Prion Proteins Without the Glycophosphatidylinositol Anchor: Potential Biomarkers in Neurodegenerative Diseases

Biomarker Insights
Volume 13: 1–5
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DOI: 10.1177/1177271918756648

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ABSTRACT: Prion protein (PrP) is a biomolecule that is involved in neuronal signaling, myelinization, and the development of neurodegenerative diseases. In the cell, PrP is shed by the ADAM10 protease. This process generates PrP molecules that lack glycophosphatidylinositol anchor, and these molecules incorporate into toxic aggregates and neutralize toxic oligomers. Due to this dual role, these molecules are important biomarkers for neurodegenerative diseases. In this review, we present shed PrP as a potential biomarker, with a focus on PrP226*, which may be the main biomarker for predicting neurodegenerative diseases in humans.

KEYWORDS: Prion disease, Alzheimer disease, neurodegenerative disease, shed PrP, PrP226*, ADAM10 protease

RECEIVED: September 14, 2017. **ACCEPTED:** November 23, 2017.

TYPE: Review

FUNDING: The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The authors gratefully acknowledge the financial support of the Slovenian Research Agency.

DECLARATION OF CONFLICTING INTERESTS: The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Introduction

Prion protein (PrP) is a naturally occurring glycoprotein. Prion protein binds to the cell membrane via a glycophosphatidylinositol (GPI) anchor[1] and is abundantly present on the cell surfaces of neurons.[2,3] In humans, PrP has many proposed functions, including the maintenance of myelinization homeostasis,[4] mitochondrial function,[5] and intercellular signaling.[6–8] For unknown reasons, the α-helix-rich 3-dimensional structure of the cellular form of PrP (PrP^C) can transform into a β-sheet-rich molecule, which is the pathological form of PrP (PrP^{Sc}). PrP^{Sc} is unable to perform the same functions as PrP^C and is prone to autocatalytic conversion and aggregation into insoluble aggregates. The accumulation of PrP^{Sc} aggregates over time results in disease.

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are rare fatal neurodegenerative disorders that occur in humans and animals. Prion diseases can be divided into 3 groups—sporadic, genetic, and iatrogenic. Sporadic diseases are the most common type, and their origins are unknown. Genetic prion diseases are the second most common type and are characterized by mutations in the PrP-encoding gene, *PRNP*. Iatrogenic prion diseases develop due to the consumption of PrP^{Sc}-infected tissue, after transplantation, after surgical interventions, and due to the use of infected growth hormone of human origin. All 3 forms have a different clinical picture but show similar symptoms of disease, such as dementia, the loss of brain function, and spongiform deformation of the brain. Prion diseases also have important clinical and neuropathological features that are similar to those of Alzheimer disease.[6] Although prion disease occurs due to accumulation of toxic PrP^{Sc} aggregates in the brain, the mechanism that underlies the conversion of PrP^C to PrP^{Sc} and the development of prion disease remains unknown.

PrP^C is linked to the cell membrane via a GPI anchor. Similar to other GPI-anchored proteins, PrP^C enters the endocytic recycling pathway.[9] As the protein revolves between the cell surface and the endosome, the protein encounters both neutral and acidic pH.[10,11] Many studies have shown that these environmental changes may cause the misfolding of PrP, leading to its transformation into the pathological form and to its accumulation.[9,12–16]

During normal metabolism, PrP^C can undergo 5 posttranslational cleavages. Cleavages α and β occur within the highly conserved hydrophobic region of PrP in the endosomes and result in the release of a 10-kDa N-terminal fragment into the extracellular matrix.[6] Cleavage γ presumably occurs during the transport of PrP through the secretary pathway before glycosylation.[17] The cleavage near the GPI anchor produces a 6- to 7-kDa unglycosylated C-terminal fragment. Prion protein is also the target of shedding. Phospholipase C cleaves PrP within the GPI anchor, which leads to the release of the whole protein into the extracellular medium.[18] Shed PrP is also produced by cleavage of the PrP^C polypeptide chain at the C-terminus by ADAM10 protease.[19] In hamsters, approximately 15% of PrP^{Sc} molecules, isolated from the prion-infected hamster brain, terminate at Gly228.[20] In addition, in a recombinant hamster PrP model, ADAM10 protease cleaves PrP between amino acid residues Gly228 and Arg229.[21] The cleavage site of ADAM10 protease in human PrP^C is not unambiguously defined. A cleavage site profile of ADAM10 was recently explored using peptide libraries and cleavage liquid chromatography-mass spectrometry analysis.[22] The authors have shown that no unique sequence exists that induces cleavage. Therefore, ADAM10 protease can produce more than one variant of shed PrP. In the proximity of

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human   NITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQRGSS 231
hamster NITIKQHTVTTTTKGENFTETDIKIMERVVEQMCTTQYQKESQAYYDGRRS 231
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Figure 1. Part of the human and hamster PrP sequence, indicating the C-terminus and potential ADAM10 protease cleavage sites. The blue arrow indicates the ADAM10 protease cleavage site in the hamster PrP, and the green arrow indicates the potential cleavage site in the human PrP, which produces PrP226*. PrP indicates prion protein.

the cleavage site, the sequence and 3-dimensional structure of human PrP differ from those of hamster PrP. Because the protein sequences are not identical, we can expect a different cleavage pattern for the ADAM10 protease. Consequently, we hypothesize that the proteolytic site in the human sequence is not located between amino acid residues 228 and 229 but is instead shifted toward the N-terminus, namely, between Tyr226 and Gln227 (Figure 1).

In addition to the brain, PrP without the GPI anchor is also found in the urine, cerebrospinal fluid, and blood of patients with different prion diseases and in controls.[23–26] Most analyses concerning PrP have focused on finding PrP^{Sc} and determining infectivity; however, studies on PrP^{C} and its variants in bodily fluids are few. Reports of PrP variants that lack the GPI anchor show that these molecules are involved in neurodegenerative diseases, where the molecules have different roles. These variants may thus be a useful tool for determining the mechanism of neurodegenerative diseases as well as for their diagnosis and therapy.

PrPs That Lack the GPI Anchor

Anchorless PrP

Analysis of aggregates in patients with sporadic Creutzfeldt-Jakob disease (sCJD)[27,28] and Gerstmann-Straussler-Scheinker (GSS) syndrome[29–32] has shown that apart from PrP^{Sc}, aggregates are built from anchorless PrP and other variants of PrP without the GPI anchor. Initially, the conversion from PrP^{C} to PrP^{Sc} was thought to occur only when PrP^{C} was bound to the cell membrane by the GPI anchor, but a study by Chesebro and coworkers showed the opposite.[33] Experiments on transgenic mice expressing only anchorless PrP showed that infection with PrP^{Sc} encouraged the accumulation of PrP in various tissues, such as the heart, kidney, and body fat.[33,34] This accumulation led to the development of prion disease without clinical signs.[33] Later, experiments on these mice confirmed that anchorless PrP could cause prion disease, even though the protein was not covalently bound to the cell surface.[35–39] Anchorless PrP aggregates were found to spread and seed through the interstitial fluid and thus enable the development of cerebral amyloid angiopathy (CAA) in the brain.[38] In addition, the recent study on transgenic mice expressing anchorless PrP showed that PrP without GPI anchor could propagate the infectivity of prion diseases within the same and among different species.[40]

Figure 2. Binding of toxic oligomers to shed PrP and PrP^{C}. (A) The toxic amyloid β (AβO) and PrP (PrP^{Sc}) oligomers are bound by the shed PrP N-terminal region and are neutralized, whereas (B) the binding of toxic oligomers to the N-terminal region of PrP^{C} on the neuronal cell membrane evokes a toxic signaling pathway (thunderbolt). PrP indicates prion protein.

PrP shed by ADAM10

ADAM proteases cleave transmembrane proteins and affect cell interactions with their surroundings, cell migration, and cell signaling.[41,42] Several research groups have shown that ADAM10 protease cleaves PrP^{C} from the cell membrane, producing shed PrP.[18,19,21] The effect of the protease was shown in 2 systems: PrP^{C}-expressing human embryonic kidney (HEK) cells that were transfected with complementary DNA encoding for ADAM10[21] and neuron-specific ADAM10 knockout mice.[43] The experiments in HEK cells showed an increase in the amount of soluble PrP^{C} in the cell supernatant, whereas the experiments in the knockout mice showed an increase in the amount of membrane-linked PrP^{C} and an increase in PrP^{Sc} formation, compared with the wild-type mice. Shedding is presumed to have an antagonistic role in the development of prion diseases. The primary product of shedding, shed PrP, is a molecule that freely floats in bodily fluids, binds PrP^{Sc} aggregates, and prevents their replication. However, when PrP^{Sc} aggregates bind to PrP^{C}, PrP^{C} is refolded into PrP^{Sc} and released from the cell membrane by ADAM10, generating new sources of infection (Figure 2).[44]

Prion protein is also involved in Alzheimer disease. Similar to PrP^{Sc} in prion diseases, toxic amyloid-β peptide oligomers (AβOs) bind to the N-terminus of GPI-anchored PrP^{C}.[45] When the expression of membrane-bound PrP^{C} increases at the neuronal surface, the level of toxic AβO binding to membrane-bound PrP^{C} also increases, causing cell damage. However, in the extracellular space, shed PrP binds to AβO and neutralizes it.[46,47] ADAM10 protease can shed both PrP^{C} and PrP^{Sc}, and therefore, the regulation of this mechanism may affect the development and occurrence of Alzheimer disease and prion diseases (Figure 2).[44]

Truncated PrP due to nonsense mutations

Nonsense mutations in the *PRNP* gene are another source of PrPs that lack GPI anchor. Due to nonsense mutations, a codon that encodes for a specific amino acid changes into a stop codon, which leads to premature termination of the protein. The product is a truncated PrP that lacks the GPI anchor at the time of synthesis. Several cases of prion diseases that were caused by the expression and accumulation of truncated PrP have been described. The nonsense mutations that caused the diseased were Y145X,[48] Q160X,[49,50] Y163X,[51,52] V203X,[53] Y226X,[30] and Q227X.[30] In the cases of Y145X, Y163X, and Y226X, the patients developed PrP-CAA. The PrP-CAA is characterized by amyloid deposits of PrP in cerebral vessels and by Alzheimer disease–like neurofibrillary tangles in the brain, predominantly in the hippocampus.[54] The distribution of PrP^{Sc} aggregates is similar to the distribution of PrP in transgenic mice expressing only anchorless PrP. The deletion of 2 base pairs in codon 178 causes a change in the protein sequence from codon 178 onward and results in a stop codon at codon 203.[53] This truncated PrP is deposited in almost all examined organs, namely, peripheral nerves, smooth muscles, and blood vessels in non-central nervous system tissues. The Q160X mutation leads to an illness that is similar to Alzheimer disease, whereas patients with the Q227X mutation develop a disease that is similar to the GSS syndrome. Interestingly, the insertion of stop mutations in the codon that codes for tyrosine results in PrP-CAA, whereas a premature stop in the codon sequence that codes for glutamine results in a different pathology. This difference may be due to the amino acid that terminates the variant's protein sequence.

*V5B2 and PrP226**

The methods used to determine the presence of PrP^{C} and PrP^{Sc} in biological samples are based on antigen-antibody interactions. To find an antibody that discriminates between Creutzfeldt-Jakob disease (CJD) and non-CJD samples, we prepared monoclonal antibodies against PrP. BALB/c mice were immunized with a KLH-bound peptide fragment of the human PrP sequence (amino acid residues between 214 and 226). Using hybridoma technology, we prepared a panel of monoclonal antibodies against PrP, one of which was V5B2.[55] V5B2 differed from the other anti-PrP monoclonal antibodies because V5B2 recognized only a specific form of PrP. We determined and confirmed the epitope of monoclonal antibody V5B2 using an alanine scan and phage display approach examining the C-terminal region between amino acid residues 214 and 228.[56] We found that monoclonal antibody V5B2 recognized the anchorless truncated form of PrP that ended with amino acid residue Tyr226, and we named the variant PrP226*.

Using monoclonal antibody V5B2, we identified PrP226* in pathological samples and found that PrP226* was a remarkable biomarker for diagnosing prion diseases. Our immunohistochemistry, dot blot, and Western blot analyses of samples from a group of patients with sCJD showed that monoclonal antibody V5B2 specifically discriminated between the CJD and non-CJD brain.[29,55] We postulated that PrP226* incorporated into PrP^{Sc} aggregates in such a manner that the C-terminus was exposed on the aggregate surface or was hidden inside the molecule. The C-terminus of PrP226* represents the epitope of V5B2. To undoubtedly determine the presence of PrP226* in the PrP^{Sc} aggregates using this monoclonal antibody, we denatured the sample before the analysis to release all V5B2 epitopes from the aggregates.[27,29] Our monoclonal antibody also enables a sensitive determination of PrP226* in the brains of TSE patients using dissociation-enhanced lanthanide fluorescence immunoassay[29] and enzyme-linked immunosorbent assay.[27] In addition to PrP-infected samples, we have shown that PrP226* is also present in the healthy brain, albeit in small amounts.[27,29] We are not the only research group to describe this PrP form. As indicated above, PrP226* was concurrently described by Jansen and coworkers.[30] The authors characterized a patient who carried a stop mutation at position Q227X and developed a disease similar to GSS syndrome.

PrP226* causes disease, and minor quantities of PrP226* are also present in the brains of healthy individuals. We suspect that this protein is produced through natural processes, such as shedding or nonsense mutations, and that under yet undefined conditions, PrP226* can act as a propagator of disease. Based on the nature of PrP226*, namely, its presence in human brain and body fluids (unpublished data), lack of GPI anchor, slight truncation at the C-terminus, and neutralization and pathological capacity, we speculate that PrP226* is produced by shedding. To determine whether PrP226* could be the result of cleavage by ADAM10, we analyzed the amount of PrP226* in the human brain. The PrP226* amount, which we determined in the brains of subjects with sCJD, is approximately 12% (unpublished data), which is similar to the amount of truncated PrP determined in the hamster.[20] Based on the properties of PrP226* *in vivo*, we have indirect evidence supporting PrP226* in humans as one of the products, if not the only product, of ADAM10 shedding (Figure 1).

*Structure and conversion properties of PrP226**

Recently, we examined the structural and biochemical properties of PrP226* and resolved the high-resolution nuclear magnetic resonance structure of the protein under acidic conditions (PDB ID 5L6R).[57] The structure of the protein reveals a disordered region between residues 90 and 125 and a structured region between residues 126 and 226. The structured region consists of 2 β-sheets and, compared with wild-type PrP, has 4 α-helices instead of 3 α-helices. As the C-terminus is truncated, new interactions within the structure are needed to stabilize the protein. To do that, the amino acid residues at the C-terminus are driven into the proximity of the amino acid residues of the flexible loop. This causes a minor change in the structure, electrostatic potential and solvent accessibility of the

C-terminus, and flexible loop compared with the wild-type molecule. Similar observations have also been made in structural studies of various pathogenic mutants that cause TSE, namely, Q212P[58] and V210I.[59]

We also determined the thermodynamic properties and *in vitro* conversion propensity of PrP226* at acidic and physiological pH.[60] We found that in both conditions, PrP226* is more thermodynamically destabilized than the wild-type protein. Furthermore, the fibrillization propensity of PrP226* was similar to that of the wild-type PrP in physiological conditions, and the fibril formation onset time at acidic pH for PrP226* was increased compared with that of the wild-type protein. Alongside PrP226*, we analyzed variants that were similar to PrP226*. One variant, PrP225*, had similar thermodynamic properties to those of PrP226*, but the fibrillization propensity was shorter than that of PrP226*. A similar trend to that observed *in vitro* was observed *in vivo*. Jansen and coworkers[30] reported that the time of onset of prion disease, which resulted from expression of only PrP225*, was 27 months, whereas the time of onset of prion disease, which resulted from PrP226* expression alone, was longer, namely, 72 months. In addition to the time of disease onset, the expression of the 2 PrP variants differentially affected the development of prion diseases, resulting in different pathologies. In conclusion, the *in vivo* and *in vitro* data suggest that a change in the protein length, even by 1 amino acid residue, can have a profound effect on *in vitro* and *in vivo* conversion.

Conclusions

Neurotoxicity occurs at the cell surface, where toxic oligomers bind to membrane-anchored PrP^{C}. Binding of PrP^{Sc} oligomers to membrane-bound PrP^{C} transforms the native molecule into PrP^{Sc}, which can be shed from the cell surface by ADAM10 protease, generating a new and infective PrP^{Sc}. However, the shedding of PrP^{C} monomers generates the shed PrP, which can act as a neutralizer of toxic oligomers, which is important in the prevention of neurodegenerative diseases such as Alzheimer disease. ADAM10 protease does not have a unique proteolytic site and can, under specific conditions, generate various shed PrPs, one of which is PrP226*. Due to the dual role of the shed PrP, particularly PrP226*, in the mechanism behind neurodegenerative diseases, these molecules can be potent biomarkers for the diagnosis of neurodegenerative diseases, and thus, these molecules can be powerful tools for the development of therapy.

Author Contributions

VK and VČŠ contributed to the writing of the manuscript, confirmed the manuscript results and conclusions, jointly developed the structure and arguments of the paper, made critical revisions and approved the final version, and have reviewed and approved the final manuscript.

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