Heterologous Stacking of Prion Protein Peptides Reveals Structural Details of Fibrils and Facilitates Complete Inhibition of Fibril Growth

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Fibrils play an important role in the pathogenesis of amyloidosis; however, the underlying mechanisms of the growth process and the structural details of fibrils are poorly understood. Crucial in the fibril formation of prion proteins is the stacking of PrP monomers. We previously proposed that the structure of the prion protein fibril may be similar as a parallel left-handed β-helix. The β-helix is composed of spiraling rungs of parallel β-strands, and in the PrP model residues 105–143 of each PrP monomer can contribute two β-helical rungs to the growing fibril. Here we report data to support this model. We show that two cyclicized human PrP peptides corresponding to residues 105–124 and 125–143, based on two single rungs of the left-handed β-helical core of the human PrPSc fibril, show spontaneous cooperative fibril growth in vitro by heterologous stacking. Because the structural model must have predictive value, peptides were designed based on the structure rules of the left-handed β-helical fold that could stack with prion protein peptides to stimulate or to block fibril growth. The stimulator peptide was designed as an optimal left-handed β-helical fold that can serve as a template for fibril growth initiation. The inhibiting peptide was designed to bind to the exposed rung but frustrate the propagation of the fibril growth. The single inhibitory peptide hardly shows inhibition, but the combination of the inhibitory with the stimulatory peptide showed complete inhibition of the fibril growth of peptide huPrP-(106–126). Moreover, the unique strategy based on stimulatory and inhibitory peptides seems a powerful new approach to study amyloidogenic fibril structures in general and could prove useful for the development of therapeutics.

Transmissible spongiform encephalopathies are neurodegenerative disorders in a wide range of mammalian species, including Creutzfeldt-Jacob disease in man, scrapie in sheep, and bovine spongiform encephalopathy in cattle. The deposition of aggregated prion protein fibrils on and in neurons is regarded to be the source of these neurodegenerative diseases and is frequently associated with occurrence of Congo red positivity (1–3). The fibrils are formed by the conformational change of the prion protein (PrPc)2 into the scrapie form (PrPSc). The misfolded conformer of the prion protein (PrPSc) is considered as the causative agent in these diseases according to the protein-only hypothesis (4). Studies have shown the toxicity of fibrils of the full-length recombinant mammalian prion protein as well as soluble β-rich oligomers to cultured cells and primary neurons (5).

It is still unknown how much of the whole PrPSc molecule is involved in the fibril growth. It is shown that the N-terminal part of PrP, specifically residues 112–141, can go through conformational changes involving β-strand formation, which subsequently triggers fibril growth (6–8), and solid state NMR studies showed that residues 112–141 are part of the highly ordered core of huPrP-(23–144) (9). It was previously shown that peptides based on the 89–143 region of the human PrP protein can form fibrils rich in β-sheet structure which are biologically active in transgenic mice (10). Within this region it is the huPrP-(106–126) peptide that is the smallest known region of PrP that forms fibrils that are toxic and resemble the physiological properties of PrPSc (11–16). The formation of PrPSc is considered to be a two-step event; first, there is the binding between PrPc and PrPSc and subsequently the conformational conversion from PrPc into PrPSc occurs. Mutation studies in a prion-infected neuroblastoma cell line showed that in mouse PrP the regions 101–110 and 136–158 are crucial for the binding and conversion events, respectively (17). Because prevention of fibril growth is the prime therapeutic target, detailed structural knowledge of the fibril is essential for understanding the mechanism of fibril growth. However, structural analysis of amyloid fibrils is hampered by insolubility, isomorphism, and aggregation. X-ray diffraction of several amyloid fibrils revealed a so-called cross-β diffraction pattern which indicates that the fibrils contain β-strands perpendicular to the fibril axis and hydrogen bonds in parallel (18, 19). Thus, for fibril growth the β-strands have to stack on top of each other. Several structures have been suggested to explain the structure of the stacked β-strands; e.g. a parallel in register organization of stacked β hairpins (24) or the comparable dry steric zipper structure (25).

The abbreviations used are: PrPc, cellular isoform of PrP; PrPSc, scrapie isoform of PrP; huPrP, human prion protein; Fmoc, fluorenylmethoxycarbonyl; LpxA, UDP-N-acetylglucosamine acetyltransferase; AU, arbitrary units; HPLC, high performance liquid chromatography; MS, mass spectroscopy; PBS, phosphate-buffered saline.

[1] The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

[2] The abbreviations used are: PrPc, cellular isoform of PrP; PrPSc, scrapie isoform of PrP; huPrP, human prion protein; Fmoc, fluorenylmethoxycarbonyl; LpxA, UDP-N-acetylglucosamine acetyltransferase; AU, arbitrary units; HPLC, high performance liquid chromatography; MS, mass spectroscopy; PBS, phosphate-buffered saline.
Previously, we and other groups suggested that the β-sheet structures in the PrPSc fibril may be similar to the topologically most simple class of β-sheets; that is, the parallel left-handed β-helix (Fig. 1A) (6, 20, 21). The left-handed β helix is formed by triangular progressive coils (rungs) of 18–20 residues. Each rung is formed by three hexapeptide motifs, which results in an approximate 3-fold symmetry. Backbone-backbone hydrogen bonding and stacking of the side chains in adjacent rungs contribute to the folding of β-helical rungs. We suggested that each PrPSc monomer contributes two left-handed β-helical rungs to the fibril, comprising residues 105–124 and 125–143 (Fig. 1A). This two-rung structural model was recently confirmed for amyloid fibrils of the HET-s prion by NMR analysis (22). In contrast to fibrils which are composed of homologous stacks of identical peptides, e.g. the Aβ peptide (23), the PrPSc fibril is more complex because it is composed of heterologous stacks of at least two peptides. For homologous stacking of two identical peptides, the complementarity issue is relatively simple because the identical side chains are in register (e.g. Ile-Ile, Val-Val stacking, and Asn ladders). However, in the case of heterologous stacking, the side chains of the additional heterologous peptide needs to be complementary with the other peptide to allow fibril growth.

To investigate whether the suggested rungs 105–123 and 125–143 from human PrP could be complementary (20), we studied the homologous stacking and the heterologous stacking of linear and cyclized prion protein peptides comprising the huPrP-(105–143) region (KTNMKGMAAAGAVVGGLGVMGSMRSPIHFGS). Qualitative and semiquantitative analysis were done by electron microscopy and Congo red staining. The quantification of the fibril formation was assessed by thioflavin S staining, in which the addition of polyanions (e.g. heparin) enhance the β-sheet formation of peptides comprising the 82–143 region of PrP and improve the reproducibility of the fibril growth (24). This study provides first evidence of heterologous stacking by two isolated putative β-strand layers (or rungs) of the human prion protein with fibril formation as a result. The left-handed β-helix structure provided insight for the “stack-and-stop” approach. With this approach a mix of a stimulatory peptide and an inhibitory peptide could completely block fibril formation. The stimulatory peptide was based on the 125–143 region that was optimized to serve as a folding template for the consecutive stacking of the 106–126 peptide. This cooperative fibril growth was completely inhibited by the inhibitory peptide based on peptides 106–126 with strategic D-amino acid and/or proline substitutions. The findings in this study support models in which the sequential strands in a fibril must somehow spiral up- or downward along the fibril axis, e.g. like the hypothetical left-handed β-helical structure of PrPSc fibrils (20). Furthermore, it allows the development of well defined small protein modules which can be used for structure studies of the 82–143 domain of PrPSc and the development of therapeutics.

**EXPERIMENTAL PROCEDURES**

**Peptides**—Peptides were synthesized by Fmoc chemistry and purified by reversed phase HPLC. For the peptide-peptide interaction studies some peptides were N-terminal-biotinylated by Fmoc chemistry via an aminohexanoic acid spacer. The peptides were analyzed for identity by electron spray desorption mass spectrometry according to previously described methods (25, 26). Peptides were cyclized by oxidation of the C- and N-terminal cysteines. For the disulfide oxidation 0.1 mg/ml peptide was incubated overnight at 4 °C in an ammonium carbonate solution (0.1%). The oxidation reaction was monitored by HPLC/MS analysis. When the cyclization was complete the product was purified by reversed phase HPLC and analyzed by HPLC/MS. All cyclized peptides yielded a purity of >95% based on UV detection at 215 nm. After completion of the cyclization reaction, trifluoroacetic acid (10% in H2O) was added until the pH was <4. The peptides were lyophilized 3 times from acetonitrile (50% (v/v) in water) and stored at −20 °C in 1.4-ml polypropylene tubes (Micronics, Lelystad, the Netherlands) in dry aliquots corresponding with amounts that make up a peptide concentration of 200 μM when dissolved in a 120 μl volume. All cooperativity experiments were performed in 50 mM phosphate buffer, pH 5.0, and 10 mM borate buffer, pH 8.5. In general, similar results were found for experiments performed in pH 5.0, but cooperativity effects were more pronounced in the pH 8.5 buffer.

**Detection of Fibril Formation by Thioflavin S Staining and Cooperativity Calculations**—The peptides were dissolved just before the start of each experiment at a concentration of 200 μM in phosphate buffer (50 mM, pH 5.0) or borate buffer (10 mM,
pH 8.5) and kept on ice. For the cooperativity studies equal volumes of the peptide solutions were mixed. Subsequently the peptide solutions were incubated for 1 h at 37 °C. For thioflavin S staining, 50-μl aliquots were transferred into microtiter wells (FluoNoNunc LuminiNunc polystyrene plates, NUNC, Roskilde, Denmark) in which 5 μl of a thioflavin S solution (1.1 mg/ml in H2O; Sigma) was already present, and the microtiter plate was agitated briefly. Plates were kept covered with microtiter cover-tape (Costar plate sealers, Corning Inc., Corning, NY) to minimize evaporation. After 90 min, the fluorescence signal was measured with a Wallac Victor 1420 Multilabel counter (PerkinElmer Life Sciences) using the following instrumental conditions: 0.1-s measurement time, normal emission aperture, excitation and emission wavelengths 450 and 535 nm, respectively, lamp energy 10,000 (arbitrary units). Before the cooperativity calculation, the background signals of the corresponding buffers were subtracted from the peptide samples. Background values were determined by thioflavin S staining of the buffers. The cooperativity factor was calculated by the following formula: measured cooperativity/theoretical cooperativity. Measured cooperativity is the fluorescence signal of the mixture (peptide 1 + peptide 2), and the theoretical cooperativity is the average fluorescence signal of the individual peptides (peptide 1 + peptide 2)/2. Cooperativity is considered to be significant if the measured cooperativity was significantly higher than the theoretical cooperativity and the fluorescence signal of the peptides individually.

**HPLC Analyses of Dimerization Studies**—Equal molar amounts of peptide huPrP-(125–143)-E4C and huPrP-(105–126)-R2C or huPrP-(106–126)-R2C (C = Cys, E = Glu, R = Arg, x = 2, 3, or 4) were mixed and incubated for 1, 6, and 24 h at 37 °C in borate buffer, pH 8.5. Subsequently samples for HPLC/MS analysis were taken, and theimerization reaction was stopped by the addition of 3 μl of trifluoroacetic acid (10% v/v in water) to a 50-μl sample. The formation of dimers was studied by HPLC analysis (Alliance, Waters Corp., Milford, MA) with a C18 reversed phase column and detected simultaneously with a photodiode array detector and mass spectrometry detector; eluent was H2O + 0.05% trifluoroacetic acid and acetonitrile + 0.05% trifluoroacetic acid with a gradient of 2%/min 5–65% acetonitrile. All reagents were at least HPLC grade.

**Electron Microscopy and Congo Red Staining**—Peptide PrP-(107–123), PrP-(127–142) and cyclized peptide rung 6 and rung 7 alone or in equimolar mixture were dissolved in borate buffer (200 μM final concentration). Peptide suspensions were incubated at 37 °C for 1, 24, and 48 h. At each time point, sample aliquots were analyzed by light and electron microscopy. For light microscopy, 10 μl of suspension were air-dried on poly-l-lysine-coated slides (Bio-Optica, Milan, Italy), stained with Congo red, and viewed under polarized light (Nikon Eclipse E-800, Japan). For ultrastructural examination, 5 μl of suspension were applied to Formvar-carbon 200-mesh nickel grids for 6 min, negatively stained with uranyl acetate, and observed with an electron microscope (EM109 Zeiss, Oberkochen, Germany) operated at 80 kV at a standard magnification (×30,000), calibrated with an appropriate grid. The samples were evaluated for the presence and relative amount of oligomers, amorphous aggregates, filamentous structures, and fibrils by two independent operators.

**Peptide-Peptide Interactions**—Peptide-peptide interactions were measured using pepscan analysis (27). Biotinylated peptides were incubated with peptides which were synthesized on a solid phase polypropylene support (minicard). The credit card-sized minicard contains 455 wells in which peptide arrays of overlapping linear and cyclized 18-mer and linear 22-mer peptides based on the PrP 97–150 region were synthesized as previously described (28). Before use, the wells with the solid phase peptides were washed with PBS-Tween (7.5 mM phosphate, 0.14 mM NaCl, 0.5% Tween 80, pH 7.4) for 30 min. Next, the wells were blocked with 5% bovine serum albumin, 5% horse serum, 1% Tween 80 in PBS for 1 h at 25 °C to reduce aspecific binding. Subsequently, the wells were washed 3 times for 5 min with 1% Tween 80 in PBS. The biotinylated PrP peptide 106–126 was dissolved in water, heated for 20 min at 90 °C, and immediately kept on ice to minimize aggregation before the incubation with the solid phase peptide arrays. After cooling of the biotinylated 106–126 peptide and shortly before use, a bovine serum albumin solution (10% bovine serum albumin, 1% Tween 80 in PBS) was added until a final concentration of 1% bovine serum albumin. The solid phase peptide arrays on the wells were incubated with the biotinylated peptide 106–126 solution (3 μl per well) for 1 h at 25 °C. After the wells were washed they were incubated for 1 h at 25 °C with streptavidin-horseradish peroxidase (1/1000) diluted in 5% bovine serum albumin plus horse serum for the detection of bound biotinylated 106–126 peptide. Next, the wells were washed, and the background signal of the wells was measured with a charge-coupled device (CCD) camera. Subsequently the wells were washed once (0.1% Tween 80 in PBS) and then incubated for 1 h at room temperature with substrate (0.5 g/liter 2,2'-azino-di[3-ethyl-benzthiazolinesulfonate(6)]]diammonium salt (ABTS), 0.006% H2O2 in 0.18 m Na2HPO4, 0.22 m citric acid, added until pH 4. The color development was detected at 405 nm with the charge-coupled device camera.

**Inhibition Studies**—The inhibition studies were performed as previously described (24). In short, fibril formation of the linear peptide huPrP-(106–126) was measured individually and in combination with a stimulating or inhibiting peptide or a mix of the stimulating and inhibiting peptides. The fibril formation of the stimulator and inhibiting peptides were also measured individually. Linear human prion protein peptides were dissolved in 30 μl of phosphate buffer (50 mM, pH 5) at a concentration of 200 μM. Also, the stimulator peptide and each inhibiting peptide were dissolved in phosphate buffer at a concentration of 200 or 400 μM. For the combination of the huPrP-(106–126) peptide with one stimulator or inhibiting peptide, the linear huPrP-(106–126) peptide was dissolved in 30 μl of 200 μM stimulator or inhibiting peptide solution, resulting in a final concentration of 200 μM concentration of each peptide. For the combination of huPrP-(106–126) peptide with a mix of the stimulator peptide and an inhibiting peptide, peptide huPrP-(106–126) was dissolved in 17.5 μl (400 μM) of stimulator peptide solution plus 17.5 μl (400 μM) inhibiting peptide solution. This resulted in a final concentration of 200 μM concentration of each peptide. Subsequently, the peptides with or without inhibiting peptide were incubated for 1 h at 37 °C. Next, 30 μl of heparin was added to the huPrP-(106–126) peptide until a final concentration of 50 μg/ml to optimize the
**Heterologous Stacking by Prion Protein Peptides**

**RESULTS**

To determine how much of the 82–143 region of the human prion protein is involved in fibril growth, peptides comprising residues 106–126 (21-mer), 106–143 (38-mer), and 82–143 (62-mer) were compared for the amount of fibril growth at equal molar concentrations. Compared with the 21-mer peptide, the fibril growth of the 62-mer and the 38-mer were ~2.4- and 1.5-fold higher, respectively (Fig. 2). The higher signal with the longer peptides suggests that residues adjacent to the 106–126 region also participate in the fibril growth and contribute at least one extra rung to the growing fibril. In our proposed left-handed β-helix model (20) this would suggest that the minimal fibrillogenic core of the PrP molecule is formed by two stacked rungs (Fig. 1A).

**N-Acetylglucosamine-1-phosphate Uridylyltransferase (LpxA) Peptides**—Because it was not known whether cooperative stacking of isolated peptides from a β-helical structure was feasible, we used a known natural left-handed β-helix structure of the enzyme LpxA as a model system. Based on regular rungs of LpxA (Fig. 1B) (29), we optimized two peptides (rung 6 and rung 7) to increase the probability of heterologous stacking. Because the rungs are taken out of the structural context and to satisfy the ideal left-handed β-helices (30). The Asn at position 3 of rung 6 forms a hydrogen bond with a residue from rung 5, which is not a part of the structure anymore; therefore, it was replaced by Thr an ideal alternative at this position. The Cys at position 15 of rung 6 was replaced by Thr, and in rung 7 the Cys at position 3 was replaced by Ala. In rung 7 the Leu at position 11 was replaced by Ile to improve the stacking with rung 6. The Asp at position 18 of rung 7 was replaced by an Ala to avoid disturbance with the polyionic extensions. For further reference in this paper the two peptides that are used for the cooperativity studies are coded rung 6 and rung 7. It was shown that peptides rung 6 and rung 7 can indeed bind thioflavin S and form fibrils (Fig. 3).

The homologous stacking of rung 7 (intrinsic fluorescence) was ~3.8 times higher than the intrinsic fluorescence of rung 6 (Fig. 3A). To enhance the probability of stacking, polyanionic (five glutamic acid residues) and polycationic (five arginine residues) extensions were added at the C terminus of the peptides; for further reference the polyionic extensions are denoted as neg and pos, respectively. Theoretically the probability of a potential stack of two peptides will be increased by the attraction of the opposite charged extensions. Indeed the addition of the polyionic extensions increased the homologous stacking of rung 6 by a factor of 2.5 (rung 6-pos + rung 6-neg) (Fig. 3A) and of rung 7 by a factor of 5 (rung 7-pos + rung 7-neg) (Fig. 3B).

To facilitate the folding of rungs 6 and 7, cyclized versions of the peptides were synthesized (Fig. 1C). The cyclizations limit the flexibility of the peptides and allow the type of stacking similar to the typical rungs of a left-handed β-helix. Peptide c-rung 6 showed no fluorescence at all (Fig. 3A), but the addition of polyionic extensions did increase the homologous stacking of c-rung 6 spectacularly by a factor of 12 compared with the linear rung 6 without polyionic extensions (Fig. 3A). In contrast to c-rung 6, the cyclized version of rung 7 (without polyionic extensions) was found to be much more fibrillogenic than its linear form as was shown by the 4-fold higher fluorescence level (Fig. 3B). In contrast to rung 6, the addition of polyionic extensions reduced the fluorescence level of the individual c-rung 7, but when the positive and negative charged c-rung 7 peptides were mixed, the homologous stacking was a factor of 3.5 higher than expected (Fig. 3B).
In contrast to heterologous sequences the ability of homologous sequences to stack in a parallel fashion has a higher probability because identical residues in the parallel strand will stack perfectly (unless they are charged). A Val-Val stack or an Asn-ladder are well known examples (31).

However, in the case of rung 6 and rung 7, a heterologous combination of two different peptides is required. For heterologous stacking there is obviously more constraint because a successful parallel alignment of the strands depends on the perfect complementarity of the side chains of both peptides. Although it is known that rungs 6 and 7 interact in the native protein, the actual experimental proof through in vitro stacking is difficult to achieve. Although heterologous cooperative effects of linear versions of rung 6 and rung 7 were not observed, the introduction of polyionic extensions resulted in a small heterologous cooperation of linear rung 6-neg combined with linear rung 7-pos (cooperativity factor = 2) (Fig. 3C). Similar results were found for the cyclized versions of rung 6 and rung 7; without the aid of polyionic extensions, heterologous stacking was not observed, but most interestingly a heterologous cooperation was found for c-rung 6-neg with c-rung 7-pos (Fig. 3C).

Also heterologous cooperativity was found with the combination of c-rung 7-neg with linear rung 6-pos and the combination of linear rung 7-pos with c-rung 6-neg (Fig. 3D). The observed heterologous cooperativity was confirmed with ultrastructural examination. Peptide c-rung-7-pos formed thin filamentous structures within 1 h of incubation, developing into few fibrils after 24 h, increasing in amount after 48 h. Peptide c-rung 6-neg showed a similar development like c-rung 7-pos; however, the amount of fibrils was less. The mix of both peptides formed thin filamentous material after 1 h, but the fibrils grew faster (24 h), and a lot of fibrils often organized in a dense meshwork, observed after 48 h of incubation compared with the peptides individually (Fig. 4, A–C). The formation of amyloidogenic fibril aggregates was confirmed by Congo red staining. The semiquantitative analysis of the amount of birefringence after Congo red staining showed that the mix of c-rung 6-neg and c-rung 7-pos formed more and faster birefringent aggregates than the peptides individually (see the supplemental figure). Our results show for the first time that peptides based on rungs of a natural left-handed β-helix are able to form fibrils with the tinctorial properties of amyloid, i.e. birefringence under polarized light after Congo Red staining.

**Prion Protein Peptides**

Dimerization of Two Prion Protein Peptides Determined by HPLC/MS Analysis—After the cooperativity studies with the idealized rung 6 and rung 7 peptides, similar studies were done with peptides corresponding to the two rungs of the hypothetical human PrP\(^{\text{Sc}}\) fibril structure (105–124 and 125–143). To
investigate the interpeptide disulfide bridge formation in heterodimers of stacked PrP peptides, we analyzed the dimerization reaction using HPLC/MS. The different peptides were synthesized with a polyionic extension and a free cysteine that would allow interpeptide disulfide formation when the peptides would form a correct heterologous parallel alignment. The tendency to heterodimerize was studied with linear peptides (125–143)-E4C, (105–126)-RxC, and (106–126)-RxC (C = Cys, E = Glu, R = Arg, x = 2, 3, or 4). HPLC/MS analysis showed heterodimerization of peptide (125–143)-E4C and (105–126)-RxC without the formation of homodimers (Fig. 5C). Peptide (105–126)-R4 can slowly homodimerize with itself (Fig. 5B), but the heterodimerization with (125–143)-E4 was preferred (Fig. 5C). The rate of heterodimerization of (125–143)-E4C with (106–126)-RxC and (105–126)-RxC (x = 2–4) depended on the number of arginine residues in the C-terminal extension (data not shown).

The heterodimerization was time-dependent, ranging from 3 to 14% after 1 h and increased after 6 h of incubation (21–73%) and 24 h (61–88%) (data not shown). The dimerization experiments indicated that, with the appropriate C-terminal extensions, linear prion protein peptides comprising regions 105–126 and 125–143 are able to heterodimerize within 1 h without the formation of homodimers.

Cooperativity of Two Prion Protein Peptides—To investigate the possibility of the heterologous stacking by prion protein peptides from the regions 105–123 and 124–143, based on the two rung model (20), these peptides were tested for thioflavin S staining, which is a measure for the amount of fibril growth. Peptides based on the 106–124 and 125–143 regions were cyclized by a disulfide bond to mimic the typical rungs of a left-handed β-helix (Fig. 1C) but also other models in which some kind of spiraling of the parallel β-strand is essential. Interestingly, the fluorescence level of the c-106–123 was much higher (3-fold) than its linear version (Fig. 6A). Also a cyclized version of the 127–143 region showed some increase in the fluorescence level compared with its linear form (Fig. 6B). Similar to the experiments with rung 6 and 7 of LpxA, also the linear and cyclized versions of the PrP peptides c-127–142 and c-107–123 showed some increase in the fluorescence level compared with their linear versions. The bar in C is applicable to all figures.
peptides did not show cooperativity without the help of polyionic extensions.

The addition of polyionic extensions to peptides comprising the PrP regions 105–123 and 124–143 reduced the fluorescence levels 2–6-fold compared with the peptides without an extension (data not shown). However, the addition of polyionic extensions resulted in a significant heterologous cooperativity as was observed between linear peptides 105–124-pos and 124–143-neg (cooperativity factor = 1.6) (Fig. 6C). The heterologous stacking improved spectacularly (cooperativity factor = 6) when a combination of polyionic extensions and cyclization was used (Fig. 6D). A significant heterologous cooperativity (cooperativity factor = 2) was also found between linear 105–123-pos and 124–143-neg, whereas c-107–123-pos combined with linear 124–142-neg showed some cooperativity (cooperativity factor 1.5) (Fig. 6E). Control experiments with peptides unrelated to prion protein sequences (acetyl-CDGAVQPDGGQPASVRN-amide and fluorescein isothiocyanate-aminohexanoic acid-LEDKIEILLSKIHLENLARLAAIRRR-amide) showed no significant cooperative fluorescence levels with any of the linear or cyclized versions of prion protein peptides with polyionic extensions based on regions 105–124 and 124–143 (data not shown). The ability to form amyloid fibrils was studied by electron microscopy, and Congo red staining for the peptides that displayed the highest cooperativity (Fig. 6D) were studied. Peptide c-127–142-neg formed round-shaped structures (oligomers) and filamentous structures within 1 h of incubation, after 24 h filamentous material and protofibrils were found, whereas fibril aggregates were found after 48 h of incubation. Peptide c-107–123-pos showed dense amorphous structures, whereas after 24 h filamentous structures were found, and after 48 h of incubation some fibrils were also evident. The mix of both peptides formed protofibrils among round-shaped structures (oligomers) and thin filamentous structures after 1 h of incubation. After 24 h the mix formed few short amyloid fibrils, whereas after 48 h aggregates of fibrils (no oligomers) were found in the mix in a much higher density than the individual peptides (Fig. 4, D–F). Furthermore, the presence of amyloid fibril was confirmed by Congo red staining. The semiquantitative analysis of the amount of birefringence after Congo red staining showed that the mix of c-107–123-pos and c-127–142-neg not only formed more amyloidogenic fibril aggregates than the peptides individually, but also faster (supplemental figure). In contrast to the electron microscopy and Congo red analysis, the thioflavine staining is maximal within 2.5 h of incubation (including 1.5 h thioflavine staining). This indicates that probably the number of thioflavine staining sites remain equal when oligomers aggregate into protofibrils and mature fibrils (data not shown).

Because the left-handed β-helix model predicted a complementary stack of two peptide rungs, it was investigated whether the influence of specific mutations in rung 1 (c-107–123-pos) and rung 2 (c-127–142-neg) could change the observed cooperativity. As a positive control, a variant of rung 1 was synthesized in which Ala115 was substituted to valine. A better cooperative stacking was expected from a valine mutation of Ala115, because the inside of the rung is better filled by a valine residue at this position according to the model. Indeed the heterologous cooperativity of rung 2 with rung 1 in which Ala115 was substituted for valine showed an increase in fluorescence (Fig. 7). Two variants of rung 2 were synthesized as negative controls; one variant comprising a scrambled 127–142 sequence (rung 2 scrambled) and one variant in which the size of the rung 2 was reduced by six residues (rung 2-small: c-130–139-neg). As expected, the heterologous cooperativity between rung 1 and rung 2 dropped from a cooperativity factor 3 to 1.5 when the size of rung 2 was reduced by six residues (rung 2-small) or when the sequence of rung 2 was scrambled (Fig. 7).

**Peptide-Peptide Interaction Studies**—To investigate which residues are involved in the heterologous stacking of prion protein peptides, binding studies were performed with peptides comprising the 97–150 region of the human prion protein. Biotinylated peptides huPrP-(106–126) (5 μg/ml) and huPrP-(106–126)-pos (0.5 μg/ml) were tested for reactivities with all overlapping linear (18- and 22-mer) and cyclized (18-mer) peptides of the PrP central domain (97–150) and as negative control peptides based on the LpxA protein (biotinylated rung 7-pos), peptide KIAALKEL-
KIAALKEKIAALKE, scrambled huPrP-(106–126) (NGAGKAG-MVGLYGAHGATAKVSLVGALA and DGAQVPDGGQPAVRNER, a peptide derived from canine parvo virus) were used.

In the linear 22-mer peptide array the linear peptide biotin-(106–126) (rung 1) showed homologous binding with the (AV)VGGLG region and showed heterologous binding with GYMLGSAMSR(PIIHFG), which is a large part of rung 2 (Fig. 8). Because the binding stretches show overlap, it is not possible to exactly determine the hotspots for binding. For example, in the case of the highest binding peptide in the 22-mer peptide array (AAAAG-AVVGGGLGGYMLGSARS), the binding contribution can be the result of the homologous binding to the sequence AAAAGAVVGGGLGG

FIGURE 7. Heterologous cooperative fibril growth of mutated cyclized prion protein peptides comprising regions 107–123 and 127–142 of the prion protein. The heterologous cooperative fibril growth of wild type rungs of cyclized peptides comprising the 107–123 (rung 1) and 127–142 (rung 2) regions of PrP combined with mutated rungs rung 1 A115V (CTNMKHMAGVAAAGAVGC-pos), rung 2-scrambled (CAGYLMHPSPSIFMC-neg), and rung 2-small (CLGSAMSPR2IC-neg). All peptides are cyclized by a disulfide bond. The measured fluorescence levels are indicated by gray bars, and the theoretical fluorescence levels of the heterologous cooperation are indicated by hatched bars. All data are the average values ± S.D. of triplicate measurements.

FIGURE 8. Peptide-peptide interaction of linear 106–126 peptide with peptides corresponding to the region 97–150 of PrP. Biotinylated linear peptide 106–126 (0.5 µg/ml) was incubated with an array of 33 solid-phase overlapping 22-mers of the PrP 97–150 region that were synthesized on a solid polypropylene support (minicard). Residues corresponding to rung 1 and 2 are indicated in cyan and blue, respectively. The shared residues in the homologous reactive regions are shown in red, and the shared residues for the heterologous reactive region are shown in pink. The reactive regions are indicated in the left-handed β-helical models with the same color coding.

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or of the heterologous binding to YMLGSAMSR and most likely both. Similar binding interactions were found with biotin-(106–126) and with biotin-(106–126)-pos in the linear 18-mer and cyclized 18-mer peptide arrays. However, these data were even more difficult to interpret because the binding stretches showed even more overlap (data not shown). No significant binding was observed with the negative control peptides (data not shown).

Inhibition of Fibril Growth of Linear Prion Protein Peptide huPrP-(106–126)—To find additional support for the hypothetical left-handed \( ^{31}H\rhd \) helical structure of human PrP\(^{Sc}\), peptides were designed based on the structure of the left-handed \( ^{31}H\rhd \) helix that could stack with prion protein peptides to stimulate or to block fibril growth. A stimulator peptide should have an optimal left-handed \( ^{31}H\rhd \) helical fold that can serve as a template for fibril growth initiation. The inhibitor peptide should be able to bind to the exposed rung but frustrate the propagation of the fibril growth. A proline is hardly ever present in the left-handed \( ^{31}H\rhd \) helical rung except for position 1 at the corner of the triangle where the occurrence is very high. Especially at the top or bottom rung the occurrence of the proline at position 1 is high because it decreases the conformational entropy and facilitates folding of the \( ^{31}H\rhd \) helical structure (32). Therefore, a \( ^{31}H\rhd \) helix stimulator peptide (peptide 1 Fig. 9) was designed with a proline at the first position of the middle hexapeptide motif of rung 2 (G131P). Because optimized rung 2 (stimulator) has a proline at both corners of the triangle (positions 131 and 137), this peptide has a higher chance of the triangular arrangement (see Fig. 11B). Indeed, a strong cooperative fibril growth was observed (47%) when rung 1 (106–126) was mixed with the optimized rung 2 (peptide 1 with the G131P mutation; Fig. 9). This is the first example of heterologous cooperative stacking without the aid of cyclization or polyionic extensions. Subsequently, several attempts were made for the design of inhibiting peptides based on rung 1 with D-amino acid substitutions at positions His111, Met112, Ala117, Ala118, and proline substitutions of Ala115 and Val122. The proline substitution of the residues at the fifth position of the middle and last hexapeptide of rung 1 frustrates the ideal fold of a \( ^{31}H\rhd \) helical rung, and the introduction of \( \sigma\)-amino acids in rung 1 would clash with the side chains of rung 2, and therefore, a reduced fibril growth cooperativity of rung 1 and rung 2 was expected. Indeed, the inhibiting peptides based on rung 1 (peptides 2–4 in Fig. 9) showed no or a low inhibition of fibril growth of rung 1, but when the stimulator peptide based on optimized rung 2 was combined with the inhibiting peptides based on rung 1, a complete inhibition of fibril growth was observed (Fig. 9). Furthermore the inhibition level was inhibitor concentration-dependent (Fig. 10). These
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**FIGURE 10.** The efficiency of fibril growth inhibitor peptide huPrP-(106–126) with d-amino acid substitutions at positions His117, Met112, and Ala115, 100 μM peptide huPrP-(106–126) was incubated with 100 μM stimulatory peptide (peptide 4), which was titrated from 100 to 5 μM. The fibril growth was determined by thioflavin S staining, and the inhibition percentages were calculated as described under “Experimental Procedures.” Subsequently the inhibition levels were standardized relative to the maximum inhibition level. All data are the average values ± S.D. of duplicate measurements.

results confirm the role of cooperativity of the heterologous chains.

**DISCUSSION**

PrPC consists predominantly of α-helix structures, whereas the conversion into PrPSc involves an increase of β-sheet content. This conformational change promotes the fibril formation. Although it is still unknown how much of the whole PrPSc molecule is involved in the fibril growth, solid-state NMR studies showed that residues 112–141 are part of the highly ordered core of huPrP-(23–144) (9). It is well accepted that the region comprising residues 89–143 can form fibrils rich in β-structures (12, 14, 15). X-ray diffraction studies of amyloid fibrils in general suggest a parallel β-strand organization (18, 19). Previously, we and others proposed that the PrPSc fibril may fold as a parallel left-handed β-helix (6, 20, 21) and that each PrPSc monomer contributes two left-handed β-helical rungs to the fibril, comprising residues 105–124 and 125–143 (Fig. 1A). This two-rung structural model was recently confirmed for amyloid fibrils of the HET-s prion by NMR analysis (22).

The smallest known region of PrP that forms fibrils is peptide huPrP-(106–126). These fibrils are toxic and resemble the physiological properties of PrPSc (11, 16, 26, 33). The left-handed β-helix model predicts, however, that the elevation of one PrPSc monomer requires at least two β-helical rungs of heterologous sequences (20). Because in this model the 106–126 region can only make up one rung, the next rung needs to be formed by residues C-terminal to the first rung. Support for this was obtained with the thioflavin S staining studies with the longer peptides that showed that flanking regions are also involved in the parallel β-sheet structure of the fibrils (Fig. 1). Subsequently we studied whether continuous heterologous stacking of the two regions would result into fibril growth. Therefore, peptides based on the 105–126 and 127–143 regions were assayed for their individual and heterologous fibril growth. Here we report experimental results supporting the model by the heterologous stacking in vitro of two peptides comprising the 105–126 and 127–143 regions of the human prion protein.

Heterologous Stacked Prion Protein Peptides Are Realistic Mimics of the Theoretical Left-handed β-Helix—Using a similar approach as for LpxA, we studied the heterologous stacking of linear and cyclized prion protein peptides comprising the PrP region 105–143. Because peptides comprising residues 105–124 and 125–143 have been suggested to form a heterologous stack in the left-handed β-helix model of PrP (20), the heterodimerization of both regions was studied with HPLC/MS analysis (Fig. 5). As expected, the electrostatic attraction of the charged polyionic extensions at the C termini favored the heterodimerization and disulfide bridge formation, favoring a parallel organization of the peptides that may facilitate parallel stacking. Also, thioflavin S staining showed that the polyionic extensions increased the heterologous cooperative fibril growth of linear peptides comprising regions 105–123 and 124–143 (Fig. 6C). Irrespective of the hypothetical nature of the left-handed β-helical model, the sequential strands in a fibril must somehow spiral up- or downward along the fibril axis, reminiscent to e.g. a left-handed β-helix. Therefore, also for PrP the cyclized peptides are more realistic mimics of the putative spiraling rungs in a fibril than the linear ones. It was found that c-106–123 was 3.2-fold more fibrilogenic than its linear version 106–126 (Fig. 6A). Furthermore, a striking 6-fold heterologous cooperativity was observed with peptides c-107–123-pos and c-127–142-neg (Fig. 6D), which is convincingly supported by the electron microscopy analysis (Fig. 4, D–F) and Congo red staining (see the supplemental figure), both indicating a faster and more intense aggregation of amyloidogenic fibrils compared with the individual peptides. Also, linear peptides can stack better with the cyclized heterologous peptide than with the linear version (Fig. 6, E and C, respectively), suggesting that a conformationally stabilized peptide increases the chance of heterologous stacking by serving as a template that can initiate fibril formation. The prion protein peptides showed even higher heterologous stacking than the positive control peptides (run 6 and 7) derived from the known left-handed β-helix LpxA (Figs. 6D and 3D, respectively). A positive control for heterologous stacking of rung 1 and rung 2 was the mutation of Ala115 into valine, which is located at the fifth position of the hexapeptide motif and is expected to fill the inside of the rung better then alanine and, therefore, would increase the stacking and fibril growth. As predicted, the A115V mutation showed high thioflavin S staining with the wild type rung 1 (Fig. 7). A negative control was the scrambled version of rung 2 including a proline at the unfavorable fifth position of the hexapeptide motif. Another negative control was a rung 2 variant, which was reduced in size by six residues (c-130–139-neg), by which the complementary stacking with rung 1 is hampered. As predicted, compared with the wild type rung 2, both negative controls showed less heterologous cooperative fibril growth with rung 1 (Fig. 7). The peptide-peptide interaction studies (Fig. 8) show that peptide 106–126 interacts homologously with sequences comprising the 106–126 region of PrP, which agrees with the known fibril growth of huPrP-(106–126). More importantly it was shown that the peptide 106–126 also interacts heterologously with the 125–143 region of PrP (Fig. 8).
Both regions agree perfectly with the proposed adjacent runs in the left-handed β-helix model, which agrees with the forecasted stacking on top of each other (Fig. 8) (20).

The structural details of the left-handed β-helical runs could assist the design of peptides that could block fibril formation by a strong binding to the exposed rung of the growing left-handed β-helix. Moreover, designs of inhibiting peptides based on the runs of the left-handed β-helix model can also give support to this model. The left-handed β-helical fold of the PrPSc model can probably be optimized because the left-handed β-helical PrPSc conformation is not a preferred fold for PrP but occurs only under particular circumstances and preferably with the aid of a pre-existing “inoculate” PrPSc β-helix that can act as a template. Especially, initiation of the fold may be stimulated by a conformationally restricted version that can act as a template, whereas in the absence of such a template alternative packing arrangements may also occur (34). The tolerance of the left-handed β-helical fold for sequence variation may allow the PrP sequence to misfold into a left-handed β-helix, but the sequence can probably be optimized to satisfy the positional preference in the hexapeptide motif even better. This knowledge was used to optimize the folding of rung 2. The left-handed β-helical runs don’t allow any proline residues, except for position 1 of the hexapeptide, where it actually has a high preference because it promotes the formation of the turn in the triangular rung. Therefore, the biggest structural impact was expected by a proline mutation of Gly131 in the first turn position of the second hexapeptide motif at the corner of the triangle (Fig. 11B). When this optimized rung 2 G131P peptide was mixed with rung 1 peptide, a very strong stimulation of fibril formation was observed. Apparently, the optimized peptide serves as a good template and increases the fibril growth of the mixture spectacularly (stimulator peptide 1 in Fig. 9). This is the first example of spontaneous heterologous cooperative stacking without the aid of cyclization or polyionic extensions. Together with the previous data, this again agrees with the left-handed β-helix model. To inhibit fibril growth, an extra element is needed that binds on the folded dimer or oligomer and blocks the binding of new rungs to the growing fibril. For that reason, a modified rung 1 was designed that would fit on rung 2 G131P but would not allow binding of the next rung. Rung 1 analogues were made with D-amino acids and prolines at various positions that can bond one rung 2, but the side chain orientation of the D-amino acid or the conformation of the proline would block binding of the next rung (Fig. 11C). Indeed, mixing the stimulatory template rung 2 G131P with the inhibitory peptides showed a dramatic inhibition of fibril formation that is higher than any other compound we have ever tested with comparable molar concentrations in our in vitro assay (Fig. 9) (24).

Conclusions—The spontaneous fibril growth in vitro by heterologously stacked human PrP peptides based on PrP regions 105–124 and 125–143 and the determination of the interacting regions provide first experimental support that human PrPSc may form a spiraling parallel β-strand resembling the left-handed β-helix structure. The cooperativity studies allowed the development of a fibril-stimulating peptide based on the PrP region 125–143, which most likely acts as a template for the heterologous binding with peptide huPrP-(106–126). The fibril growth of peptide huPrP-(106–126) was completely inhibited when it was incubated with a mix of the stimulating peptide and an inhibiting peptide based on sequences of the PrP region 106–126 containing D-amino acid and proline substitutions at strategic locations. The inhibiting peptide may act as a “stopper” rung inhibiting the fibril growth. The stack-and-stop approach, in which an optimized rung 2 is used as a stabilizing template and a modified rung 1 peptide is used as a stopper rung (Fig. 11C), may be used to design small protein modules with a limited number of stacked peptides. Such well-defined protein modules may be used for the development of a therapeutic strategy for the reduction of PrPSc fibril formation. Whether the inhibition of fibril growth will also inhibit toxicity remains to be proven. Furthermore, the defined protein modules may also be used to study part of the structural details of the 82–143 domain of the PrPSc protein by NMR or crystallography.

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