CHARACTERIZATION OF THE PRION PROTEIN IN HUMAN URINE

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Running title: PrP in human urine

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The presence of the prion protein (PrP) in normal human urine is controversial and currently inconclusive. This issue has taken a special relevance because prion infectivity has been demonstrated in urine of animals carrying experimental or naturally occurring prion diseases but the actual presence and tissue origin of the infectious prion have not been determined. We used immunoprecipitation, one- and two-dimensional electrophoresis and mass spectrometry to definitely prove the presence of PrP in human urine and its post-translational modifications. We show that urinary PrP (uPrP) is truncated mainly at residue 112 but also at other residues up to 122. This truncation makes uPrP undetectable with some commonly used antibodies to PrP. uPrP is glycosylated and carries an anchor which, at variance with that of cellular PrP, lacks the inositol-associated phospholipid moiety indicating that uPrP is probably shed from the cell surface. The detailed characterization of uPrP reported here definitely proves the presence of PrP in human urine and will help determine the origin of prion infectivity in urine.

The normal or cellular prion protein, PrP\textsuperscript{C}, is predominantly a cell surface protein that is sensitive to proteases, and is soluble in non-ionic detergents (1–4). In humans, the posttranslational modified, full-length PrP\textsuperscript{C} is made of 209 amino acids (residues 23-231), which include two sites of N-glycosylation, and carries a glycosylphosphatidylinositol (GPI) anchor (Fig. 1A) (5,6).

Because glycosylation is not obligatory, three PrP\textsuperscript{C} glycoforms, diglycosylated, monoglycosylated and unglycosylated, are commonly observed (6). In addition to the full-length PrP\textsuperscript{C}, truncated forms of PrP\textsuperscript{C} have been found in brains and cultured cells (7-9). A major PrP C-terminal fragment named C1 is generated during normal PrP processing by a cleavage at residues 111/112 that has been suggested to take place in the endosomal recycling (10), or in a late compartment of the secretory pathway such as the Golgi apparatus (11). Like the full-length PrP\textsuperscript{C}, the C1 fragment is mostly glycosylated with an electrophoretic mobility of approximately 28kDa which is reduced to approximately 18kDa following deglycosylation (8). The GPI anchor links both full-length PrP\textsuperscript{C} and the C1 fragment to the external surface of the plasma membrane of the cell. The structure of the GPI anchor, which is thought to be identical for the full-length and the C1 fragment, is made of a glycans core attached to the PrP\textsuperscript{C} C-terminus through a phosphodiester linkage of phosphoethanolamine, and a hydrophobic phospholipid component made of phosphatidyl-inositol and fatty acid that mediates the attachment to the membrane (12,13). Although the GPI-linked membrane isoform accounts for most of the PrP, minor trans-membrane and cytosolic forms of PrP have also been detected (14).

As the GPI anchor is subject to a variety of natural cleavages, secretion or shedding of various...
isoforms of PrP C in the medium of cultured cells, cerebrospinal fluid, serum, plasma and milk has been reported (6,15-18).

A rogue post-translational modification of PrP C can trigger prion diseases, a group of fatal encephalopathies that affect both humans and animals (19). The basic pathogenic mechanism underlying prion diseases is the conversion of the normal PrP C into a misfolded, disease-associated isoform, termed prion or scrapie prion protein (PrP Sc), which accumulates predominantly in the brain and to a lesser extent in other organs (19-21).

Although PrP Sc and PrP C share the amino acid sequence and the normal posttranslational modifications, they differ in that PrP Sc is insoluble in non-denaturing detergents, mostly resistant to proteases and aggregated (22,23). It is thought that the conversion of PrP C to PrP Sc is associated with a transition from α-helical to β-sheet–rich conformations. Prion transmissions between animals, from cattle to humans through food ingestion, and from humans to humans through blood transfusion, solid tissue grafting and injection of tissue-extracted hormones have been widely documented (24-25).

The presence of infectious PrP in human urine would pose serious risks to public health because of the medicinal use of urine-extracted proteins, hormones and urokinase and the risk of environmental prion contamination. The search for PrP Sc in urine has yielded controversial results. Shaked and colleagues (26) originally reported the detection of PrP Sc by Western blotting with the monoclonal antibody 3F4 in the urine of prion-affected Syrian hamsters and human subjects. They also described the presence of protease-sensitive PrP, apparently full-length PrP C, in controls that were free of prion disease, which suggests that normal urine contains PrP C while urine from individuals affected with prion disease also contains PrP Sc. However, three subsequent studies using the same antibody failed to detect PrP in urine from normal and prion-disease-affected Syrian hamsters and human subjects. They also described the presence of protease-sensitive PrP, apparently full-length PrP C, in controls that were free of prion disease, which suggests that normal urine contains PrP C while urine from individuals affected with prion disease also contains PrP Sc. However, three subsequent studies using the same antibody failed to detect PrP in urine from normal and prion-disease-affected individuals and demonstrated that the false positive results arose from the cross-reaction of anti-mouse IgG with either contaminating bacterial proteins (27) or urinary IgG fragments (28,29).

Infected PrP in urine was observed in urine from prion-infected mice affected by concomitant nephritis but not in prion-infected but nephritis-free mice (34). Thus, despite numerous reports of prion infectivity in urine in the course of prion diseases, the nature of the infectious prion agent in urine as well as the presence of PrP Sc in urine remain to be determined.

Since PrP C serves as substrate and precursor in the formation of infectious PrP Sc, a critical step in understanding the mechanism of prionuria is to definitively establish the presence and characteristics of PrP C in urine. We previously reported the detection of a PrP-immunoreactive protein in human urine (35). Here we report on the use of advanced mass spectrometry combined with other techniques to definitively demonstrate the presence, primary structure and posttranslational modifications of PrP in human urine.

**EXPERIMENTAL PROCEDURES**

**Antibodies.** A panel of five antibodies recognizing epitopes spanning the entire length of human PrP C was used (Fig. 1A). They included two rabbit antibodies to the N- and C-termini (36), and the monoclonal antibodies 3F4 (37) (Signet Laboratories, Dedham, MA), 6H4 (Prionics, Zurich), and 8H4 (38) to internal sequences of PrP (Fig. 1A).

**Immunoprecipitation.** Brain homogenate was prepared at 4°C in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.5) containing protease inhibitors (Roche Applied Science). Human urine was concentrated 100 fold by ultrafiltration with a 10-kDa cut-off membrane filter (Centriprep, Millipore). Anti-PrP monoclonal antibodies 3F4, 6H4, 8H4 and two polyclonal antibodies to the N- and C-termini of PrP were conjugated to tosyl-activated magnetic beads (M-280 Dynabeads, Dynal) and immunoprecipitation was performed as described previously (39). The resulting immunoprecipitates were used for immunoblotting with Anti-C antibody.

**Immunoblotting.** Samples were subjected to one-dimension (1-D) or 2-dimension (2-D) sodiumdodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting using anti-C antibody, as described elsewhere (36,40).
**Purification and mass spectrometric detection of urinary PrP.** Urine collected from healthy subjects was concentrated 200-folds by ultra filtration followed by deglycosylation with PNGase F (New England Biolabs). Deglycosilated proteins were then separated in 14% Tris-glycine tube gel (BioRad), PrP-containing fractions were detected with the anti-C antibody and further purified by two-dimensional chromatofocusing and reverse phase HPLC. After each purification step, the fractions were collected and analyzed. Urinary PrP (uPrP) was detected by immunoblotting with anti-C and the purity of the fractions was assessed by silver staining. The final preparations were subjected to 2-D SDS-PAGE and Coomassie-stained spots matching those identified on immunoblots were excised and digested in-gel with trypsin. Tryptic peptides were separated by reverse phase HPLC followed by nanoESI MS using a hybrid ion trap Fourier-transform ion cyclotron resonance (ICR) mass spectrometer (LTQ-FT) equipped with a 7 T superconducting electromagnet and a packed-Tip nanospray ionization probe (LTQ-FT, Thermo Electron Corp). Chromatographic separation of the protein digest was performed by an Ultimate 3000 nano HPLC (Dionex, Germering, Germany) with a trapping precolumn (C18, PepMap100, 300 µm ×5 mm, 5 µm particle size, 100 Å, Dionex, Germering, Germany) followed by a reverse phase column (C18, 75µm×150 mm, 3 µm, Dionex), using a mobile phase A (0.1 % formic acid in a water) and B (80 % acetonitrile, 0.04 % formic acid in water) with a linear gradient of 2% B per min. The peptides were electrosprayed at a flow rate of 300 nL/min via the silica noncoated PicoTip emitter (FS360-20-10-C12, New Objective Inc., Woburn, MA) at the voltage of 2.2 kV. The capillary temperature was kept at 200º C. Full MS spectra were recorded in the FT ICR cell and data dependent tandem MS spectra of 6 highest intensive ions were simultaneously recorded by the linear ion trap LTQ at collision energy of 35 eV, isolation width of 2.5 Da and activation Q of 0.250. Peptide assignments were made by searching tandem MS spectra against NCBI protein database using the BioWorks software (Thermo Electron).

**Characterization of the GPI anchor.** N-linked glycons were removed by PNGase F (New England Biolabs) according to the manufacturer’s protocol and urinary proteins were dissolved in Tris-buffered saline (pH 7.5) containing 2% Triton X-114 at 4 ºC . The sample was subjected to phase transition for 15 min at 35 ºC, followed by centrifugation. The resulting aqueous phase and detergent phase were collected and prepared for immunoblotting analysis.

The lipid component of GPI anchor was removed from the urinary proteins by incubating the deglycosylated samples with phosphatidylinositol-specific phospholipase C (PI-PLC) (Sigma) 1 U/ml for 1h at 37 ºC.

To remove the entire GPI anchor from urinary proteins, the deglycosylated samples were treated with 48% HF for 48 h at 4 ºC. The HF-treated sample was neutralized by the addition of 4M unbuffered Tris. Samples equivalent of one tenth of the starting material and its fractions were loaded onto 16% SDS-PAGE, and immunoblotted with anti-C.

**RESULTS**

**Antibody mapping of urinary PrP (uPrP).** Parallel immunoprecipitations of concentrated urine and normal brain homogenate with a panel of antibodies (Abs) to three regions of full-length PrP revealed that, while all the Abs immunoprecipitated PrP-immunoreactive proteins from normal brain homogenate, in urine preparations the immunoprecipitations were positive only with the two Abs to the more C-terminal region of PrP (Fig. 1). Immunoblotting with the Anti-N antibody also failed to detect the PrP-immunoreactive protein in urine. As expected, the electrophoretic mobilities of the PrP immunoprecipitated from the brain spanned from 35 kDa to 18 kDa, and deglycosylation resulted in two PrP-reactive immunoblot bands of about 27 kDa and 18 kDa corresponding to the full-length and C1 fragment of PrP, respectively (8). In contrast, only one PrP-immunoreactive band of ~28 kDa was detected in the urine immunoprecipitates, which, following deglycosylation shifted to about 18 kDa (Fig. 1C). These findings strongly suggest that normal human urine contains an isoform of PrP that is truncated at the N-terminus at residues that are at, or more C-terminal than, the 106-110 epitope of 3F4 (Fig. 1A).

On two-dimensional immunoblots, the 28 kDa uPrP was distributed as at least nine spots with
acidic pIs of 3.8-5.3 likely reflecting the major heterogeneity conferred to uPrP by the glycans (Fig. 1D). After deglycosylation only three, less acidic spots of 18 kDa were detected at pIs 5.4-6.0. This remaining heterogeneity might derive from the presence of different GPI anchors (41) and/or the presence in urine of PrP isoforms with slightly ragged ends which would carry different charges.

### Peptide sequencing of uPrP by mass spectrometry

To prove that the PrP-immunoreactive band observed in the human urine indeed was PrP, we subjected purified and deglycosylated uPrP to tryptic digestion and peptide sequencing using highly accurate Fourier-transform mass spectrometry (MS). The peptides detected by tandem MS were matched with the sequence of human PrP C (see Methods). Except for a few small peptides, all other tryptic peptides were identified by MS with high degree of confidence as belonging to the C-terminal region of PrP. This region comprised residues 112-228 with the PrP112-136 sequence being the most N-terminal peptide detected (Table; Fig. 2A). Additional evidence identifying PrP was the observation that peptides 157-185 and 195-204, which carries two predicted sites of N-glycosylation, contained aspartic acid (D) rather than asparagine (N) as residue 181 and 197 of the human PrP sequence (Table 1 and Fig. 2B and C). These results indicate that deglycosylation of uPrP by PNGase F had resulted in the substitution of asparagine with aspartic acid at the two glycosylation sites, as expected (42).

### Characterization of the GPI anchor in uPrP

To investigate the presence and the characteristics of the GPI anchor, we first subjected urinary proteins to phase transition following deglycosylation and treatment with 2% Triton X-114. Following these procedures uPrP was recovered mostly in the aqueous phase rather than in the detergent phase (Fig. 3A) suggesting that the anchor associated with uPrP, if present, is soluble and largely hydrophilic thus likely lacking the phospholipid component. As second step, we digested uPrP with the phospholipase C (PIPL-C) that cleaves off the phospholipid moiety of the GPI anchor. In brain tissue, used here as control, it is well known that the cleavage of the anchor phospholipid moiety paradoxically causes a slower migration of the anchor-cleaved PrP (15,40,43). We reproduced this phenomenon in both full-length PrP and the C1 PrP C-terminal fragment (Fig. 3B). In contrast, this effect was not observed when PI-PLC digestion was performed on uPrP (Fig. 3B). Combined the phase transition and PIPL-C cleavage experiments suggested that the phospholipid component of the anchor is not present in uPrP. To verify whether the glycan core of the anchor was retained in the uPrP, we used hydrofluoric acid (HF) which hydrolyzes the phosphodiester bonds and releases the entire anchor from the PrP (40,43,44). HF treatment of uPrP indeed generated a faster migrating fragment with a reduction of molecular mass of about 3-4 kDa, consistent with the removal of the anchor (Fig. 3C). Again, following HF digestion the C1 PrP fragment from brain preparations showed precisely the same change in migration (Fig. 3C). This set of studies clearly indicates that uPrP lacks the phospholipid component but retains the hydrophilic glycan core of the GPI anchor (Fig. 3D). Primary and secondary structures, and posttranslational modifications of uPrP are shown diagrammatically in Fig. 4.
DISCUSSION

The present study unequivocally demonstrates that PrP is present in normal human urine. Our MS analyses provide the first description of the primary structure of uPrP and show that human uPrP is truncated at N-terminus residue 112. Although other N-terminus forms, truncated at residues 113, 117, 118, 120 or 122, might also exist, the major uPrP backbone matches that of the PrP112-231 fragment called C1 that is generated by a metabolic pathway of PrP. This fragment as the full-length PrP is mostly tethered by a complete GPI anchor to the cell plasma membrane (8,13). In contrast, our analyses of the uPrP C-terminal region and anchor show the presence of an incomplete anchor. Our inability to demonstrate the C-terminal sequence 229-231 by MS is very likely due to the trypsin treatment that cleaved PrP at residues R or K before MS analysis, thereby releasing the most C-terminal three residues attached to the anchor. Similar findings were reported in a study on hamster brain PrP in which trypsin treatment of the 221-231 C-terminal PrP linked to the GPI anchor ESQAYYDGRRS-GPI generated the C-terminal truncated sequence 221-229 ESQAYYDGR ending with R as expected (44). This conclusion is strongly supported by the set of studies we carried out which includes Trion X-114 phase transition, PI-PLC digestion and HF treatment. The results show that uPrP, carries an anchor which, unlike that of C1 fragment, lacks the phospholipid component. Therefore, uPrP likely results from the shedding that has been observed in cell culture and body fluids such as cerebrospinal fluid, serum, sperm and milk (6,16,17,45-48). Two mechanisms of PrPC shedding have been proposed (15). The first postulates that PrPC is shed by proteolytic cleavage, possibly zinc metalloprotease, resulting in a PrP isoform truncated at C-terminus 228 lacking the GPI anchor (15,40,43). According to the second mechanism, that matches our observations in uPrP, shedding would occur following either disruption of the lipid rafts or GPI cleavage by a phospholipase, or both. Either of these two latter processes would generate PrP linked to a soluble anchor lacking the phospholipid component (15,47). It has been reported that in cultured cells, the PrP shed by either of these two mechanisms has the full length N-terminus or is truncated at or around residue 112 as PrP fragment C1 (16). Whether uPrP originates from the shedding of the C1 fragment, as has been shown in BHK cells, or from the shedding of the full length PrP that is N-terminus truncated following the shedding, remains to be determined.

Our findings raise a few considerations related not only to the normal metabolism of PrP in the urinary system but also to the origin of the PrP isoform(s) that carry the prion infectivity demonstrated in the urine of animals affected by prion diseases. First, if uPrP results from shedding, it is important to determine whether it originates from tissues involved in producing and storing urine or it directly derives from PrP shed into the blood. Our finding of a large overrepresentation of C1 over the full-length PrP in the urinary bladder and, to a minor extent, in the kidney (Notari et al., unpublished data) supports the possibility that uPrP derives from one or both these tissues. On the other hand, if uPrP originates in blood, it likely has the same characteristics of blood plasma PrP, which has not been fully characterized (49,50). These considerations raise the question of whether soluble, hydrophilic and shed uPrP are capable of sustaining prion infectivity in urine. This possibility may not be ruled out despite at least three lines of evidence that suggest that C1 is not a good substrate for PrPSc replication. First, the C1 fragment is generated from a PrP N-terminal cleavage that breaks the 100-130 residue domain (the so-called amyloidogenic region of PrP), considered important for the conversion of PrPC into PrPSc (8,51-53). Second, C1 also lacks two of the three regions recently shown to be important for PrP-PrPSc interaction, specifically 23/33, 98/110 and 136/158 (54). Finally, the conversion of C1 to PrPSc has never been reported.

Unquestionably, to elucidate the origin of prion infectivity in urine it is necessary to characterize the infectious PrP present in urine and related tissues. The present characterization of uPrP will likely facilitate future studies towards these goals and help develop a possible pre-mortem diagnostic test of prion diseases.
REFERENCES

FOOTNOTES

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The abbreviations used are: PrP<sup>C</sup>, cellular PrP; PrP<sup>Sc</sup>, scrapie PrP; uPrP, urinary PrP; PrP, prion protein; GPI, glycosylphosphatidylinositol; PNGase F, N-glycosidase F. PI-PLC, phosphatidylinositol-specific phospholipase C; MS, mass spectrometry; Abs, antibodies.

FIGURE LEGENDS

**Figure 1. Immunoprecipitation and immunoblotting of brain and uPrP.** (A) Diagram of the primary and secondary structure, and post-translational modifications of human PrP. Beta sheet (β): 128-131, 161-164, alpha helix (α): 144-154, 173-194, 200-220 are indicated. Sugars at N-181, N-197 residues, disulfide bond C79-C214 and GPI anchor are represented. Antibodies used in immunoblot and immunoprecipitation with relative epitopes are indicated. (B) Immunoblot detection of PrP from brain (B) and urine (U) in crude homogenate and following PrP immunoprecipitation with the antibodies indicated (IP Abs). The Anti-N and Anti-C antibodies were used for detection (IB Abs). (C) Comparative immunoblotting of uPrP and brain PrP before and after deglycosylation with PNGase F. While upon PNGase F treatment brain PrP, which untreated migrates to 27-35 kDa, shifts to two major bands of 27 kDa and 18 kDa, untreated uPrP shows a mobility of 28 kDa that shifts to 18 kDa upon treatment. (D) Two-dimensional gel electrophoresis of uPrP. Upper panel: uPrP not treated with PNGase F; at least 9 spots likely corresponding to PrP glycoforms with acidic isoelectric points (pIs) of 3.8-5.3 are detected. Lower panel: in uPrP treated with PNGase F the spots are reduced to 3-4 with less acidic pIs of 5.4, 5.7, 6.0.

**Figure 2. Mass Spectra of tryptic peptides of uPrP.** (A) Mass spectrum of the N-terminal peptide 112-136. The 112-136 peptide is identified based on the parent ions with isotopic resolution as they are detected in the full scan MS spectrum (insert). The monoisotopic peak of the 112-136 peptide with m/z value of 1137.551 is selected for fragmentation via collision-induced dissociation. This dissociation produces daughter b and y ions which are then detected in the tandem MS/MS spectrum. The b and y ions results from the sequential cleavage of peptide bonds corresponding to N-terminal and C-terminal fragments of the 112-136 peptide, respectively. The detected b and y ions are consistent with the 112-136 peptide sequence shown on the top part of the panel. The b ions are indicated by their positions in the peptide sequence (subscript) while the y ions indicate the charges (superscript). * indicates oxidized methionine. (B) and (C) mass spectra of the peptides 157-185 and 195-204 containing the 181 and 197 N-glycosylation sites, respectively. The peptides are identified as described in (A) based on isotopic parent ions detected in the full scan MS spectra (inserts). The monoisotopic peaks with m/z values of 910.418 (B) and of 570.758 (C) are selected for fragmentation via collision-induced dissociation, producing daughter ions detected in the tandem MS/MS spectrum (see A for more details). The b and y ions are consistent with the peptide sequences shown on the top of panels (B) and (C). * and # indicate oxidized methionine and carboxyaminomethylated cysteine, respectively. Note that residues 181 and 197 are detected as D (aspartic acid) and not N (asparagine), consistent with the PNGase F deglycosylation, carried out before MS analyses, which converts asparagine into aspartic acid by de-amidation. In all the sequences, dots indicate the residues immediately preceding and following the sequence examined.

**Figure 3. Characterization of the GPI anchor of uPrP.** (A) Phase transition. The immunoblots of deglycosylated Triton X-114 (Tx-114)-treated urine fractions show that uPrP is recovered mostly in the aqueous phase (Aq) instead of the detergent phase (Det). The Triton X-114-untreated urine used as control is indicated (lane -). (B) PI-PLC cleavage. In the brain preparation, full-length PrP and, to a lesser extent, the C1 fragment show the paradoxical slower electrophoretic migration effect caused by the cleavage of the GPI phospholipid component by PI-PLC. This effect is not detectable in uPrP. (C) Hydrofluoric acid (HF) treatment of uPrP results in a 3-4 kDa electrophoretic down shift, which matches
that of the PrP C1 fragment PrP from the brain. (D) Diagrammatic representation of GPI anchor. The GPI anchor is represented with its glycan core and lipid component. Hydrolysis sites of phosphodiester bonds by HF and of phospholipid portion by PI-PLC are indicated.

**Figure 4. Linear sequences and locations of the amino acids definitely identified by FT-MS with diagram of the GPI anchor in uPrP.** (A) The sequences of uPrP definitely identified by MS are underlined. Letters in bold indicate possible N-termini. The two D residues generated by PNGase F deglycosylation at N-glycosylation sites 181 and 197 (see text) are shown in bold italic. (B) Diagrammatic representation of the primary and secondary structures of uPrP with post-translational modifications including N-linked glycans (o), disulfide bonds and hydrophilic GPI anchor. The linear sequences definitely identified by MS are aligned on the top. Secondary structures are hypothetical based on the assumption that they are the same as those of PrPc.
Table: uPrP peptides identified by LTQ-FT-MS.

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¹ MH⁺, theoretical mass of the protonated molecule; ² z, number of charges; ³ m/z (ion)exp, experimentally determined mass to charge ratio of the ionized molecule; ⁴ m/z (ion)Th, theoretical mass to charge ratio of ionized molecule; ⁵ Xcorr, cross correlation score, a parameter for the quality of peptide matches in the Sequest software. The higher the score, the better the confidence of peptide; ⁶ Position, location of the peptide in the PrP sequence. Symbols * and # in the peptide sequences indicate, oxidation of methionine and alkylation of cysteine, respectively. Dots identify residues immediately preceding or following the indicated sequence.
Characterization of the prion protein in human urine
Ayuna Dagdanova, Serguei Ilchenko, Silvio Notari, Qiwei Yang, Mark E. Obrenovich, Kristen Hatcher, Peter McAnulty, Lequn Huang, Wenquan Zou, Qingzhong Kong, Pierluigi Gambetti and Shu G. Chen

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