Integrity of Helix 2-Helix 3 Domain of the PrP Protein Is Not Mandatory for Prion Replication*

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Background: Prions are self-propagating β-sheet-rich conformers of PrP protein, however, domains involved in conformational modification remain undetermined.

Results: Peptide insertions in the H2-H3 inter-helix segment or C-terminal part of H2 do not prevent prion replication.

Conclusion: The center of the H2-H3 domain of PrP is not critical for prion conversion.

Significance: These data improve current knowledge on structural features of prions.

The process of prion conversion is not yet well understood at the molecular level. The regions critical for the conformational change of PrP remain mostly debated and the extent of sequence change acceptable for prion conversion is poorly documented. To achieve progress on these issues, we applied a reverse genetic approach using the Rov cell system. This allowed us to test the susceptibility of a number of insertion mutants to conversion into prion in the absence of wild-type PrP molecules. We were able to propagate several prions with 8 to 16 extra amino acids, including a polyglycine stretch and His or FLAG tags, inserted in the middle of the protease-resistant fragment. These results demonstrate the possibility to increase the length of the loop between helices H2 and H3 up to 4-fold, without preventing prion replication. They also indicate that this loop probably remains unstructured in PrPSc. We also showed that bona fide prions can be produced following insertion of octapeptides in the two C-terminal turns of H2. These insertions do not interfere with the overall fold of the H2-H3 domain indicating that the highly conserved sequence of the terminal part of H2 is not critical for the conversion. Altogether these data showed that the amplitude of modifications acceptable for prion conversion in the core of the globular domain of PrP is much greater than one might have assumed. These observations should help to refine structural models of PrPSc and elucidate the conformational changes underlying prions generation.

Prions cause fatal neurodegenerative diseases such as Creutzfeldt-Jakob disease, Gerstmann-Sträussler-Scheinker syndrome, and fatal familial insomnia in humans, scrapie in sheep and goats, bovine spongiform encephalopathy in cattle, and chronic wasting disease in cervids. These disorders are associated with the conformational conversion of a monomeric cellular prion protein, PrP\textsuperscript{C}, to an aggregated, pathological form, PrP\textsuperscript{Sc} (1, 2). Although the structure of PrP\textsuperscript{C} is well characterized (3–5), that of the abnormally folded conformer PrP\textsuperscript{Sc} remains a critical challenge. PrP\textsuperscript{C} is a conserved cell surface, GPI-anchored glycoprotein that comprises an unstructured NH\textsubscript{2}-proximal half followed by a globular part arranged in three β-helices and a short antiparallel β-sheet pleated sheet. A single disulfide bond keeps helices 2 and 3 closely arranged. Although PrP\textsuperscript{C} and PrP\textsuperscript{Sc} appear to have identical primary structures, they differ profoundly in secondary structure and biophysical properties. PrP\textsuperscript{C} is largely helical and sensitive to proteinase K (PK) digestion, whereas PrP\textsuperscript{Sc} is enriched in β-structure, insoluble, and partially resistant to PK (6–11). The conformational dynamic changes underlying PrP\textsuperscript{Sc} formation remain enigmatic, as well as the structural features conferring PK resistance to the infectivity associated, prion protein core, usually starting at residue 82–97 and extending up to the C terminus (12–15).

There is evidential consensus that the N-terminal moity, removed by PK treatment in vitro or lysosomal proteases in cells (16, 17), is not essential for the replication because prions were generated from the N-terminal-deleted PrP (either Δ23–88 or Δ32–93) in infected transgenic mice (18, 19). Descriptive experimental data about which region of the PrP PK-resistant domain carries the information for structural transition are still lacking; yet a number of theoretical models have been proposed. Some models consider either the N- (20, 21) or C-terminal part (22–25) of the PK-resistant domain as the minimum essential region that supports structural modifications, whereas others involve both parts of the PK-resistant domain as being essential for prion conversion (11, 26). It is worth noting that the punctual missense mutations associated with inherited forms of prion diseases are clustered between residues 97 and 148 and between 178 and 238 (27). Reverse genetic approaches to express modified PrP sequences have provided some clues but are constrained by the fact that mini-

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3 The abbreviations used are: PrP\textsuperscript{C}, normal prion protein; PrP, prion protein; PrP\textsuperscript{Sc}, scrapie-associated PrP; PK, proteinase K; PrP\textsuperscript{PK}, PK-resistant PrP\textsuperscript{Sc}; IMAC, immobilized metal affinity chromatography; HSQC, heteronuclear single quantum coherence; PNGase, peptide-N-glycosidase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
Prions with Insertions in the H2–H3 Domain

Mal amino acid substitutions in the PrP protein can hamper prion replication and/or may have a dramatic effect on the susceptibility to infection (28–31). Deletions within the protease-resistant domain, whereas inducing neuropathology (32–35), generally do not allow prion replication, except for PrP106, i.e. PrP with two deletions (Δ23–88, Δ141–176), the so called miniprion (18). However, subsequent studies led these authors to propose the participation of the 141–176 region in the structural changes (20). Recently Myc or tetracysteine tags were introduced by insertion or sequence modification at one each end of the PK-resistant domain (36–38), and shown to be compatible with prion conversion. However, introduction of such tags farther within this domain had so far prevented prion replication (36, 38). Here we report the conversion into prion of several PrP mutants with an insertion of 8–16 amino acids in the middle of the H2–H3 region, hence demonstrating an unexpected tolerance to sequence modification in a central part of the PK-resistant core.

EXPERIMENTAL PROCEDURES

Ethic Statement—All animal experiments have been performed in accordance with the European Community Council directive 86/609/EEC.

Generation of PrP Insertion Mutants—Insertions and substitutions were introduced by site-directed mutagenesis (QuickChange II mutagenesis kit, Stratagene) into sheep PrP (allotype Val136–Arg154–Gln171) cloned into pTRE plasmid (Clontech) using mutagenic primers (supplemental Table S1). The plasmid encoding PrP-ins203, i.e. with an in-frame insertion of 24 bp, was isolated during screening of an experiment originally intended to modify the second site of glycosylation (31). The sequences of all the mutant constructs were verified by sequencing. Numbers indicated for each insertion refer to the position of the first amino acid of the insert according to the sheep PrP numbering.

Cell Culture and Isolation of Rov Cells—Rov cells are epithelial RK13 cells that stably express either wild-type (WT) or mutant ovine PrP using a tetracycline-inducible system (31, 39). They were obtained by transfection and puromycin selection and were grown in Opti-MEM medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) and antibiotics, and split at 1:4 after trypsinization once a week. To assess PrPC expression, cells were cultivated in the continuous presence of 1 mg/ml of doxycycline (Sigma).

Antibodies—Several mouse anti-PrP monoclonal antibodies (mAb) were used. The 4F2 mAb (40) is an IgG2b directed to the octarepeat domain (54–92, sheep PrP numbering) and was used to detect PrPSC; Sha31 mAb (epitope 148–159) (40) was used to detect denatured PrP present in 50 μg/ml of PK per 1 mg of protein, for 2 hours at 37 °C, then centrifuged for 30 min at 22,000 × g. Pellets were dissolved in Laemml sample buffer and boiled for 5 min at 100 °C. When needed, 500 units of PNGase F (New England Biolabs, MA) and 1% Nonidet P-40 were added to denatured proteins that were further incubated at 37 °C (17).

Transgenic Mice, in Vivo Infections, and Tissue Homogenate Preparation—To test for the infectivity of PrPSc-ins203, 5 × 106 Rov-ins203 or RK13 cells were harvested by scraping at passage 8 post-infection. Cell pellets were resuspended in 100 μl of medium containing 5% glucose and antibiotics, then frozen and thawed three times before sonication for 1 min. A 20-μl aliquot of these cellular lysates was injected intracranially to tg338 mice overexpressing the VRQ allele of ovine PrP (44). Brains and spleens of infected animals were harvested at the terminal stage of the disease and analyzed for PrPSc content and distribution by immunoblots and histoblots, respectively, as described previously (45).

Immunoblotting Detection of PrPC and PrPSc—Either 12 or 4–12 NuPage BisTris polyacrylamide gels (Invitrogen) were used for SDS-PAGE. For PrPSc analysis, 50 μg of protein were loaded on the gel. For PrPSc, samples corresponding to the PK-resistant PrP present in 50 μg of protein of the cellular lysate were loaded on the gel, except for PrPSc-ins(Gly) and PrPSc-ins(FLAG), which an equivalent of 250 μg of protein were loaded as indicated in the figure legends (Figs. 4D and 5D). The transfer of proteins, their detection and revelation were described previously (17).

Conformational Stability Assay—Cellular lysates were brought to the desired concentration of guanidine HCl, incubated for 1 h at 20 °C under agitation, then diluted to reach the same volume and guanidine HCl concentration (800 μl, 0.5 M). 200 μl of 5 × digestion buffer (750 mm Tris-HCl, pH 7.4, 10% Sarkosyl and 20 μg/ml of PK) was added and samples were incubated for 1 h at 37 °C under agitation, before methanol precipitation and analysis by immunoblotting.

Cu2+–Imobilized Metal Affinity Chromatography (IMAC) Hitrap Chromatography—The AKTA Purifier100 FPLC chromatographic system was used (GE Healthcare) as previously described (17, 46). One milliliter of cell lysates (1 mg of protein) made in TNT buffer (50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Nonidet P-40, 100 μg/ml of aprotinin, and 100 μg/ml of leupeptin) was loaded onto a Hitrap Cu2+ column (GE Healthcare). Fractions of 2 ml were collected and the column was equilibrated with 50 mM Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 0.5% Triton X-100, and whole cell lysates were prepared at 4 °C in TL1 buffer (50 mm Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 0.5% Triton X-100). Lysates were clarified by centrifugation for 2 min at 800 × g and protein concentrations were determined by microBCA assay (Pierce). For PrPSc, lysates were incubated with 4 μg of PK per 1 mg of protein, for 2 h at 37 °C, then centrifuged for 30 min at 22,000 × g. Pellets were dissolved in Laemml sample buffer and boiled for 5 min at 100 °C. When needed, 500 units of PNGase F (New England Biolabs, MA) and 1% Nonidet P-40 were added to denatured proteins that were further incubated at 37 °C (17).

Prion Infection of Cell Cultures—Rov cells were infected with the cloned 127S scrapie strain as previously described (31) using 1% (w/v) brain pool homogenates of terminally ill tg338 mice (44). Two days postexposure cells were washed, incubated for 2 additional days, trypsinized, and split at 1:10 dilution. Cells were further incubated for 1 week before analysis (passage 1) and split at 1:4 dilution at each following passage. To test the infectivity of cultures propagating mutant PrPSc cells were harvested at passage 6–8 postexposure, pelleted, frozen and thawed, then sonicated, and used as inoculums.

Cell Lysis, Proteinase K Digestion, and PNGase Treatment—Cells were washed twice with cold phosphate-buffered saline and whole cell lysates were prepared at 4 °C in TL1 buffer (50 mm Tris-HCl, pH 7.4, 0.5% sodium deoxycholate, 0.5% Triton X-100). Lysates were clarified by centrifugation for 2 min at 800 × g and protein concentrations were determined by microBCA assay (Pierce). For PrPSc, lysates were incubated with 4 μg of PK per 1 mg of protein, for 2 h at 37 °C, then centrifuged for 30 min at 22,000 × g. Pellets were dissolved in Laemml sample buffer and boiled for 5 min at 100 °C. When needed, 500 units of PNGase F (New England Biolabs, MA) and 1% Nonidet P-40 were added to denatured proteins that were further incubated at 37 °C (17).

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and 1% Triton X-100) supplemented with protease inhibitors (Roche Applied Science) was injected into the column. Columns were washed and a linear gradient of 3–200 mM imidazole in TNT buffer was applied to elute column-bound proteins at a flow rate of 1 ml/min for 10 min; 0.5-ml fractions were collected. To solubilize aggregates of PrPSc-ins(His6), all buffers were complemented with 4M urea. Eluted fractions were either treated with 5 g/ml of PK and precipitated with cold methanol after addition of 20 g of BSA as a protein carrier or methanol precipitated without PK treatment for total PrP. Individual columns were dedicated to each type of lysate.

**Immunofluorescence**—Cells were fixed with 4% paraformaldehyde and PrPC was detected as previously described (31, 47). For PrPSc immunodetection, fixed and permeabilized cells were treated for 5 min with 3.5M guanidium thiocyanate and washed three times before exposure to ICSM33 mAb as previously described (42, 47), or to both ICSM33 and M2 anti-FLAG mAbs and then to fluorescent anti-IgG2b and anti-IgG1. Images were acquired with an Axio observer Z1 microscope (Zeiss) and a coolsnap HQ2 camera (Photometrics) driven by the Axiovision imaging system software.

**NMR Measurements**—Recombinant proteins (WT PrP, PrP-ins191, and PrP-ins193) were produced and purified from *Escherichia coli* as published previously (25). HSQC experiments were run at 25 °C on 0.5–0.7 mM 15N-labeled samples of wild-type PrP and mutants using a VARIAN INOVA 600 MHz. The samples were in 5 mM MOPS buffer, pH 7.0. Water suppression was achieved with a standard Watergate pulse sequence.

**Circular Dichroism**—CD measurements were carried out on a Jasco-715 spectropolarimeter equipped with a thermostated cell holder controlled by a Jasco Peltier element. Far-UV CD spectra were recorded from 260 to 195 nm at 25 °C in a 1-mm path-length quartz cuvette at a protein concentration of 12 μM in 5 mM MOPS buffer, pH 7.0. Each CD spectrum was obtained by averaging 15 scans collected at a scan rate of 200 nm/min. Baseline spectra obtained with buffer were subtracted for all spectra. The thermal denaturation scans were obtained by heating a 12 μM sample from 20 to 97 °C at 2 °C/min and by monitoring the ellipticity at 220 nm with a 4-s averaging time and a 0.2 °C data pitch. The thermogram was analyzed assuming a two-state model.

**RESULTS**

**PrPSc with Extended H2-H3 Loop Can Convert into Prion**—In a previous study aimed at producing PrP glycosylation mutants (31), we identified a mutant that contained an insertion of 8 amino acids downstream from the second glycosylation site, within the unstructured loop joining helices H2 and H3 of sheep PrP (Fig. 1, A and B). This mutant, denoted PrP-ins203, arose fortuitously probably due to a misalignment of the mutagenic primers used. To examine the effects of this mutation on PrP glycosylation and permissiveness to prion infection, PrP-ins203 was stably expressed in RK13 cells. Immunoblotting analysis revealed the presence of mono- and diglycosylated PrP-ins203 compared with WT PrP on a resolute gel (left, 4F2 mAb). Analysis of a similar blot with the V14 mAb reveals only the WT PrP (right).

![Figure 1](https://example.com/figure1.png)

**FIGURE 1. Characteristics of a PrP with 8 amino acids inserted in front of helix-3. A, the sequences of mammalian PrP included between the 2 cysteines are highly conserved. Identical amino acids are boxed and those corresponding to the H2-H3 loop are underlined. B, nucleotide (top line) and primary sequences of PrP-ins203. In this and subsequent figures, the insert is in red and helices H2 and H3 are highlighted in green and blue, respectively. The asparagine of the second site of glycosylation is marked. C, glycoforms profiles of WT PrP and PrP-ins203 as shown by immunoblotting (mAb 4F2). Molecular masses markers (in kDa) are indicated. D, ratios of the unglycosylated form as quantified from four immunoblots. E, PNGase treatment of samples showing the lower mobility of deglycosylated PrP-ins203 compared with WT PrP on a resolute gel (left, 4F2 mAb). Analysis of a similar blot with the V14 mAb reveals only the WT PrP (right). F, immunofluorescence with anti-PrP antibody shows a similar cell surface expression of the WT and mutant protein (4F2 mAb).**
Recognizes a conformational epitope (amino acids 191–199) encompassing a region located nearby upstream of the site of insertion of the octapeptide ins-203 (5, 41), was unable to recognize PrP-ins203 (Fig. 1E and supplemental Fig. S1). This suggested that the presence of additional amino acids just downstream of the V14 epitope somehow modifies the local conformation of the PrP. Yet the insertion mutant was correctly routed to the cell surface, as ascertained by PrP immunofluorescence labeling of intact cells (Fig. 1F) and presence in lipid rafts (supplemental Fig. S2).

Cells expressing either WT or mutant PrP were infected with the 127S strain of ovine prion and analyzed for their PrPres content at subsequent passages of cultures. The immunoblot in Fig. 2 shows that PrP-ins203 was converted into a self-propagating, protease-resistant species with amounts of PrPres comparable with those in cells expressing WT PrP (Fig. 2). After PNGase treatment, the PrPres fragment of the insertion mutant was shown slightly bigger in size than WT PrP, thus indicating that the inserted amino acid stretch was protected from protease digestion in PrPSc. To further characterize the PrP-ins203 mutant we subjected it to a conformational stability assay using guanidine hydrochloride as a denaturing agent (48). The insertion did not appreciably modify the resistance to denaturation (Fig. 3A), because half-denaturation of the mutant and WT PrPSc occurred at 1.4 ± 0.2 and 1.3 ± 0.4 M, respectively.

PrP-ins203 conferred cells similar susceptibility to prion infection as WT PrP in cell cultures exposed to serial dilutions of 127S-infected mouse brain homogenate. Both Rov-wt and Rov-ins203 produced large amounts of PrPres after exposure to inoculum at the 10⁻³ dilution, whereas no PrPres was detected beyond the 10⁻⁴ dilution (Fig. 3B). To confirm that PrP-ins203 led to the generation of infectious prion, the cell homogenate from 8 passages on Rov-ins203 cultures was inoculated to naive Rov-ins203 or Rov-wt cells, and to tg338 mice. Both homotypic and allotopic cell cultures were successfully infected based on PrPres accumulation (Fig. 3C). Mice inoculated with the PrP-ins203-derived homogenate died at 61 ± 2 days with typical TSE signs (Fig. 3D) and showed the presence of PrPins203 with a glycoform pattern and regional deposition in the brain typical of the 127S strain (supplemental Fig. S3). Altogether these results demonstrate that a mutant PrP with an 8-amino acid insertion in the PK-resistant core is efficiently convertible into bona fide prion.

**Insertion of Glycine Residues, or Extension of the H2-H3 Loop Up to 16 Amino Acids Do Not Prevent Prion Replication**—We modified the original insertion already containing 3 glycine residues to create a segment containing 6 glycines (Fig. 4A). Because a feature of the polyglycine stretch is to be incompatible with the formation of secondary, either α-helix or β-sheet structures, it was assumed that the insert ins203(Gly6) would neither participate to elongation of H3 nor convert into a β-sheet segment if PrPSc were to be produced. When expressed in cells, PrP-ins203(Gly6) showed a glycoform pattern with a low level of the unglycosylated form as for the original PrPins203, and some decrease in the proportion of the diglycosylated form (Fig. 4B). PrP-ins203(Gly6) was efficiently routed to the cell surface and found to be associated with lipid raft fractions (supplemental Fig. S2) and was converted into PK-resistant species upon infection with 127S prion (Fig. 4, C and D). PrPSc-ins203(Gly6) was readily propagated along with five sub-passages, although the amount of PrPSc was reduced by at least...
10-fold compared with that in WT PrP Rov cells (Fig. 4D). Thus, extension of the loop between H2 and H3 by a polyglycine stretch remained compatible with PrPSc formation, suggesting that this part of the protein is unstructured in misfolded conformers.

We also introduced an 8-amino acid FLAG sequence into the original ins203 octapeptide, enabling us to recognize the presence of the insert using a relevant antibody (Fig. 5A). As for other ins203 insertions, the unglycosylated forms of PrP-ins(FLAG) were produced in low amounts in RK13 cells (Fig. 5B), and FLAG-PrP was efficiently expressed at the cell surface (Fig. 5C). Rov-ins203(FLAG) were susceptible to prion infection and generated self-propagating PrPSc, however, the PrPres levels were markedly reduced compared with WT PrP (Fig. 5D). Cells in which PrPSc had accumulated efficiently exhibited numerous aggregates labeled, after guanidium thiocyanate denaturation, by anti-PrP and anti-FLAG antibodies (Fig. 5E). We further showed that PrPSc-ins(FLAG) autopropagated in cell culture was also infectious for Rov-wt cells (Fig. 5F). Altogether these results established that a PrPC with a 4-fold increase, i.e. from 5 to 21 amino acids, of the size of the sequence between H2 and H3 can be converted into bona fide prions.

Production of His-tagged PrPSc—As a means to facilitate PrP purification, the first 6 amino acids of ins203 were substituted by histidine residues and the resulting PrP-ins203(His6) was expressed in RK13 cells (Fig. 6A). The functionality of the tag was tested by loading cellular lysates onto Cu2⁺-IMAC columns. Because PrP already contains an N-terminal octarepeat sequence, a strong binding site for divalent ions, both WT and the His-tag PrPSc were retained on such columns (Fig. 6B). However, PrP-ins(His6) was eluted at a much higher concentration of the Cu²⁺ competitor imidazole (150 mM) than WT PrP (90 mM), indicating that the His tag actually increased the avidity for immobilized copper ions of PrP (Fig. 6C), including diglycosylated molecules where complex N-glycan chains are attached close to the tag. Interestingly, the fractions in which His tag PrP was eluted contained at least 10-fold less contaminant proteins than the fractions containing WT PrP (not shown).

![FIGURE 4. PrP-ins203(Gly6) is convertible into prion. A, amino acid sequence of the mutated region of PrP-ins203(Gly6). B, immunoblot showing that the unglycosylated species are markedly under-represented for the insertion mutant compared with WT PrP. (4F2 mAb). C, immunofluorescence with anti-PrP antibodies showing that the insertion mutant is properly routed to the cell surface (4F2mAb). D, Western blot analysis of PK-digested samples. The gel was loaded with 250 or 50 µg of PK-digested protein for the mutant PrP and WT PrP, respectively. PrP containing the polyglycine stretch was successfully converted into a self-propagating PK-resistant protein but levels of PrPres accumulated in cells were lower than for the WT PrP (Sha31 mAb).](image-url)

![FIGURE 5. PrP-ins203(FLAG) with a H2-H3 loop extended by 16 additional amino acids is convertible into a true prion. A, primary sequence of theFLAG-tagged mutant PrP. The FLAG sequence (blue) was inserted in the middle of the original ins203 sequence (red). B, analysis of Rov203(FLAG) expression by immunoblotting (4F2 mAb). C, cell surface expression of PrP-ins203(FLAG) on nonpermeabilized cells. D, PrPSc detection by immunoblot (left panel, Sha31 mAb) for infected and mock-infected (m) cells. Presence of the FLAG was verified by a specific antibody (right panel). E, detection of PrPSc by immunofluorescence. Prion-infected Rov-ins203(FLAG) cells were immunolabeled with anti-PrP and anti-FLAG antibodies. Merge images with nuclei labeled by DAPI (blue) showed colocalization of PrPSc and FLAG signals in infected cells (yellow). F, de novo infection of WT Rov cells challenged with homogenate from Rov-ins203(FLAG) cells propagating a PrPSc for five passages. The third passage post-infection is shown (left panel).](image-url)
Prions with Insertions in the H2-H3 Domain

To determine whether the H2-H3 segment was accessible to interaction into PrPSc molecules as well, Rov-ins(His6) cells were subjected to prion infection. Infected cells produced PrPres from the first passage on, the amount of which increased gradually on subpassaging to reach a level comparable with that produced by Rov-wt after 7–8 passages. His-tag PrPSc was able to infect cultures expressing homotypic as well as WT PrP sequence (Fig. 6, C and D). Anti-His antibodies recognized the PK-resistant fragment (Fig. 6C, right panel). The half-denaturation value of PrPSc-ins(His6) in conformational stability assay was 1.3 ± 0.25 M, similar to WT and ins203 PrPres (see above).

We also asked whether a His tag inserted in the PK-resistant core would still bind to copper-immobilized ions. Although we did show binding of the His tag PrPSc to IMAC Cu2+ columns (Fig. 7), the bulk of PrPSc did not bind and was either excluded in the flow-through (Fig. 7, top panel) or retained on the column even after application of imidazole at high concentrations (not shown). To improve the purification of His-tagged PrPSc, cellular lysates were incubated with 4 M urea prior to application on the columns. Proteins recovered by elution with increasing imidazole concentrations were PK-digested and analyzed by immunoblotting. WT PrPSc, present in Rov cellular lysates essentially as N-terminal truncated species (17), bound to Cu2+ columns with low avidity and started to elute at 40 mM imidazole (Fig. 7, lower panel). His-tagged PrPSc was eluted at higher concentrations of imidazole, starting at 88 mM (Fig. 7, middle panel), indicating that the tag could be functional for the PrPSc species under adequate conditions of solubility.

Integrity of C-terminal Part of Helix-2 Is Not Essential for Prion Conversion—To determine how variable the exact location of the 8-amino acid insertion could be, we moved it upstream and downstream of the original position (Fig. 8A). The insert was introduced at the other end of the inter helix loop (position 198), or in helix H2 (195 to 187) or helix 3 (207 and 210). These mutants were expressed and efficiently reached the cell surface (supplemental Fig. S4A). PrP-ins210, which was not detectably expressed at the membrane but showed intracellular accumulation, essentially at a juxtanuclear position (supplemental Fig. S4B). PrP-ins198 did not confer Rov cells susceptibility to prion infection, indicating that even in the unstructured segment considered, the site of insertion might be critical. Cells expressing H3 mutants, Rov-ins207 and Rov-ins210, were not permissive to the infection, yet the convertibility of PrP-ins210 could not be adequately tested due to impaired trafficking.

In contrast, PrP with insertion into H2, 3 amino acids before the end of the helix (position 195) and then further upstream with 1 amino acid increment up to 193 conferred permissiveness to the cells and led to reproducible and robust infection as attested by the formation of self-propagating PrPSc (Fig. 8B). Demonstration of the infectivity of each of the three mutant PrPSc was ascertained by using cellular homogenates to challenge naive Rov-wt and homotypic cells (supplemental Fig. S5). Insertion further upstream markedly affected (PrP-ins192) or even hampered (PrP-ins191 and PrP-ins187) prion replication (Fig. 8B). These results showed that insertion of extra amino
acids in the part of the protein corresponding to last two turns of H2 (Fig. 9) did not prevent prion formation, whereas introduction of the same insertion further upstream in the helix impaired conversion.

**Insertions in H2 C Terminus Do Not Interfere with PrP-fold**

To understand whether the inserted sequences affect the fold of PrP, we recorded NMR $^{15}$N HSQC experiments, which are sensitive to protein tertiary fold. As insertions in H2 could be more destabilizing for mutants than insertion in the loop, we specifically analyzed PrP-ins193 and PrP-ins191. The former is competent for prion conversion but not the latter, although they are both expressed at the cell surface and found associated with...
In this study, we expressed a number of sheep PrP mutants displaying a peptide insertion at various places and we show that nine mutants with exogenous sequences introduced in the central part of the H2-H3 region remain convertible into prions, thus questioning how critical is the integrity of this region for the conversion into PrPSc. This issue is important particularly in view of several recent reports proposing the crucial involvement of the H2-H3 region in misfolding events leading to prion formation (11, 22, 24, 26, 49, 50).

PrP Proteins with Extended H2-H3 Interhelix Segment Remain Convertible into Prions—Four PrP mutants were built with insertions at position 203 that increased about 2–4-fold the size of the loop joining H2 and H3. The resulting proteins were glycosylated and properly routed to the cell surface, whereas sharing a notable reduction of unglycosylated PrP species. The latter feature might result from an increased glycosylation efficiency (51) or a reduced stability of this form, or both, as observed for the F198S mutation in human (52–54) and for its equivalent in the sheep sequence, F201S (31), both of which are close to the insertion site. Upon prion infection ins203 mutants produced self-propagating PrPSc albeit at different levels. The original PrP-ins203 generated as much PrPSc as WT by PrP, whereas cells expressing the His-tagged PrP had to undergo several passages before reaching similar levels, suggesting an adaptation of the prion strain to this particular substrate or selection of most performing components (55). The other two PrP mutants produced lower quantities of PrPSc, which may reflect reduced replication efficiency, or a lower stability of the misfolded protein in the cells. Nevertheless, these insertion mutants of PrPSc were de novo infectious for naïve cells or for transgenic mice expressing the sheep PrP. Altogether these results proved that bona fide prions could be generated despite a substantial increase in size of the segment separating H2 and H3, the heart of structured PrPSc. The magnitude of acceptable changes in this region was impressive because not only was the size of the H2-H3 connecting segment increased, but the physicochemical characteristics of the loop were also drastically altered. In PrP-ins(His6) and PrP-ins-(FLAG), in particular, the calculated isoelectric point and charge at pH 7 of the loop segment were markedly modified.

PrPSc and PrPSc Conformations of Insertion Mutants in Position 203—The loss of the conformational V14 epitope in PrP-ins203 might suggest some local dynamic change of the conformation of the region immediately upstream of the insert (56), as the epitope was reported to cover the domain between the two glycosylation sites, from the middle of H2 to the beginning of the H2-H3 loop (5, 41). However, another possibility is that the size of the segment encompassing the epitope would have been previously underestimated. PrPSc-ins203 had a similar resistance to guanidium denaturation as WT PrPSc, suggesting no major structural variation between the two misfolded proteins, despite dramatic under-representation of aglycosylated species in PrPSc-ins203. Moreover, the strain features in tg338 mice of PrPSc-ins203 were typical of the 127S strain propagated on WT PrP cultures. Importantly, the introduction of six consecutive glycines in the insert did not preclude prion conversion. This...
finding strongly suggests that the corresponding segment was not changed into the β-sheet in PrPSc, and comes in support of the structurally based prediction that the loop between H2 and H3 remains unstructured in PrPSc (20, 22).

**Prions Can Be Tagged inside the PK-resistant Core**—The insertion of tags might be useful to study the biology of prion infection. Previous observations tended to indicate that the ends of the PK-resistant fragment could be modified, whereas the inside part could not (36–38). In fact, as shown here, tags can be introduced within the protease-resistant segment without blocking prion replication. Despite their location between two heavy N-glycan chains, the His tag and FLAG tag were easily accessible in PrPSc and proved to be useful for purification and immunodetection experiments. Yet, purification or detection of His-tagged PrPSc could not be achieved without partial solubilization of aggregates by urea. Also, PrPSc-ins(FLAG) was not recognized by anti-FLAG antibodies without prior guanidine treatment of the cells. Both results are consistent with previous observations on other tagged PrPSc and the inaccessibility of all these tags to proteolytic enzymes in misfolded PrP (36–38).

**Integrity of the Last Two Turns of H2 Is Not Mandatory for Prion Replication**—Insertion of the octapeptide (AQQGGGFT) either at the beginning of the H2-H3 loop (position 198) and in the N-terminal part of H3 (position 207) impaired the formation of prion. Nevertheless, the integrity of the extremity of H3 or of the first residue of the H2-H3 loop is unlikely to be essential for prion conversion because insertion of another sequence at close proximity appeared to be compatible with PrPSc formation (57). In contrast, introduction of the octapeptide in the C-terminal part of H2 allowed the generation of four PrP mutants that all remained prion-compatible as attested by self-propagation of PrPSc and de novo infectivity of the mutant PrPSc. Of note, the levels of newly formed PrPSc decreased while moving the insertion from position 195 to 192, with no conversion occurring beyond this position. As the thread of helix comprises a little more than 3 amino acids, it means that insertion in the last two C-terminal turns of H2 allowed the formation of bona fide prions.

Furthermore, a double mutant with an insert in the last turn of H2 and another in the H2-H3 loop was competent for prion
conversion and propagation, showing that the simultaneous modification of both regions was tolerated. Like the PrP-ins(FLAG) mutant mentioned above, PrP-ins(195 + 203) harbors 16 additional amino acids, strengthening the view that neither the size of the middle part of the H2-H3 region nor the amino acid sequence are critical for 127S prion replication. Altogether these findings were unexpected as the H2-H3 region is proposed to be essential for prion conversion according to several, recently published models (22, 26, 49), in which two helices are proposed to undergo drastic conformational change and to adopt a β-sheet conformation in the prion state (11, 23, 50). Other models, however, consider that it is mainly the N-terminal moiety of the PK-resistant fragment that supports structural modification (20, 21, 58). Also, a recent paper has reported productive infection in cell cultures expressing a PrP molecule with two point mutations substituting original amino acids by cysteines, one in the H2 end and the other in the loop (59).

Insertions in H2 End Preserve Overall Fold of the H2-H3 Domain—We wonder whether the insertions could have destabilizing effects on the structure of PrP\textsuperscript{C} and particularly on the H2-H3 domain. This question concerned both the PrP construct competent for prion conversion and those that are not. We thus turned to structural analysis of the recombinant insertion mutant PrP assuming that their structures would reflect characteristics of the corresponding glycosylated PrP anchored at the membrane of mammalian cells. The conclusions from these works that insertions ins193 are compatible with prion conversion and ins191, are not compatible, do not induce significant alteration of the overall fold of PrP.

As a whole, if we assume the increasingly supported hypothesis of a conformational change of the H2-H3 domain in prions (22–26), the comparison of infection data with structural analysis of PrP-ins193 and PrP-ins191 may suggest that when approaching the disulfide bridge, which could induce specific constraints to surrounding areas, modifications of H2 are less important for the overall fold of PrP\textsuperscript{C} than for its conversion into PrP\textsuperscript{Sc}.

In conclusion we showed that it is possible to generate bona fide prions with 8–16 additional amino acids inserted in the middle of the PK-resistant domain, revealing an unexpected tolerance to PrP sequence change in the prion core. More specifically we showed that octapeptide insertions in either the H2-H3 loop or the last two C-proximal turns of H2 were not critical for prion conversion. These findings indicate that the integrity and sequence specificity of the loop and the two adjacent turns of H2 are not determinant for the structure of PrP\textsuperscript{Sc} and will help to validate and refine structural models.

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REFERENCES

1. Prusiner, S. B. (1998) Prions. Proc. Natl. Acad. Sci. U.S.A. 95, 13363–13383
2. Bühler, H., Aguzzi, A., Sailer, A., Greiner, R. A., Autenried, P., Aguet, M., and Weissmann, C. (1993) Mice devoid of PrP are resistant to scrapie. Cell 73, 1339–1347
3. Riek, R., Hornemann, S., Wider, G., Billette, M., Glockshuber, R., and Wüthrich, K. (1996) NMR structure of the mouse prion protein domain PrP(121–231). Nature 382, 180–182
4. Donne, D. G., Viles, J. H., Groth, D., Mehlihorn, I., James, T. I., Cohen, F. E., Prusiner, S. B., Wright, P. E., and Dyson, H. J. (1997) Structure of the recombinant full-length hamster prion protein PrP(29–231). The N-terminal moiety of the PK-resistant fragment that supports structural integrity and sequence specificity of the loop and the two adjacent turns of H2 are not critical for prion conversion and propagation, showing that the simultaneous modification of both regions was tolerated. Like the PrP-ins(FLAG) mutant mentioned above, PrP-ins(195 + 203) harbors 16 additional amino acids, strengthening the view that neither the size of the middle part of the H2-H3 region nor the amino acid sequence are critical for 127S prion replication. Altogether these findings were unexpected as the H2-H3 region is proposed to be essential for prion conversion according to several, recently published models (22, 26, 49), in which two helices are proposed to undergo drastic conformational change and to adopt a β-sheet conformation in the prion state (11, 23, 50). Other models, however, consider that it is mainly the N-terminal moiety of the PK-resistant fragment that supports structural modification (20, 21, 58). Also, a recent paper has reported productive infection in cell cultures expressing a PrP molecule with two point mutations substituting original amino acids by cysteines, one in the H2 end and the other in the loop (59).

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18. Supattapone, S., Bosque, P., Muramoto, T., Wille, H., Aagaard, C., Peretz, D., Nguyen, H. O., Heinrich, C., Torchia, M., Safar, J., Cohen, F. E., DeArmond, S. J., Prusiner, S. B., and Scott, M. (1999) Prion protein of 106 residues creates an artificial transmission barrier for prion replication in transgenic mice. *Cell* 96, 869–878

19. Flechsig, E., Shmerling, D., Hegyi, I., Raeber, A. J., Fischer, M., Cozzio, A., von Mering, C., Aguzzi, A., and Weissmann, C. (2000) Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron* 27, 399–408

20. Govaerts, C., Wille, H., Prusiner, S. B., and Cohen, F. E. (2004) Evidence for assembly of prions with left-handed β-helices into trimers. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8342–8347

21. Wille, H., Bian, W., McDonald, M., Kendall, A., Colby, D. W., Bloch, L., Ollesch, J., Borovinskiy, A. L., Cohen, F. E., Prusiner, S. B., and Stubbs, G. (2009) Natural and synthetic prion structure from x-ray fiber diffraction. *Proc. Natl. Acad. Sci. U.S.A.* 106, 16990–16995

22. Cobb, N. J., Sönnickshen, F. D., McHaourab, H., and Surewicz, W. K. (2007) Molecular architecture of human prion protein amyloid. A parallel, in-register β-structure. *Proc. Natl. Acad. Sci. U.S.A.* 104, 18946–18951

23. Lu, X., Wintrobe, P. L., and Surewicz, W. K. (2007) β-Sheet core of human prion protein amyloid fibrils as determined by hydrogen/deuterium exchange. *Proc. Natl. Acad. Sci. U.S.A.* 104, 1510–1515

24. Tycko, R., Savchenko, R., Ostapchenko, V. G., Makarava, N., and Baskakov, I. V. (2010) The α-helical C-terminal domain of full-length recombinant PrP converts to an in-register parallel β-sheet structure in PrP fibrils. Evidence from solid state nuclear magnetic resonance. *Biochemistry* 49, 9488–9497

25. Adrover, M., Pauwels, K., Prigent, S., de Chiara, C., Xu, Z., Chapuis, C., Pastore, A., and Rezaei, H. (2010) Prion fibrillization is mediated by a native structural element that comprises helices H2 and H3. *J. Biol. Chem.* 285, 21004–21012

26. Kunes, K. C., Clark, S. C., Cox, D. L., and Singh, R. R. (2008) Left-handed β-helix models for mammalian prion fibrils. *Prion* 2, 81–90

27. Lloyd, S., Mead, S., and Collinge, J. (2011) Genetics of prion disease. *Top. Curr. Chem.* 305, 1–22

28. Priola, S. A., and Chesebro, B. (1995) A single hamster PrP amino acid blocks conversion to protease-resistant PrP in scrapie-infected mouse neuroblastoma cells. *J. Virol.* 69, 7754–7758

29. Kaneko, K., Zulianello, L., Scott, M., Cooper, C. M., Wallace, A. C., and James, T. L., Cohen, F. E., and Prusiner, S. B. (1997) Evidence for protein X binding to a discontinuous epitope on the cellular prion protein during scrapie prion propagation. *Proc. Natl. Acad. Sci. U.S.A.* 94, 10069–10074

30. Atarashi, R., Sim, V. L., Nishida, N., Caughey, B., and Aguzzi, A. (2009) Specific biarsenical labeling of cell surface proteins allows fluorescent- and biotin-tagging of amyloid precursor protein and prion proteins. *Mol. Biol. Cell* 20, 233–244

31. Rutishauser, D., Mertz, K. D., Moos, R., Brunner, E., Rülliche, T., Calella, A. M., and Aguzzi, A. (2009) The comprehensive native interactome of a fully functional tagged prion protein. *PLoS One* 4, e4446

32. Goołd, R., Rabbabian, S., Sutton, L., Andre, R., Arora, P., Moonga, J., Clarke, A. R., Schiavo, G., Jat, P., Collinge, J., and Tabrizi, S. J. (2011) Rapid cell-surface prion protein conversion revealed using a novel cell system. *Nat. Commun.* 2, 281

33. Vilette, D., Andreoletti, O., Archer, F., Madelaine, F. M., Vilotte, J. L., Lehmann, S., and Laude, H. (2001) *Ex vivo* propagation of infectious sheep scrapie agent in heterologous epithelial cells expressing ovine prion protein. *Proc. Natl. Acad. Sci. U.S.A.* 98, 4055–4059

34. Féraudet, C., Morel, N., Simon, S., Volland, H., Frobot, Y., Crémoin, C., Vilette, D., Lehmann, S., and Grassi, J. (2005) Screening of 145 anti-PrP monoclonal antibodies for their capacity to inhibit PrPSc replication in infected cells. *J. Biol. Chem.* 280, 11247–11258

35. Moudjou, M., Treguer, E., Rezaei, H., Sabuncu, E., Neuendorf, E., Groschup, M. H., Grosclaude, J., and Laude, H. (2006) Glyc-can-controlled epitopes of prion protein include a major determinant of susceptibility to sheep scrapie. *J. Virol.* 78, 9270–9276

36. Paquet, S., Langevin, C., Chapuis, J., Jackson, G. S., Laude, H., and Vilette, D. (2007) Efficient dissemination of prions through preferential transmission to nearby cells. *J. Gen. Virol.* 88, 706–713

37. Krasemann, S., Groschup, M. H., Harmeyer, S., Hunsmann, G., and Bodemer, W. (1996) Generation of monoclonal antibodies against human prion proteins in PrP0/0 mice. *Mol. Med.* 2, 725–734

38. Vilotte, J. L., Soulier, S., Essalmani, R., Stinnakre, M. G., Vainman, D., Lepour-lluy, O., Da Silva, J. C., Besnard, N., Dawson, M., Buschmann, A., Groschup, M., Petit, S., Madelaine, M. F., Rakotobe, S., Le Dur, A., Vilette, D., and Laude, H. (2001) Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine PrP. *J. Virol.*, 5977–5984

39. Langevin, C., Andréolotti, O., Le Dur, A., Laude, H., and Bérinque, V. (2011) Marked influence of the route of infection on prion strain apparent phenotype in a scrapie transgenic mouse model. *Neurobiol. Dis.* 41, 219–225

40. Moudjou, M., Bernard, J., Sabuncu, E., Langevin, C., and Laude, H. (2007) Glycan chains modulate prion protein binding to immobilized metal ions. *Neurochem. Int.* 50, 689–695

41. Dron, M., Dandy-Dron, F., Farooq Salamat, M. K., and Laude, H. (2009) Proteasome inhibitors promote the sequestration of PrPSc into aggresomes within the cytosome of prion-infected CAD neuronal cells. *J. Gen. Virol.* 90, 2050–2060

42. Peretz, D., Scott, M. R., Groth, D., Williamson, R. A., Burton, D. R., Cohen, F. E., and Prusiner, S. B. (2001) Strain-specific relative conformational stability of the scrapie prion protein. *Protein Sci.* 10, 854–863

43. Chakroun, N., Prigent, S., Dressis, C. A., Noinville, S., Chapuis, C., Fraternali, F., and Rezaei, H. (2010) The oligomerization properties of prion protein are restricted to the H2H3 domain. *FASEB J.* 24, 3222–3231

44. Prigent, S., and Rezaei, H. (2011) PrP α-helix assemblies. Spotting the responsible regions in prion propagation. *Prion* 5, 69–75

45. Ben-Dor, S., Esterman, N., Rubin, E., and Sharon, N. (2004) Biases and complex patterns in the residues flanking protein N-glycosylation sites. *Glycobiology* 14, 95–101

46. Dlouhy, S. R., Hsiao, K., Farlow, M. R., Foroud, T., Conneally, P. M., Johnson, P., Prusiner, S. B., Hodes, M. E., and Ghetti, B. (1992) Linkage of the Indiana kindred of Gerstmann-Sträussler-Scheinker disease to the prion protein gene. *Nat. Genet.* 1, 64–67

47. Zaidi, S. L., Richardson, S. L., Capellari, S., Song, L., Smith, M. A., Gambetti, P., and Petersen, R. B. (2005) Characterization of the F1985 prion protein mutation. Enhanced glycosylation and defective refolding. *J. Alzheimers Dis.* 7, 159–171; discussion 173–180

48. Meli, M., Gasset, M., and Colombo, G. (2011) Dynamic diagnosis of familial prion diseases supports the β2-α2 loop as a universal interference target. *PLoS One* 6, e19093

49. Li, J., Browning, S., Mahal, S. P., Oelschlegel, A. M., and Weissmann, C. (2010) Darwinian evolution of prions in cell culture. *Science* 327, 869–872

50. van der Kemp, M. W., and Daggett, V. (2010) Pathogenic mutations in the
hydrophobic core of the human prion protein can promote structural instability and misfolding. J. Mol. Biol. **404**, 732–748

57. Geissen, M., Mella, H., Saalmüller, A., Eiden, M., Proft, J., Pfaff, E., Schätzel, H. M., and Groschup, M. H. (2009) Inhibition of prion amplification by expression of dominant inhibitory mutants. A systematic insertion mutagenesis study. *Infect Disord. Drug Targets* **9**, 40–47

58. DeMarco, M. L., and Daggett, V. (2004) From conversion to aggregation. Protofibril formation of the prion protein. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 2293–2298

59. Hafner-Bratkovic, I., Bester, R., Pristovsek, P., Gaedtke, L., Veranic, P., Gaspersic, I., Mancek-Keber, M., Avbelj, M., Polymenidou, M., Julius, C., Aguzzi, A., Vorberg, I., and Jerala, R. (2011) Globular domain of the prion protein needs to be unlocked by domain swapping to support prion protein conversion. *J. Biol. Chem.* **286**, 12149–12156