and spleen (14). The distribution of the Dpl protein has never before been extensively studied in mammals, and the protein has only been reported to be present in mouse testicular extracts (28). In this study, large-scale screening of human tissues and fluids for Dpl by Western blotting showed a very restricted distribution in humans, with the protein limited to the male genital tract. This pattern was confirmed by an enzyme-linked immunosorbent assay of HuDpl in the same samples.³ The amount of HuDpl present was estimated to correspond to ~ 50 μ g/g of total protein in the testicular extracts and ~ 10 μ g/g of total protein in seminal plasma. HuDpl levels in the brain are at most one-thousandth of those in the testis. HuDpl was also detected in spermatozoal extracts and in seminal fluid as a heavily N- and O-glycosylated protein. Mouse Dpl does not seem to be subject to O-glycosylation,⁴ suggesting that this post-translational modification is not crucial for the physiological function of this protein. The strong signal observed on Western blots of testicular homogenates corresponded to intense immunohistochemical staining of the Ser-

toli cells on sections of normal and pathologic testes. Sertoli cells are large columnar cells that are present in the seminiferous tubules and that are involved in the development of the germinal cells and establishment of the blood-testis barrier, defining two compartments. The basal compartment contains the diploid spermatogonia, whereas the adluminal compartment contains meiotic cells (spermatocytes, spermatids, and spermatozoa). In immunohistochemical staining experiments, HuDpl was detected in the seminiferous tubules, at the adluminal pole of Sertoli cells. The only GPI-anchored protein described to date on Sertoli cells is the rat apical protein ceruloplasmin (29). As Sertoli cells communicate with the germ cells either directly (cell-cell interaction) or indirectly (paracrine interaction) throughout gametogenesis, it seems likely that Dpl is involved in spermiogenesis.

During the course of our study, *Prnd*^{0/0} mice were established by an other group (30). These mice were characterized by a spermiogenesis defect leading to a lower number of spermatozoa with decreased motility and inability to fertilize oocytes *in vitro*. Interestingly, the Dpl immunostaining pattern observed in the testis seems to differ between humans and mice, in which Dpl is expressed in spermatids (30).⁵ It therefore remains to be determined whether an impaired Sertoli cell function is linked to the *Prnd*^{0/0} phenotype.

Interestingly, HuDpl was also found on mature ejaculated spermatozoa. HuDpl, as a highly glycosylated protein, may therefore be involved in active protection of the spermatozoa, reducing interactions between the cells (31). Further characterization showed that HuDpl was present on the flagella of ejaculated spermatozoa, suggesting that this protein may be involved in the motility of spermatozoa. Spermatozoa mature and become motile during their transit through the epididymis (32). HuDpl was not detected on testicular spermatozoa, but was detected on epididymal spermatozoa, suggesting that this protein is acquired after passage of the maturing spermatozoon through the epididymis. Interestingly, spermatozoa from the cauda epididymis of *Prnd*^{0/0} male mice display poor motility (30). Many proteins have been shown to be acquired during the maturation of spermatozoa, and these proteins are involved in the post-testicular remodeling of the membrane. Notably, some GPI proteins are produced by the epithelia of the efferent ducts, epididymis, and possibly other accessory glands (32), and these proteins become associated with the spermatozoa during transit through and storage in the male genital tract (33, 34). Two mechanisms of GPI transfer between seminal plasma and spermatozoa involving either vesicular organelles acting as GPI carriers or GPI-containing aggregates have been described (34). The presence of HuDpl with an intact GPI anchor in seminal plasma strongly suggests that the protein is transferred to the membrane of spermatozoa during their maturation.

As interactions between PrP^c and Dpl may occur, we also focused on PrP^c production in the human male reproductive system, for which few data are available. We found that PrP^c was produced in human testis, as expected for a protein considered to be ubiquitous. It was detected in germinal cells, spermatocytes, and spermatids, but not in spermatogonia. PrP^c was also detected on human mature spermatozoa.

HuPrP^c has been reported to be produced as a C-terminally truncated isoform in ejaculated spermatozoa (35). This isoform is resistant to PI-PLC and is not associated with lipid rafts in the cell membrane. As HuDpl was also found on spermatozoa, we thought that the presence on these cells of HuPrP^c devoid of the domain of greatest similarity to HuDpl might be of physi-

³ J. Grassi, personal communication.

⁴ K. Peoc'h and J.-L. Laplanche, unpublished data.

⁵ K. Peoc'h, C. Serres, and J.-L. Laplanche, unpublished data.

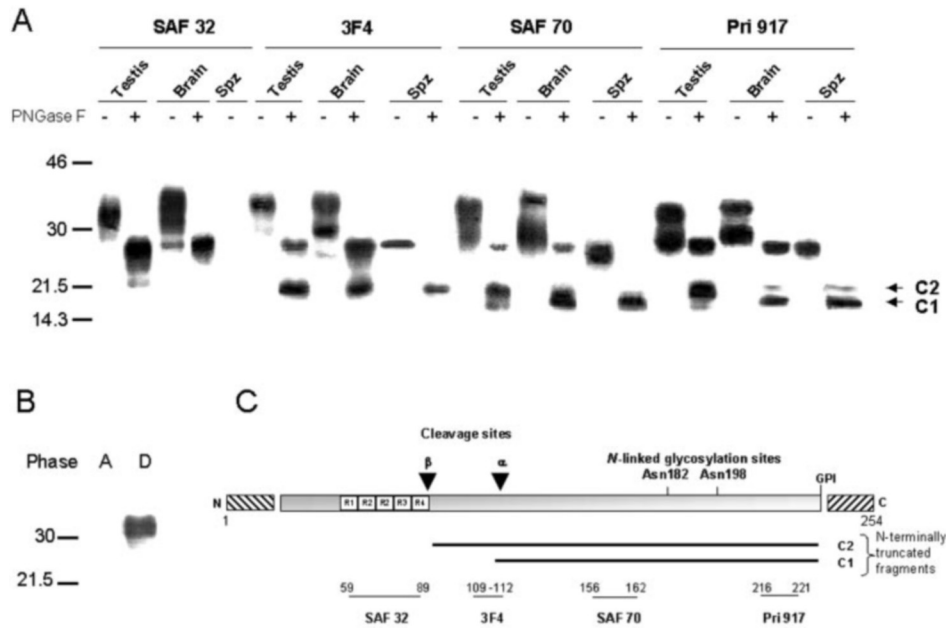


FIG. 6. Comparison of testicular and sperm HuPrP^c distributions with brain HuPrP^c distribution. *A*, pattern of PrP^c expression in human testis, brain, and spermatozoa. This pattern was investigated, before and after PNGase F treatment, with four different anti-PrP antibodies: SAF32 (1:1000 dilution), 3F4 (1:5000 dilution), SAF70 (1:1000 dilution), and Pri917 (1:5000 dilution). The pattern observed was obtained with 60 μ g of total protein from human testis, 60 μ g of total protein from human brain, and 25 μ g of total protein equivalent to 12.5×10^6 mature ejaculated spermatozoa (*Spz*). Molecular masses are indicated in kilodaltons. *B*, HuPrP^c from seminal plasma partitions into the detergent phase. After Triton X-114 partitioning of cell-free seminal plasma, the detergent (*D*) and aqueous (*A*) phases were analyzed by Western blotting with the SAF70 antibody (1:1000). Molecular masses are indicated in kilodaltons. *C*, schematic representation of HuPrP^c and locations of the epitopes corresponding to the various antibodies used for mapping. Cleavage α is located at residues 111 and 112, and cleavage β is located close to residue 90. The N-terminally truncated forms of HuPrP^c indicated by lines are the result of cleavages occurring near codon 90 (site β) and at codon 111/112 (site α), as shown in the brain (24, 25).

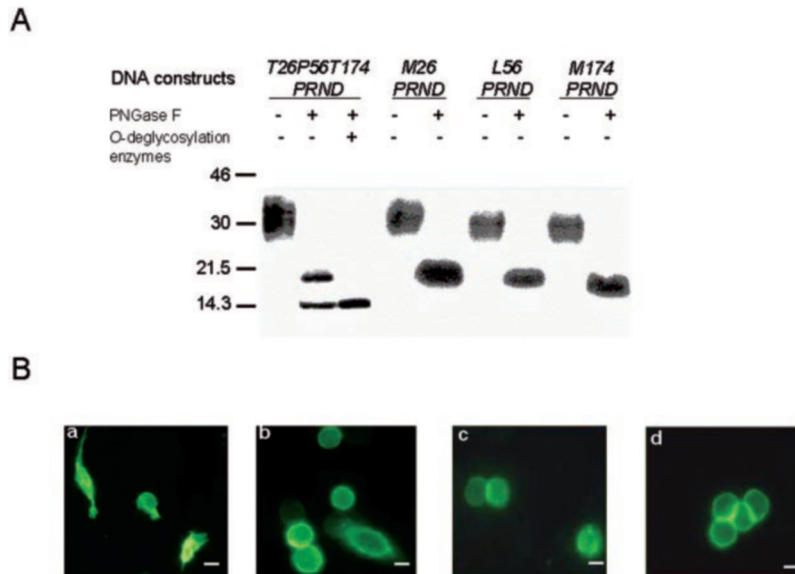


FIG. 7. Analysis of wild-type and variant PRND genes in transiently transfected CHO cells. *A*, wild-type and variant forms of HuDpl are both *N*- and *O*-glycosylated. Cell lysates transfected with either wild-type (Thr²⁶/Pro⁵⁶/Thr¹⁷⁴ PRND) or variant forms of PRND were subjected to Western blotting. 48 h after transfection, lysates of transiently transfected CHO cells (30 μ g of total protein) were run on the gel and analyzed with the DDC39 antibody (1:10,000). Molecular masses are indicated in kilodaltons. For deglycosylation experiments, 20 μ g of total protein were treated with PNGase F (10,000 IU/ml) or with the glycoprotein deglycosylation kit as recommended by the manufacturer. *B*, wild-type and variant forms of HuDpl are present at the surface of CHO cells. CHO cells were transfected with constructs containing either wild-type (Thr²⁶/Pro⁵⁶/Thr¹⁷⁴ (T26P56T174) PRND, panel *a*) or variant (T26M (M26) PRND, panel *b*; P56L (L56) PRND, panel *c*; or T174M (M174) PRND, panel *d*) forms of PRND and were processed for indirect immunofluorescence with Dop151 (1:100) 1 day after transfection. Bars = 10 μ m.

ological relevance. Surprisingly, we detected on these cells only N-terminally truncated isoforms of HuPrP^c, similar to C1 and C2 in the human brain. These forms appeared to be GPI-anchored according to the results of flow cytometric experiments. The two truncated fragments of HuPrP^c detected on ejaculated spermatozoa probably result from proteolytic cleav-

ages, which begin in epididymal fluid and greatly increase during the ejaculation process (36). The 18-kDa isoform was the most abundant and may, like C1 (37), be considered to be a final cleavage product of full-length HuPrP^c. The spermatozoa are unable to synthesize new proteins due to their condensed chromatin. Consequently, the absence of detectable full-length

HuPrP^c on spermatozoa, in contrast to the full-length GPI-linked forms detected in seminal plasma, indicates either the absence of transfer of HuPrP^c from seminal plasma to the spermatozoa or, if this transfer occurred, the rapid proteolysis of the newly transferred molecules. Finally, we did not detect the C-terminally truncated isoform of HuPrP^c described in a previous study (35). This may be due to differences in the protocol for preparing spermatozoal extracts, which may affect the extent of proteolysis of the spermatozoal proteins, or to the use of different antibodies.

A protective role against copper-induced damage has been suggested for PrP^c in mouse spermatozoa (35), but the absence at the cell surface of the high affinity N-terminal copper-binding region of HuPrP^c (residues 51–96) (38) does not support this hypothesis in humans. It is unclear whether the presence of both HuDpl and only copper binding-deficient forms of HuPrP^c at the surface of mature spermatozoa is of physiological relevance. Analyses with recombinant proteins and a 45-mer peptide have documented an unexpected property of mouse Dpl to bind copper⁶; and if confirmed for HuDpl, it is tempting to speculate that the truncation of HuPrP^c on spermatozoa would prevent possible competition between the two proteins for copper binding.

As abnormal Dpl gene expression may be involved in human male sterility, we investigated whether the polymorphisms at codons 26 (T26M), 56 (P56L), and 174 (T174M) of *PRND*, the human Dpl gene, would affect processing of the protein. We detected no major abnormality in cellular trafficking of the HuDpl protein and no cell toxicity effect resulting from variations at codons 26, 56, and 174 in comparisons with the wild-type Thr²⁶/Pro⁵⁶/Thr¹⁷⁴ HuDpl protein. Notably, the Thr-to-Met substitution at residue 26, the first N-terminal amino acid of mature HuDpl according to the results of N-terminal sequencing, did not prevent cleavage of the signal peptide, in contrast to predictions (26). However, although these *PRND* polymorphisms did not seem to impair cellular trafficking of HuDpl, it remains unclear whether they could modify structural and/or functional aspects of the protein, impairing spermatogenesis and/or sperm motility.

In conclusion, the high level of Dpl production in both Sertoli cells and spermatozoa in humans strongly suggests that the physiological function of HuDpl is related to human reproduction and may affect the quantitative or qualitative production of male gametes. The localization of HuDpl at the surface of spermatozoa opens up new possibilities for immunocontraception, and increasing our knowledge of Dpl may improve our understanding of the basis of human male sterility.

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