Molecular Interactions between Prions as Seeds and Recombinant Prion Proteins as Substrates Resemble the Biological Interspecies Barrier In Vitro

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Abstract

Prion diseases like Creutzfeldt-Jakob disease in humans, Scrapie in sheep or bovine spongiform encephalopathy are fatal neurodegenerative diseases, which can be of sporadic, genetic, or infectious origin. Prion diseases are transmissible between different species, however, with a variable species barrier. The key event of prion amplification is the conversion of the cellular isoform of the prion protein (PrPC) into the pathogenic isoform (PrPSc). We developed a sodiumdodecylsulfate-based PrP conversion system that induces amyloid fibril formation from soluble α-helical structured recombinant PrP (recPrP). This approach was extended applying pre-purified PrPSc as seeds which accelerate fibrillization of recPrP. In the present study we investigated the interspecies coherence of prion disease. Therefore we used PrPSc from different species like Syrian hamster, cattle, mouse and sheep and seeded fibrillization of recPrP from the same or other species to mimic in vitro the natural species barrier. We could show that the in vitro system of seeded fibrillization is in accordance with what is known from the naturally occurring species barriers.

Introduction

Prion diseases are fatal progressive neurodegenerative diseases of spontaneous, genetic, or infectious origin. The conversion of the host encoded prion protein (PrPC) into the disease causing isoform PrPSc is the key molecular event in prion disease. The common hypothesis is, that the amplification of PrPSc is achieved by the conversion of the α-helical dominated cellular isoform PrPC into β-sheet rich and insoluble PrPSc while PrPSc acts as template and catalyst for PrPC conversion [1]. The pool of PrPSc is replenished by the cellular synthesis of PrPC. Some mechanistic models have been suggested, including the heterodimer model [2], the cooperative model [3], and the model of seeded polymerization [4]. Most experimental data support the seeded polymerization. Prion diseases are not only transmissible within one species, but in some cases also between different species [5]. This so called “interspecies transmission” is of special interest since the origin of bovine spongiform encephalopathy is suspected to be caused by feeding meat and bone meal from Scrapie infected sheep to cattle, i.e. an interspecies transmission might have lead to the BSE-epidemic in the UK [6]. In an experimental approach it was indeed shown that cattle are susceptible to infection by sheep Scrapie isolates [7]. On the other hand it is known that interspecies transmission can fail e.g. Syrian hamster Scrapie is not transmissible to mice [8]. In that case the interspecies barrier would be too high for experimental observation. The molecular mechanism of prion disease transmissibility between different species is still not understood.

A molecular model for the infectious process of prions is the conversion of PrPC to PrPSc induced by the invading PrPSc. As mentioned above, the widely accepted mechanistic model is that of PrPSc acting as seed for polymerization of PrPC [9,4,10]. To study the mechanism of spontaneous and seed-depended fibrillization of recPrP different in vitro conversion assays were introduced, which lead to the formation of amyloid fibrils [11,12,13]. The in vitro conversion into amyloid led to the first generation of synthetic prions utilizing only murine recPrP [14], i.e. without PrPSc as seed, which would represent a model for the sporadic case of prion diseases.

In the present study we use the SDS PrP conversion system to simulate intra- and interspecies transmission in vitro. It is a minimal system in the sense, that only recPrP as substrate, buffer, and purified PrPSc as seed, but no cellular extract is needed. We extend the spontaneous and seed depended conversion, established for recombinant Syrian hamster PrP [11] and bovine recPrP [15], to recombinant ovine and murine PrP. It has been shown earlier that well balanced concentrations of sodium chloride and SDS have to be chosen for every substrate PrP in order to guarantee a suitable...
extent of partial denaturation, which is a prerequisite for fibrillization, spontaneous as well as seeded fibrillization.

To monitor amyloid formation of recPrP we used the amyloid specific marker Thioflavin T (ThT). We are able to mimic the species barrier in vitro in complete accordance with the species barrier as found with the in vivo infection of prions.

**Results**

In our previous studies we analyzed spontaneous and PrPSc-seeded fibril formation of recombinant PrP (recPrP). Buffer conditions were established which consist of well selected SDS and NaCl concentrations, so that recPrP forms spontaneously amyloid fibrils within weeks, but the fibril formation was accelerated by seeding with PrPSc to hours or days. It is important to note, that the optimal buffer conditions had to be selected for the species of recPrP during spontaneous fibril formation, and these conditions were used for seeded fibril formation. It was discussed earlier that the buffer conditions allow a particular extent of partial denaturation of recPrP in a well characterized pre-amyloid state. Spontaneous and seeded fibril formation of recPrP was analyzed with bovine and hamster recPrP as substrate and the homologous NaPTA-precipitated PrPSc as seeds [11,15]. In the present study, we established conditions for spontaneous and seeded fibril formation of recPrP from sheep (aminoacids: 25–233) and mouse (aminoacids: 89–231). The aim of this study was to combine recPrP-substrates and PrPSc-seeds of different species to study inter- and intraspecies transmission results resemble exactly the well known species barrier between Syrian hamster Scrapie and mouse as recipient [8]. In all seeded fibrillation experiments, i.e. intraspecies as well as interspecies combinations, NaPTA-precipitate of brain tissue from non-infected animals served as controls and did not lead to an increased fluorescence read out within the timescale of the experiments (fig. 2 blue lines).

Figure 3 gives an overview of the fluorescence readouts of various seed - substrate combinations. The ThT-fluorescence intensities in the time period of 24 to 48 hours of incubation and after seeding were summarized. Interspecies transmission is obvious for five different combinations of PrPSc-seeds and recPrP substrate: OvPrPSc to SHaPrP(90–231), OvPrPSc to BovPrP(25–241), SHaPrPSc to OvPrP(25–233), SHaPrPSc to BovPrP(25–241) and BovPrPSc to OvPrP(25–233). In our system, approach two well known in vivo interspecies barriers [8] [16] could be simulated with our in vitro conversion system namely the species barrier of BSE to Syrian Hamster as well as of Syrian Hamster Scrapie to mouse. In summary, all of our in vitro intra- and interspecies transmission results resemble exactly the in vivo situation (table 1).

**Discussion**

Conversion of the cellular prion protein (PrPc) to PrPSc is the key event in prion infection [2]. Although many studies with
recPrP and with different conversion systems have been carried out the molecular mechanism is still not well understood [17] [18,19]. These studies are based mainly on the simulation of the structural properties of PrPSc like β-sheet content, PK-resistance or morphology of amyloid fibrils. However these systems have not generated infectious PrP or only very low titers of infectivity were generated spontaneously [14]. Recently, the group of S.B. Prusiner and colleagues could show that subtle variations in the structure of in vitro generated fibrils give rise to a variety of infectious preparation with distinct strain properties [20]. In contrast to spontaneous fibrillization seed-dependent assays like protein misfolding cyclic amplification (PMCA) [21] and quaking-induced conversion (Quic) [22] were established and are in very good agreement with the infectious etiology of prion diseases. Both assays are carried out in cellular extracts from uninfected animals or cells, which cannot exclude the involvement of cellular compounds in the conversion reaction.

Our group established a SDS-based conversion assay that works without cellular extracts, using solely purified compounds, like phosphate buffer, recPrP as substrate and prepurified PrPSc as seed [11]. The use of partially denaturing conditions in conversion of recombinant PrP into amyloid with physiochemical properties reminiscent of PrPSc seems to be a general concept in in vitro conversion studies of PrP and were studied in greater detail [18,22]. Beside those classical protein denaturants other compounds have been identified (e.g. glycosaminiclycans and oligonu-

Figure 1. Characterization of spontaneous OvPrP(25–233) fibril formation within the in vitro conversion system. A: Dependence of OvPrP(25–233) amyloid formation on SDS-concentration. OvPrP(25–233) was incubated in 10 mM NaPi pH 7.4, 250 mM NaCl and 0.01–0.05% SDS. The amount of fibril formation was measured by ThT-fluorescence. Thioflavin T was added to a final concentration of 5 μM to 10 ng/μl OvPrP(25–233). B: Electron micrographs show the typical structure of amyloid fibrils after 7 days of incubation of OvPrP(25–233) in 10 mM NaPi pH 7.4, 250 mM NaCl 0.02% SDS (bar = 20 nm). C: Secondary structure analysis of the pre-amyloid state. CD-spectra were measured directly after adapting the SDS conditions, with a final concentration of 150 ng/μl OvPrP(25–233) in 10 mMNaPi pH 7.4 and 250 mMNaCl. D: Sedimentation equilibrium centrifugation of OvPrP(25–233) after 7 days of incubation. (left) Experimental data overlaid by the fitted curves (right) residuals.

doi:10.1371/journal.pone.0014283.g001
cleotides) to promote PrP conversion in vitro but their structural influence on PrP remains unknown [23,24].

PMCA and Quic were mainly developed for diagnostic purposes, our system with the well defined components was developed to describe quantitatively the prion propagation mechanism using hamster PrP. This SDS-based conversion assay was applied to additional species, in order to test if our conversion system can be used with prion proteins of different species and if the pre-amyloid state described with hamster PrP represent a general mechanism for amyloid formation in our in vitro conversion system. Furthermore, by combining seed and substrate from different species we have the opportunity to simulate the phenomena of species barriers for the first time on the level of a direct molecular interaction of prion seed and recPrP substrate without the influence of any other cellular component. In the presented study we were able to show that amyloid fibrils of recPrP can be formed within the SDS-based conversion system for all species investigated (cattle, sheep, mouse, hamster). Only the SDS concentration had to be adapted. These results and the properties of the intermediate state will be discussed on a more detailed level later. The phenomenon of species barrier for prion transmission was successfully modeled as seed dependent in vitro fibrillization. Consequently, also a strict species barrier was found in the in vitro simulation (fig. 3). For murine substrate, only one positive transmission (MuPrPSc to MuPrP(89–231)) and one negative (SHaPrPSc to MuPrP(89–231)) could be performed for reasons of shortage of mouse prions in our lab. As a main result of our work we found that the in vitro simulation of the species barrier is in complete agreement with the experimental data from in vivo transmission studies (Tab. 1). Similar results on interspecies transmission and species barrier were reported by studies with PMCA [25] and by Cashman and co-workers [26], whereby both systems use cellular extracts. Because our conversion system does not include cellular extracts we conclude that the species barrier is encoded within the direct interaction of PrPSc and PrPC [27].

The interpretation of our results on the molecular level shows the importance of partial denaturation of recPrP as substrate as described by Stöhr et al. [11]. PrP in this intermediate or pre-amyloid state is soluble for weeks, suggesting that it is in a state of low free energy. Since it is present in a monomer-dimer-equilibrium one can argue that the partially denatured PrP is prone to intermolecular interactions possibly also with PrPSc. However in our conversion conditions PrP is in the state of lowest

Figure 2. Kinetics of intra- and interspecies seeded recPrP amyloid formation. Amyloid formation of recPrP was monitored using ThT-fluorescence assay in 10 mM NaPi (pH 7.4) with 250 mM NaCl (at 37 °C). In vitro intraspecies transmission: (A) Amyloid formation of 1.8 mM (40 ng/μl) OvPrP(25–233)-substrate seeded with OvPrPSc in 0.02% SDS (red line), (B) of 1.8 mM (30 ng/μl) MuPrP(89–231)-substrate seeded with MuPrPSc in 0.04% SDS (red line). In vitro interspecies transmission: (C) Amyloid formation of BovPrP(25–241)-substrate seeded with OvPrPSc in 0.02% SDS (red line), (D) in presence of SHaPrPSc no MuPrP(89–231) amyloid formation occurs (red line). NaPTA-precipitate of corresponding same treated brain tissue from healthy animals: blue line.

doi:10.1371/journal.pone.0014283.g002
free energy if it is refolded for attachment to the fibrillar seed. We assume that this state is not present in measurable amounts in solution but only attached to the seed. The degree of denaturation in the intermediate state is critical; for different recPrP sequences, i.e. for different species, different SDS concentrations are needed. More than the optimal SDS concentration would lead to a more unfolded PrPC whereas less SDS leads to a more refolded PrPC state as compared to the optimal intermediate state. In both cases the conversion of PrP to fibrillar PrP would be too slow to be observable. For an interspecies transmission a partially unfolded state of the substrate PrP is required, but ΔG for substrate (PrP refolded in the complex) with the seed from the other species in the same way as for intraspecies transmission has to be sufficiently low, i.e. lower than in the intermediate state, to guarantee the transition. In summary not the intermediate state of substrate PrP is critical for transition but its potency to refold into a conformation well adapted to the PrPSc-seed.

In future experiments we will extend our experimental approach to other species like human CJD and cervid CWD. Due to the high occurrence of CWD in Northern America, the combination of CWD-seed and human PrP as substrate would be of particular interest.

**Materials and Methods**

**Recombinant Prion Proteins**

The recombinant prion protein (recPrP) was prepared and purified as described previously [28,29]. The recPrP with the amino acid sequence of Syrian Hamster (90–231) (SHaPrP) PrP as well as recPrP with the amino acid sequence of cattle (25–241) (BovPrP) PrP was used in our studies before [15]. We adopted the purification protocols to full length recombinant ovine PrP (25–233) (OvPrP). Recombinant murine PrP (89–231) (MuPrP) was acquired from Allprion (Schlieren, Switzerland).

**NaPTA precipitation of PrPSc**

PrPSc from brain tissue of different species was purified by NaPTA (Sodium phosphotungstate dibasic hydrate) precipitation [30,31] Additionally to Syrian hamster and bovine PrPSc (SHaPrPSc and BovPrPSc) [11,15] we adopt the purification protocol to ovine PrPSc (OvPrPSc) and to Murine PrPSc (MuPrPSc).

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![Table 1. In vivo vs. In vitro intraspecies and interspecies transmission and species barrier.](image)
The resulting pellet was resuspended in 10 mM NaPi by brief sonification (Sonicator, Labsonic U, Braun Diessel, Melsungen).

Circular dichroism spectroscopy
Circular dichroism (CD) spectra were recorded with a J-715 spectropolarimeter (Jasco, Easton, MD, USA) in a 0.1 cm quartz cuvette at room temperature. The scanning speed was 50 nm/min with resolution of 1 nm. For each sample 10 spectra were accumulated between 195 and 260 nm. The protein concentration was 150 ng/μl. Background spectra of buffer samples were subtracted from the respective protein spectra.

Analytical Ultracentrifugation
Sedimentation-diffusion equilibrium experiments were performed, as described in ref [11], in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter) applying standard 12-mm double-sector cells at 20°C. The data were analyzed by using the Global Fit procedure, which is implemented in the UltraScan II software package (Version 5.0 for UNIX) of B. Demeler (University of Texas Health Science Center, San Antonio, TX).

Electron microscopy, negative stain
A droplet of 5–10 μl containing the recPrP was placed on glow discharged grid and left to adsorb for 2 minutes. After adsorption to the grid surface the sample was washed briefly (50 μl of 0.1 and 0.01 M NH₄ acetate) and stained with 2% ammoniummolybdate (50 μl). The samples were analyzed with a Zeiss EM910 microscope at 80 kV.

Thioflavin T-Assay
Fluorescence emission spectra of Thioflavin T (ThT) were measured at a concentration of 5 μM ThT and 10 ng/μl recPrP in 150 μl 10 mM NaPi pH 7.4. The emission spectra were recorded from 460 nm to 630 nm with a fixed excitation wavelength of 455 nm, average of λₘₐₓ 495 to 505 is shown for a point time. Fibrillation kinetics were followed in 96 well plates according to Stöhr et al. [11]. All measurements were performed in a Tecan sapphire plate reader (Tecan Group, Männedorf, Switzerland).

The chosen regression line is polynomial fitted to original data points.

Spontaneous and seeded amyloid formation of recPrP
Spontaneous and seeded amyloid fibril formation of recPrP of the different species was monitored by ThT-assay as described above. The buffer conditions especially the SDS-concentrations were adjusted according to the amyloid forming conditions of the spontaneous case for each species. The results are displayed as sum of the average fluorescence intensities of 495 to 505 nm in the saturation phase (24 h to 48 h in 30 min interval) of the curve. To determine the specificity of the seeding effect in interspecies transmission or interspecies barrier NaPTA-precipitated PrPSc from brain tissue of infected and non-infected animals was compared.

Supporting Information
Text S1 Including supporting figures and tables. Found at: doi:10.1371/journal.pone.0014283.s001 (0.13 MB DOC)

Acknowledgments
We would like to thank our collaborators for providing us brain tissue of prion infected as well as non-infected animals of different species: Stanley B. Prusiner (UCSF, San Francisco, USA) provided hamster brain, Martin Groschup (Friedrich-Loeffler-Institut, Institute for Novel and Emerging Infectious Diseases, Greifswald/Insel Riems, Germany) provided cattle brain, Carsten Korth (University of Duesseldorf Medical School, Department Neuropathology, Germany) provided mouse brain, Olivier Andréddetti (UMR INRA-ENV, Physiopathologie Infectieue et Para-sitaire des Ruminants, Ecole Nationale Vétérinaire, Toulouse, France) provided sheep brain. We are thankful for the guidance and help in cloning and expression of recombinant PrP by Tommy Agynnin and Bernd Esters.

Author Contributions
Conceived and designed the experiments: GP DR EB. Performed the experiments: GP LL JS LNS. Analyzed the data: GP LNS DR DW EB. Contributed reagents/materials/analysis tools: JW. Wrote the paper: GP DR DW EB.

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