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Microdeletions within the hydrophobic core region of cellular prion protein alter its topology and metabolism

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The cellular prion protein (PrP^C) is a GPI-anchored cell-surface protein. A small subset of PrP^C molecules, however, can be integrated into the ER-membrane via a transmembrane domain (TM), which also harbors the most highly conserved regions of PrP^C, termed the hydrophobic core (HC). A mutation in HC is associated with prion disease resulting in an enhanced formation of a transmembrane form (^{Ctm}PrP), which has thus been postulated to be a neurotoxic molecule besides PrP^{Sc}. To elucidate a possible physiological function of the transmembrane domain, we created a set of mutants carrying microdeletions of 2–8 aminoacids within HC between position 114 and 121. Here, we show that these mutations display reduced propensity for transmembrane topology. In addition, the mutants exhibited alterations in the formation of the C1 proteolytic fragment, which is generated by α-cleavage during normal PrP^C metabolism, indicating that HC might function as recognition site for the protease(s) responsible for PrP^C α-cleavage. Interestingly, the mutant G113V, corresponding to a hereditary form of prion disease in humans, displayed increased ^{Ctm}PrP topology and decreased α-cleavage in our *in vitro* assay. In conclusion, HC represents an essential determinant for transmembrane PrP topology, whereas the high evolutionary conservation of this region is rather based upon preservation of PrP^C α-cleavage, thus highlighting the biological importance of this cleavage.

Introduction

Prions are the causative agents of transmissible spongiform encephalopathies (TSEs). These fatal neurodegenerative diseases, including BSE in cattle, Scrapie in sheep and Creutzfeldt–Jakob disease (CJD) in humans, can be of infectious or genetic etiology or arise sporadically. They are characterized by the conversion of PrP^C into a disease-associated isoform (PrP^{Sc}), which shows resistance to proteinase K (PK) [1].

PrP^C is mainly expressed in neurons and is attached to the outer surface of the cell membrane via a C-terminal glycosyl-phosphatidylinositol (GPI) anchor. In addition to the cell-surface anchored version of PrP^C, termed ^{sec}PrP, transmembrane topologies with either the N-terminus (^{Ntm}PrP) or the C-terminus (^{Ctm}PrP) facing the lumen of the endoplasmic reticulum (ER) have been described [2]. These are inserted into the ER-membrane via the TM of PrP^C, a hydrophobic stretch of aminoacids 111–134 encompassing the highly conserved HC (residues 112–119). The mutation A117V is associated with heritable prion disease and displays increased proportion of ^{Ctm}PrP topology. It causes spontaneous neurodegeneration in transgenic mice, but brain tissue of the affected animals lacks infectivity. It has thus been hypothesized that ^{Ctm}PrP represents the neurotoxic species during prion infection [2,3]. The importance of the TM region for disease is reflected by the fact that a peptide encompassing aminoacids 105–125 has widely been

Abbreviations: α1-3, α-helical structure; β1-2, β-sheet structure; aa, aminoacids; Ab, antibody; ADAM, A disintegrin and metalloproteinase; AP, acceptor peptide; APP, amyloid precursor protein; C1, C-terminal fragment of PrP after α-cleavage; Cu, Copper binding sites; C-Ab, antibody directed against the C-terminus of PrP; ^{Ctm}PrP, C-terminal transmembrane topology of PrP; HC, hydrophobic core; GPI, glycosyl-phosphatidylinositol anchor signal sequence; IP, immunoprecipitation; Mem, microsomal membranes; MoPrP, mouse PrP; N1, N-terminal fragment of PrP after α-cleavage; N-Ab, antibody, directed against the N-terminus of PrP; ^{Ntm}PrP, N-terminal transmembrane topology of PrP; Octa-R, octa-repeats; PK, proteinase K; PrP^C, cellular, physiological prion protein; SS, signal sequence; TM, transmembrane (domain); wt, wild-type.

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used as a neurotoxic model peptide [4,5]. Furthermore, various interaction partners such as stress-inducible protein 1 (STI-1) and also PrP^{Sc} bind to TM [6,7]. The latter binding is essential for the conversion of PrP^{C} into PrP^{Sc} [8]. This is exemplified by PrP mutants with deletion of the aminoacids 114–121 or 112–119 [7,9].

During its normal metabolism, mature PrP^{C} can be cleaved at two sites, *i.e.* β-cleavage at around residue 90 or α-cleavage at position 109–111, resulting in small N-terminal (N1 and N2, respectively) and larger C-terminal fragments (C1 and C2) [10]. Such cleavage reactions are reminiscent of cleavage events in the Alzheimer amyloid precursor protein (APP) [11].

Here we constructed a set of microdeletions within TM of mouse PrP (MoPrP) to investigate the role of TM in PrP^{C} topology. Our data reveal that these deletions lead to alterations both in membrane topology and in α-cleavage.

Materials and methods

Unless stated otherwise, all aminoacid numbering refers with the murine PrP sequence. All chemicals were purchased from Sigma–Aldrich, Germany.

Plasmids. Plasmid pUC19::MoPrP-wt [9], comprising the coding sequence for MoPrP, was used to generate a set of eight deletion mutants. For *in vitro* topology assays, the various coding sequences were cloned into pTNT vector (Promega) containing the SP6 promoter. The G113V mutation was created by mutagenesis of pTNT::MoPrP-wt using the Quick change site directed mutagenesis kit (Stratagene) by using the primers G113V-forward (5′ CAACCT CAAGCATGTGGCAGTGGCCGCGGCAGCTGGGGCAGTAG 3′) and G113V-reverse (5′ CTACTGCCCCAGCTGCCGCGGCCACTGCCACATGC TTGAGGTTG 3′).

Transgenic mice. The generation of the F902 line of transgenic mice expressing MoPrP-114Δ8 has been described [12]. In parallel another transgenic line termed M630 was created. Offspring of the two lines were backcrossed four times on a $Prnp^{-/-}$ background by mating with $Prnp^{-/-}$ FVB mice.

In vitro topology assay and determination of α-cleavage in vivo. Transcription and translation of the various PrP mutants was carried out essentially as described [2]. A detailed description is provided under Supplementary Data.

Statistical analysis and similarity calculations. For each PrP-construct, the proportion of any PrP^{C} fragment was calculated by dividing its band intensity, obtained by densitometry, by the sum of the band intensities of all fragments generated. The difference in the proportion between PrP-wt and the deletion mutant was then analyzed by One-way ANOVA in combination with Tukey's multiple comparison test. Two groups were compared by Student's *t*-test. All statistical analyses were performed using Prism GraphPad software. The similarity of HC from aminoacid 114–119 between PrP-wt and the different deletion mutants was assessed by assigning to each aminoacid a value depending on its similarity to the original aminoacid in the wt sequence, using the BLOSUM100 matrix [13]. The higher this value, the more similar is the character of the sequence at this position to PrP-wt. An identical alanine for example, is given the value 8, whereas its substitution to glycine changes this value to −1, and with lysine to −4. The sum of the respective values (= similarity index) is a measure of the similarity of the character of the respective residues 114–119 to the wt sequence. The correlation of transmembrane forms and α-cleavage with the different deletions of the TM was assessed using linear regression.

Results

Characterization of PrP^{C} polypeptides generated in the in vitro topology assay

During cellular trafficking and protein maturation, subpopulations of PrP insert into the membrane of the ER via TM. Using a set of mutants bearing microdeletions within its HC region (Fig. 1), we asked whether these deletions would affect the ability of PrP^{C} to adopt transmembrane topology. We therefore, translated the deletion mutants *in vitro* in the presence of microsomal membranes, followed by PK digestion in the absence of detergents. This leads to digestion of the cytosolic protein parts and thus truncation of the transmembrane forms of PrP^{C}, either at the N-terminus (for ^{Ctm}PrP) or at the C-terminus (for ^{Ntm}PrP), resulting in a distinct fragment size for each topological conformation [2].

First, we confirmed the identity and further characterized the properties of the PrP fragments from topology assays by immunoprecipitation with antibodies either directed against the N- or the C-terminus of PrP^{C} (Supplementary Fig. 1). As expected, an N-terminal antibody precipitated the normal full-length ^{sec}PrP (secretory PrP) as well as the transmembrane fragment ^{Ntm}PrP. Fragments that comprise an intact C-terminal region, such as ^{sec}PrP and ^{Ctm}PrP, on the other hand, were immunoprecipitated with the

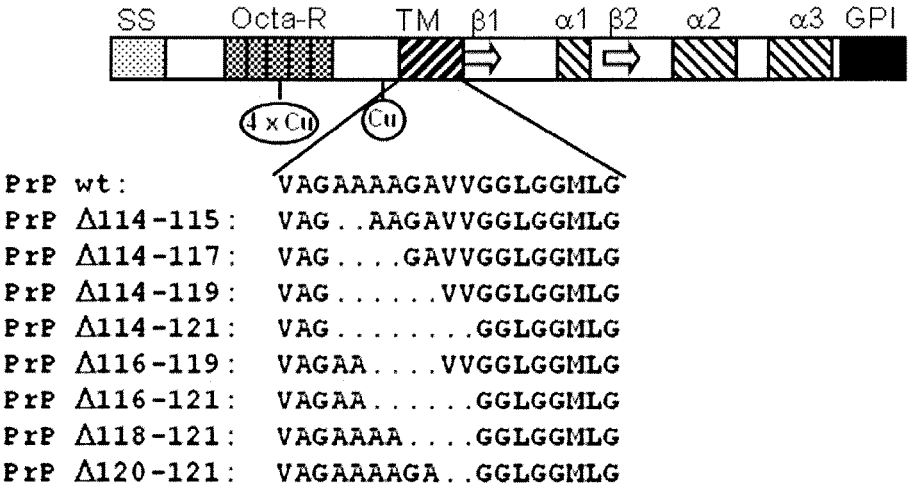


Fig. 1. Mouse PrP deletion mutants. Schematic drawing of mouse PrP^{C}. Underneath the scheme, the aminoacid sequence displays the deletions within HC for each mutant. α1–3: α-helix; β1–2: β-sheet; Cu: Copper binding sites; GPI-anchor signal sequence; Octa-R: octa-repeats; SS: signal sequence; TM: transmembrane domain.

C-terminal antibody 6H4. In addition to these expected fragments, immunoprecipitation with the 6H4 revealed a fragment of 17–18 kDa. This N-terminally truncated fragment already appeared in the absence of PK (Supplementary Fig. 1A, lane 1) and was therefore not generated by PK digestion. Size and truncation rather corresponded to the C1 fragment created by α-cleavage during metabolism of PrP^C [10]. The deletion mutant PrPΔ114–121 displayed almost complete loss of ^{Ctm}PrP and ^{Ntm}PrP topologies as well as of C1 (Supplementary Fig. 1).

The 18 kDa fragment lacks the 3F4 epitope

In the original paper describing the C1 fragment [10], C1 was identified by the lack of immunostaining with antibody 3F4 recognizing residues 109–112 in human and hamster brain as this epitope overlaps with the α-cleavage site. Since 3F4 does not recognize MoPrP, we analyzed hamster PrP-wt in the topology assay, immunoprecipitating the fragments generated after PK digestion either with 3F4 or with a C-terminal antibody 6H4 (Supplementary Fig. 2). Whereas the 18-kDa band was visible as one of the fragments precipitated with 6H4, the antibody 3F4 was unable to precipitate this fragment. This observation further substantiates that the C-terminal 18-kDa fragment is C1 as originally defined by Autilio-Gambetti and colleagues by the loss of the 3F4 epitope after PrP α-cleavage.

Deletions in the hydrophobic core region of PrP^C influence transmembrane topology and α-cleavage

To study the influence of HC on PrP^C membrane topology and α-cleavage in more detail, the deletion mutants (Fig. 1) were analyzed in the topology assay and most showed a significant decrease in transmembrane topology and also marked decrease in α-cleavage as demonstrated by decreased C1 levels (Supplementary Fig. 3; Fig. 2).

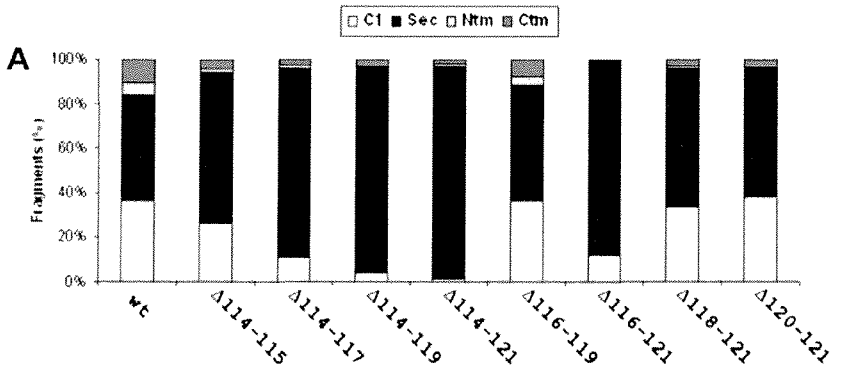
Deletion of aminoacids 114–121 causes the loss of α-cleavage in vivo

The effect of HC on PrP^C α-cleavage is also present *in vivo*. We have created two lines of transgenic mice expressing the deletion mutant PrPΔ114–121 on a $Prnp^{-/-}$ background, one of which (F902) has been published [12]. While an 18-kDa band was clearly detectable in non-transgenic $Prnp^{+/+}$ mouse tissue using antibody 6H4 (Fig. 3A), the intensity of this fragment was dramatically reduced in both transgenic mouse lines expressing PrPΔ114–121.

In order to clearly demonstrate that the 18 kDa band was indeed C1, we re-probed the blot with an antibody directed against the N-terminus of PrP^C. The lower band was no longer present in the PrP-wt samples, confirming the identity of the 18-kDa band as the C1 fragment (Fig. 3C).

In both transgenic lines, the level of PrPΔ114–121 expression was markedly reduced compared to PrP^C in wt-mice [12] (Fig. 3A). To rule out that this low level of mutant protein expression caused the respective C1 band to be below the level of detection threshold, the blot was overexposed, resulting in a faint C1 band (Fig. 3B). Thus, the reduction of the C1-fragment in the transgenic mice is an authentic result and not an artifact due to the limited quantity of PrP loaded on the gel.

The data from the densitometric analysis of the Western blot were consistent with the *in vitro* topology results (Fig. 2). In both experimental setups, PrPΔ114–121 displayed a near-complete loss of α-cleavage. The residual C1 levels *in vivo* were 2.0% in transgenic line M630 and 3.4% in line F902; *in vitro* the residual C1 level was 1.5% (Fig. 3D).



B

	Wt	Δ114-115	Δ114-117	Δ114-119	Δ114-121	Δ116-119	Δ116-121	Δ118-121	Δ120-121
Ctm		***	***	***	***	*	***	***	***
Ntm		**	**	***	***	n.s.	***	***	***
Sec		***	***	***	***	n.s.	***	**	n.s.
C1		**	***	***	***	n.s.	***	n.s.	n.s.

Fig. 2. Quantification of topology assessment of the different PrP^C deletion mutants. (A) Quantification of the ratios of each topological form (black: full-length ^{Sec}PrP; light grey: ^{Ntm}PrP; dark grey: ^{Ctm}PrP) and the C1-fragment (white) for each PrP^C mutant. Signal intensities of the different fragments were quantified by densitometry, $n > 3$ independent experiments. (B) Statistical significance of the differences in fragment abundance for each mutant compared to PrP^C-wt was calculated using one-way ANOVA/Tukey's multiple comparison test (* denotes $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant).

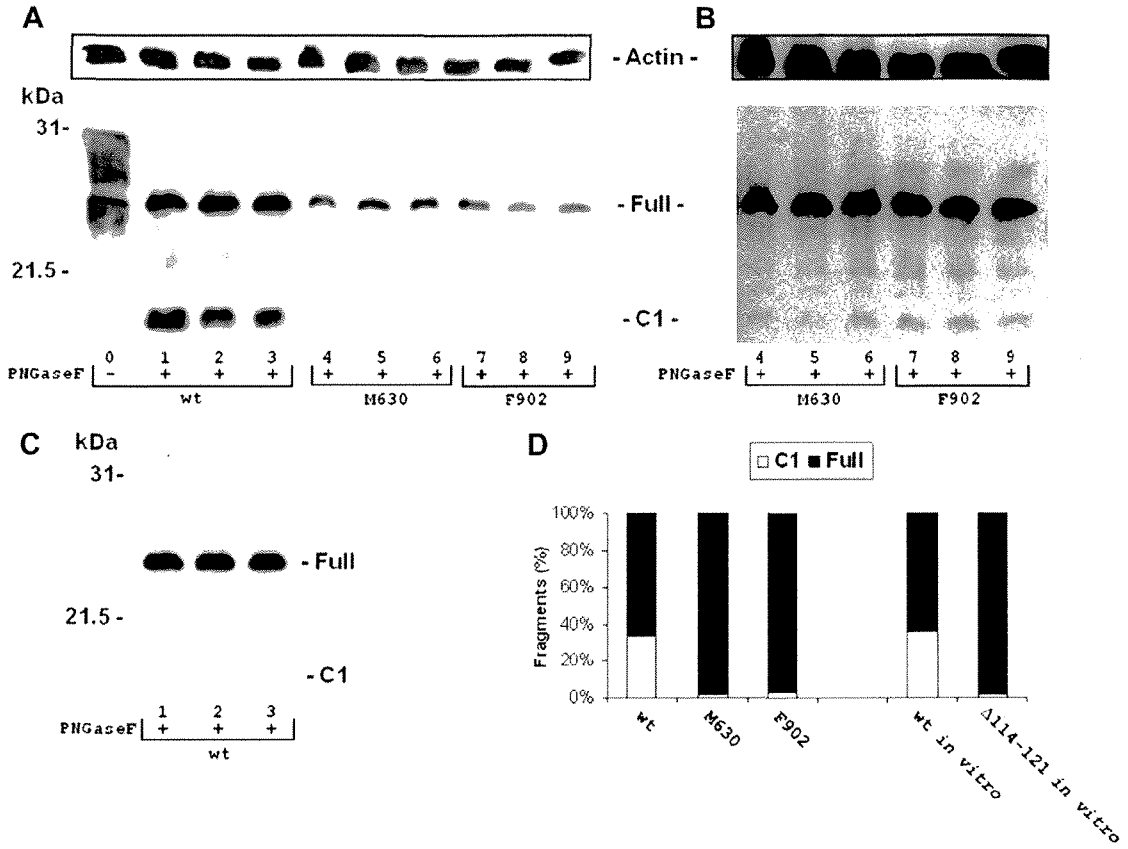


Fig. 3. PrP^C α-cleavage analysis *in vivo*. (A) Western blot analysis of brain homogenates from PrP-wt (lanes 0–3) and transgenic mice expressing PrPΔ114–121 on a *Prnp* $^{-/-}$ background (M630; lanes 4–6) and (F902; lanes 7–9). For each genotype, equal amounts of brain homogenates from 3 mice were either deglycosylated by PNGaseF (lanes 1–9) or left untreated as a control (PrP-wt; lane 0). Actin was used as a loading control. Non-cleaved full-length PrP^C ("Full") and the C1 fragment were visualized by probing the blot with antibody 6H4. A strong C1 band was only present in the PrP-wt samples. (B) Section showing the mutant samples from the blot shown in panel A. In this overexposed blot, very low levels of C1 indeed become visible in brains from mice expressing PrPΔ114–121. (C) Section of the PrP-wt samples from the blot of panel (A). After removal of the C-terminal antibody 6H4, the blot was re-probed with an N-terminal antibody. Only full-length PrP^C was visible. After cleavage, the resulting C1 fragment has lost the epitope for the N-terminally binding antibody. This result further validates the identity of the lower band as C1. (D) Quantification of the PrP^C fragments *in vivo*. Signal intensities in panel (A) were quantified by densitometry. The two bars on the right-hand side indicate the amount of C1 versus non-cleaved fragments (^{sec}PrP plus the transmembrane forms ^{Ntm}PrP and ^{Ctm}PrP) from the *in vitro* topology assay (see also Fig. 2). The *in vitro* and *in vivo* results showed a comparable decrease in α-cleavage for PrPΔ114–121 (*in vivo*: 2.0% in transgenic line M630, 3.4% in line F902; *in vitro*: 1.5 %) compared to 33.7% C1 for PrP-wt *in vivo* and 33.6% *in vitro*.

Alterations in PrP^C α-cleavage correlate with the aminoacid sequence of HC

The extent of loss of PrP^C α-cleavage in the different mutants is not simply a function of deletion size. The four-aminoacid deletion in PrPΔ114–117, for example, reduced the level of C1 to 30%, whereas a different four-aminoacid deletion in PrPΔ116–119 had no effect on α-cleavage. Based on this striking observation we systematically compared sequence similarity between the mutants and PrP-wt between positions 114 and 119 (Fig. 4) and observed that the efficiency of PrP α-cleavage is directly correlated with the similarity of this segment to the wt sequence. The last two aminoacids of the deletion region, *i.e.* residues 120–121, have no influence on cleavage efficiency, demonstrated by unchanged α-cleavage efficiency in PrPΔ120–121. Furthermore, the comparison of segments 114–120 or 114–121 between mutants and wt exhibited a much weaker correlation than the comparison of segment 114–119 (data not shown).

The amount of transmembrane topology and α-cleavage is altered in a pathologic mutation of PrP

Two hereditary forms of human prion diseases are associated with missense mutations in HC, *i.e.* G114V and A117V [14]. It is known that A117V is associated with an increase in ^{Ctm}PrP [2], suggesting that the pathologic impact of mutations within HC could be associated with alterations in the metabolism of PrP^C, such as the upregulation of a toxic transmembrane PrP form, *i.e.* ^{Ctm}PrP. We asked whether G114V (corresponding to G113V in MoPrP) may also impact physiologic PrP^C metabolism. Our *in vitro* topology assay indeed revealed a significant, threefold increase of ^{Ctm}PrP topology from 10.5% to 32.7%, as well as a decrease in α-cleavage by 50% (Supplementary Fig. 4).

Discussion

HC supports the integration but not the orientation of PrP^C into the lipid bilayer

We have created a series of mutants bearing microdeletions within the most highly conserved region of PrP^C, *i.e.* the HC region. We could demonstrate that these mutants are inserted less efficiently into microsomal membranes, thereby leading to a pronounced reduction of the transmembrane PrP^C forms ^{Ctm}PrP and ^{Ntm}PrP. This decrease demonstrates that also the N-terminal part of TM, *i.e.* the hydrophobic core, plays a considerable role in transmembrane topology. This region is necessary for membrane integration of both ^{Ntm}PrP and ^{Ctm}PrP. The pronounced effect of these

A

	Amino acid position								Similarity	TM fragments (%)	C1 fragment (%)
	114	115	116	117	118	119	120	121	114-119		
wt	A	A	A	A	G	A	V	V	49	16 ± 2	37 ± 7
Δ114-115	A	A	G	A	V	V	G	G	13	6 ± 2	26 ± 5
Δ114-117	G	A	V	V	G	G	L	G	11	4.0 ± 2	11 ± 5
Δ114-119	V	V	G	G	L	G	G	Y	-10	4 ± 1	4 ± 1
Δ114-121	G	G	L	G	G	Y	M	L	-3	3 ± 2	2 ± 1
Δ116-119	A	A	V	V	G	G	L	G	20	11 ± 2	36 ± 4
Δ116-121	A	A	G	G	L	G	G	Y	5	1 ± 1	12 ± 5
Δ118-121	A	A	A	A	G	G	L	G	40	4 ± 1	33 ± 5
Δ120-121	A	A	A	A	G	A	G	G	49	4 ± 2	38 ± 5

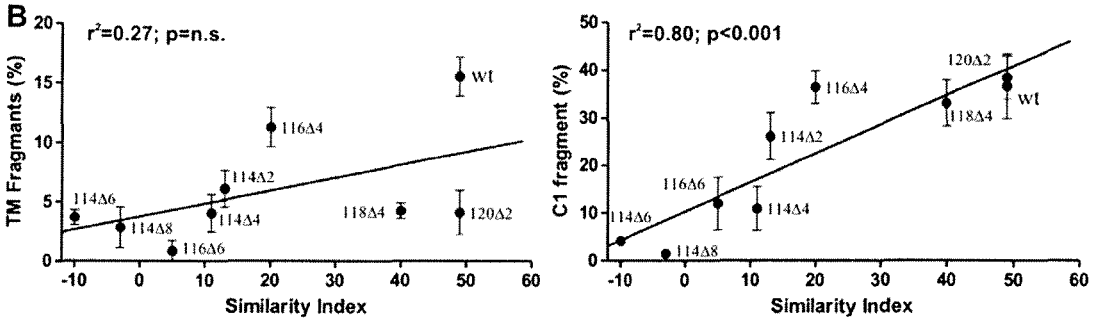


Fig. 4. Impact of the HC aminoacid sequence on PrP^C topology and α-cleavage. (A) For each PrP^C mutant, the aminoacid sequence within the region of the deletion is shown. Aminoacids in boldface are identical with PrP-wt. The similarity of aminoacids 114–119 is shown in the next column. High scores indicate high similarity to PrP-wt. The two columns on the right display the proportion of the transmembrane versions and of the C1 fragment. (B) In contrast to an insignificant correlation of the changes in transmembrane topology (^{Ctm}PrP and ^{Ntm}PrP; left), C1-fragment abundance (right) did correlate with similarity with the HC wt-sequence.

microdeletions, affecting only one specific part of the TM domain, is in accordance with previous results demonstrating that TM is subdivided in distinct topogenic entities, which regulate protein integration and orientation. In a recent study, the middle and C-terminal part of TM has been shown to counteract ^{Ctm}PrP topology [15]. In contrast to the determination of PrP orientation in the ER membrane by the middle TM-segment, also HC plays a considerable role for topology determination. However, this segment does not influence PrP orientation; as we show here, it rather influences the integration of PrP^C into the ER-membrane, as demonstrated by the marked decrease of both transmembrane forms in the various mutants.

Deletions in HC have also an impact on the generation of the C1 fragment

Deletions within HC also exhibited notable changes in the level of an additional fragment migrating at 18-kDa. Immunoprecipitation analysis of the deletion mutants using N- or C-terminal PrP antibodies revealed that this fragment was N-terminally truncated. Both, size and N-terminal truncation were perfectly compatible with the C1 fragment [10]. Furthermore, the topology assay with hamster PrP^C demonstrated that this fragment had lost the 3F4 epitope, which is located exactly at the α-cleavage site. All these results match the characteristics of the C1 fragment as defined by Autilio-Gambetti and colleagues using similar experimental approaches [10]. The analysis of transgenic mice expressing the deletion mutant PrPΔ114–121 in the absence of PrP-wt confirmed the impact of HC on PrP^C α-cleavage.

HC is necessary for PrP^C α-cleavage

The site of α-cleavage of PrP^C had been mapped to residues 109–111. α-Cleavage efficiency of our mutants correlated with the similarity of the HC sequence between residues 114 and 119. This stretch is part of one of the most highly conserved regions of the PrP [16]. Hence, the evolutionary conservation of the hydrophobic core might result from its function as a recognition site for the proteases catalyzing PrP^C α-cleavage, underlining the importance of this cleavage for PrP^C function.

Strikingly, α-cleavage of PrP^C is mediated by the same proteases of the ADAM family that are responsible for the α-cleavage of APP [17]. α-Cleavage of APP prevents formation of the toxic Aβ fragment and furthermore elicits various trophic stimuli. It was thus proposed that α-cleavage of PrP^C might also have cytoprotective effects [11]. Since HC is crucial for the conversion of PrP^C to PrP^{Sc} [9,18,19], PrP^C α-cleavage destroys the region, thereby preventing the conversion process, analogous to α-cleavage in APP. This notion is further supported by the finding that all PrP^{Sc} fragments studied so far comprise the intact C1-cleavage site [20].

Since the *in vitro* topology assay only comprises cell lysate and microsomal membranes, lacking an intact plasmalemma, the generation of C1 fragment under these conditions substantiates the view that α-cleavage of PrP^C already occurs during the trafficking of the protein through intracellular compartments, such as ER or Golgi [21].

Involvement of HC in prion pathology

Although the evidence for a crucial role of PrP in some neurodegenerative diseases such as GSS or CJD is overwhelming, the exact cause for its neurotoxicity remains enigmatic. The transmembrane PrP^C form ^{Ctm}PrP was shown to be upregulated in certain prion diseases, and transgenic mice expressing high levels of ^{Ctm}PrP develop neurodegeneration [3,22]. In addition to the function of certain topologic forms, also the α-cleavage of PrP has been implicated

in specific PrP function, including protection against oxidative stress [23] or against PrP^{Sc} formation [9,11,20].

Interestingly, we discovered both, an increase of ^{Ctm}PrP as well as a decrease in C1-cleavage in a hereditary form of human prion disease, G114V [14], suggesting that both mechanisms, *i.e.* the increase of toxic PrP forms, such as ^{Ctm}PrP, and the decrease of protective PrP α-cleavage might be relevant for PrP function.

Irrespective of the exact physiological role of the PrP^{C} and its associated pathological mechanisms we have been able, by using mutants bearing microdeletions in HC, to further characterized this most highly conserved part of the protein as a crucial regulatory element by showing that it exerts at least two distinct functions, *i.e.* integration (but not orientation) of nascent PrP^{C} polypeptides into the ER membrane and α-cleavage of PrP^{C}.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.02.015.

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