Quaternary structure convergence and structural diversification of prion assemblies at the early replication stage

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**Abstract**

Aggregation of host-encoded peptides or proteins into amyloid fibrils is key to the pathogenesis of a number of neurodegenerative disorders. In prion diseases, the cellular prion protein (PrPC) can misfold and auto-organise into conformationally diverse assemblies (PrPSc). While this structural variability is at the origin of the strain phenomenon, there is strong evidence for further PrPSc structural heterogeneity within a single strain, suggesting co-propagation of distinct PrPSc subsets. Accessing the processes of PrPSc formation and self-diversification has remained challenging, although these problematics are central to the prion replication paradigm.

Here, by using several prion strains, we found that the early replication steps *in vivo* and in cell-free amplification assays lead invariably to the formation of two structurally distinct PrPSc subsets. Exploring their kinetics of formation revealed the existence of two processes. The first one comprises a decrease of parental PrPSc diversity to generate solely these two subsets. The second one permits diversification by assisting the transformation of one subset into the other through PrPC consumption by an autocatalytic reaction. Our findings provide a mechanism for prion replication and diversification based on the dynamic character of PrPSc assemblies. Pour la présentation des résultats, je préfère la deuxième version (barrée) que j’ai un peu modifiée. Elle me parait plus précise en terme mécanistique que la première qui reste assez vague Here by using several prion strains, thus leading

FL : plus clair dans intro : The exploration of their kinetic of formation by PMCA and mathematical modeling reveal the existence of two processes of prion replication. The first process reduces the parental polydispersity present in the seed/template to generate mostly the small-sized A assemblies. The second one allows diversification of A assemblies into B assemblies. This secondary process is autocatalytic where B assists the transformation of A into B through the consumption/conversion of monomeric PrPC. Our findings reveal the dynamic character of the prion replication process with important implications for prion propagation, selection and adaptation to different environment.

**Introduction**

The prion paradigm unifies a number of age-related, incurable neurodegenerative disorders due to protein misfolding and aggregation in term of pathogenic mechanisms ([*1-4*](#_ENREF_1)). These disorders include human and animal forms of prion diseases, Alzheimer’s disease, Parkinson’s disease and Huntington’s disease. In principle, host-encoded monomeric proteins or peptides are converted into misfolded and aggregated assemblies, which serve as seed or template for further autocatalytic conversion. In prion diseases, the ubiquitously expressed, host-encoded prion protein PrPC is converted into a misfolded, β-sheet rich conformer termed PrPSc ([*5*](#_ENREF_5)). In susceptible host-species and in laboratory rodent models, PrPSc assemblies show a remarkable ability to form stable, structurally distinct PrPSc conformers ([*6-9*](#_ENREF_6)), known as prion strains, and encoding unique stereotypical biological phenotypes ([*10-13*](#_ENREF_10)). The strain-specific structural differences can be observed at the secondary structure level in terms of local structural variation but also at the quaternary level with strain-specific size distribution ([*11*](#_ENREF_11)*,* [*14*](#_ENREF_14)*,* [*15*](#_ENREF_15)). A large body of evidence supports the view for further structural diversity within specific prion populations and strains, including i) the selection of prion substrain during the transmission of natural isolates ([*16-18*](#_ENREF_16)) or experimental prion strains ([*19*](#_ENREF_19)) with species or transmission barrier, ii) size- or density-fractionation studies supporting the existence of a heterogeneous spectrum of PrPSc assemblies with distinct tertiary/quaternary structures ([*14*](#_ENREF_14)*,* [*15*](#_ENREF_15)*,* [*20-25*](#_ENREF_20)) and biological activity (templating activity and infectivity) ([*14*](#_ENREF_14)*,* [*15*](#_ENREF_15)*,* [*20*](#_ENREF_20)), iii) kinetic studies of prion pathogenesis suggesting that the formation of neurotoxic PrPSc species ([*26*](#_ENREF_26)) would be produced at late stage of prion infection, whereas replicative PrPSc assemblies would be formed at earlier stages ([*27*](#_ENREF_27)*,* [*28*](#_ENREF_28)). The prion replication process thus intrinsically allows a structural diversification of PrPSc assemblies.

While the kinetic aspects of prion replication ‘as a whole’ have been comprehensively described by measuring infectivity or PrPSc levels in the brain (see references ([*29*](#_ENREF_29)*,* [*30*](#_ENREF_30)) as examples), the mechanisms by which PrPSc structural diversification and formation of different subpopulations occur within a given strain remain undescribed in the actual framework of prion paradigm. The autocatalytic conversion model proposed by Griffith in 1967 ([*31*](#_ENREF_31)), the nucleated-polymerization model by Lansbury and Caughey in 1995 ([*32*](#_ENREF_32)) and other derived models as examples) merely assumed the existence of structurally homogenous assemblies having absolutely identical propensity to replicate all along the disease progression. Recent high-resolution structural analysis of the N-terminal domain of the yeast prion SuP35 tend to suggest that conformational fluctuations in natively disordered monomeric Sup35 were responsible for structural diversification of Sup35 aggregates ([*33*](#_ENREF_33)). This idea could be extrapolated to mammalian prion PrP in order to explain strain structural diversification and strain mutation ([*8*](#_ENREF_8)). However, based on the best replicator selection concept ([*34*](#_ENREF_34)*,* [*35*](#_ENREF_35)) it will not explain the coevolution of at least two structurally distinct PrPSc subassemblies within the same environment ([*36*](#_ENREF_36)*,* [*37*](#_ENREF_37)) .

In order to go deep inside into the molecular mechanisms of PrPSc structural diversification, we explored by sedimentation velocity (SV) based methods the early stage of the prion conversion *in vivo* and in a cell-free system called protein misfolding cyclic amplification (PMCA). This technique mimics *in vivo* prion replication with accelerated kinetics ([*38*](#_ENREF_38)). By using several prion strains as template, we demonstrate that the early stage of prion replication generates invariably two subsets of assemblies termed A and B, differing in proportion, size and structure. The exploration of their kinetic of formation by PMCA and mathematical modeling reveal the existence of two processes of prion replication. The first process reduces the parental polydispersity present in the seed/template to generate mostly the small-sized A assemblies. The second one allows diversification of A assemblies into B assemblies. This secondary process is autocatalytic where B assists the transformation of A into B through the consumption/conversion of monomeric PrPC. Our findings reveal the dynamic character of the prion replication process with important implications for prion propagation, selection and adaptation to different environment.

**Results**

Low-sized PrPSc assemblies are formed at early stage of prion replication

The early phases of prion replication are commonly thought to consist in an elongation/growing process ([*39*](#_ENREF_39)), the template serving as base. We studied the size distribution of proteinase K (PK)-resistant PrPSc (PrPres) assemblies at the early step of prion replication in the brain by sedimentation velocity (SV) in an iodixanol gradient, using a previously published methodology ([*15*](#_ENREF_15)*,* [*20*](#_ENREF_20)*,* [*40*](#_ENREF_40)). PrPSc size distribution at disease end stage served as control. Three different host PrP/strains combinations were studied, 127S cloned scrapie prion strain in ovine PrP tg338 transgenic mice ([*29*](#_ENREF_29)), 139A cloned mouse prion strain in mouse PrP tga20 mice ([*41*](#_ENREF_41)) and vCJD cloned human prion strain in human PrP tg650 mice ([*42*](#_ENREF_42)*,* [*43*](#_ENREF_43)). As shown in Figure 1A-C, small-sized assemblies sedimenting between fractions 1 to 4 were preferentially detected at the early stage of the pathogenesis, independently of the strain considered. A second population with a larger size distribution and peaking in fraction 8-10 and fraction 18 was detected for 127S. At disease end stage and for the 3 strains, the small size assemblies have mostly disappeared at the expense of larger size assemblies.

To determine if the early formation of small assemblies in the brain could be reproduced by an *in vitro bona fide* amplification method, we used a high-throughput variant of PMCA (i.e. mb-PMCA), generating in one unique round of 48h as much infectivity as in the brain at terminal stage of the disease with high reproducibility in terms of limiting dilution and amplification yield ([*44*](#_ENREF_44)*,* [*45*](#_ENREF_45)). The same three strains were used as seeds while brain homogenates from healthy mice in which these strains were passaged served as PrPC-containing substrates. The prion seeds were >105-diluted to avoid detection of input PrPSc in the PMCA products. When the size distribution of the amplified products was analysed by SV after one mb-PMCA round (4 independent mb-PMCA reactions), two discrete distributions were observed, for the three strains (Figure 1D). The post-PMCA sedimentograms revealed the existence of a major set of small assemblies (named A) sedimenting between fractions 1 to 3 and a minor set of larger assemblies (named B) well-defined by a gaussian distribution centred on fraction 15. The relative proportions of A and B varied amongst the three strains, B being barely detected in 139A amplicons. These data indicate that during mb-PMCA amplification, two populations of PrPSc assemblies are generated with a predominance of small assemblies.

The bimodal (i.e. generation of two sets of assemblies) and discrete behaviour of the size distribution as well as the formation of predominantly small assemblies A in the mb-PMCA condition could be a consequence of shearing forces during the sonication step ([*46-48*](#_ENREF_46)) rather than an intrinsic consequence of the replication process. To distinguish between these two possibilities, undiluted 127S seeds (i.e. 20% brain homogenate) were incubated and sonicated in identical mb-PMCA-conditions but without PrPC substrate (i.e. in PrP0/0 brain lysate). As shown in Figure 1E, the size distribution analysis of the sonicated 127S seeds in PrP0/0 substrate revealed mostly the presence of larger-sized assemblies, as observed upon solubilization at 37°C ([*20*](#_ENREF_20)), thus ruling out the PMCA conditions being mostly at the origin of small size assemblies during mb-PMCA with PrPC substrate.

Altogether, these observations suggest that *in vivo* the early phase of replication for 127S, 139A and vCJD prion strains generate mainly small-sized assemblies. The mb-PMCA amplification method leads to the generation of two sets of assemblies with a predominance of small size assemblies as at early stage of the *in vivo* replication.

The formation of A and B assemblies follows a cooperative process

We next asked whether B subassemblies results from a simple condensation of the A subset (Oswald ripening process , ([*49*](#_ENREF_49))) or are generated by an alternative templating pathway. We first examined the influence of the amplification rate on the formation of these two species by varying the concentration of the seed used to template the mb-PMCA reaction. We compared the SV-sedimentograms of the mb-PMCA products seeded with 10-3 to 10-10 diluted 127S brain homogenate. As shown in Figure 2A, the relative amounts of A assemblies decreased as those from B increased as a function of the seed concentration. The variation of A and B peak surface area as function of the logarithm of dilution factor revealed a quasi-linear decrease of A assemblies when B assemblies followed a sigmoidal increase (Figure 2B). The sigmoidal increase in B assemblies to the detriment of the quasi-linear decrease of A assemblies indicates that: i) the formation of B assemblies follows a seed-concentration dependent cooperative process and ii) B assemblies do not result from the simple condensation of A assemblies (Figure 2C (un schéma de processus sequential?)); in that case the increase in B would correlate with a decrease of A subset according to the same kinetic law.

To further explore the entanglement between A and B assemblies, we next fixed the mb-PMCA regime favouring the formation of A assemblies with high dilutions of the inoculum-seed followed by quiescent incubation at 37°C for variable periods (Figure 2D). As shown with 127S prions, the SV analysis at defined incubation time-points post-PMCA reaction revealed a decrease in the population of A assemblies in favour of B assemblies (Figure 2D). At 4h post-incubation, there was an equal proportion of A and B when at 24h and further most of the PrPres assemblies were in the form of B assemblies. The A to B transformation followed a bimodal behaviour (i.e. absence of assemblies of intermediate size), suggesting that the formation of B assemblies resulted from the association with a specific number of A assemblies. ~~The non-significant variation in the size of B assemblies at the early stage of their formation during the quiescent phase strongly suggests that B assemblies are the product of a reaction between A assemblies and other PrP conformer?? PrPSc, suPrP, PrPC?? co-sedimenting with A~~. Furthermore, the variation in the proportion of A and B assemblies as function of the logarithm of the time showed a sigmoidal inverse variation of A and B populations, suggestive of a catalytic formation of B assemblies during the quiescent phase (Figure 2E). Similarly, 139A and vCJD prions showed a bimodal evolution of A to B assemblies during a 24h-quiescent phase (Figure 2F), arguing in favour of a generic process of transformation from A to B.

To determine if the higher size translation shift from A to B was concerted with a structural rearrangement in the PrPSc assemblies, we determined the specific infectivity of A and B. A 127S-PMCA product was fractionated at the end of the reaction or after 48h of quiescent incubation. Pools of fractions corresponding to A and B peaks were inoculated to reporter tg338 mice. The specific infectivity (infectivity per PrP molecule) was calculated from the mean survival time by using 127S dose-response curves ([*15*](#_ENREF_15)). As shown in figure 2G, the specific infectivity of A assemblies was 50-100-fold higher than that of B assemblies. This value did not change on longer period of quiescent incubation (7 days, Figure 2G).

To determine whether B assemblies could further evolve, we extended the quiescent phase up to 30 days. For the three 127S, 139A and vCJD prion strains, the sedimentogram curves at 7 and 30 days showed a translational-shift of the B assemblies peak to higher fractions, indicative of an isokinetic-increase in their mean average size (Figure 3, left curves). The difference in the specific infectivity values of A and B assemblies did not change on longer period of quiescent incubation (7 days, Figure 2G, 127S strain). This size translation thus contrasts with the bimodal phase and highlights a change in the kinetic regime.

Collectively, these observations indicate A and B assemblies are structurally distinct. The formation of B assemblies is cooperative and result from a complex kinetic pathway. On longer quiescent incubation, a change in the kinetic regime occurred.

The formation of B assemblies requires the presence of PrPC during the quiescent phase

Our previous studies revealed that only ~30% of the PrPC substrate was converted into PrPSc after a complete round of mb-PMCA ([*44*](#_ENREF_44)*,* [*45*](#_ENREF_45)). To determine if the remaining 70% still participates to the transformation of A to B assemblies during the quiescent phase, PMCA products from 139A, 127S and vCJD prions were treated with PK to eliminate PrPC, before quiescent incubation at 37°C. As shown in Figure 3 (right curves), the amount of B assemblies generated during the quiescent incubation was drastically decreased for the three prion strains in the absence of PrPC substrate. Further quiescent incubation for 7 and 30 days in the absence of PrPC allowed the formation of low amounts of B assemblies for 127S and 139A prion strain. The fact that the transformation of A to B assemblies is strongly facilitated by the presence of PrPC  suggests that B assemblies result from the integration/conversion of PrPC into A assemblies. Apparition of B after a long time of incubation in context without PrPC may result of a leakage of momoner from a conformer co-sedimenting with A.

Kinetical scheme describing the transformation of A to B assemblies

To setup a kinetic mechanism and provide a molecular interpretation of the assemblies’ dynamics during the quiescent phase, a number of elementary steps were identified based on experimental observations and were used as unavoidable constraints (ref?). The first constraint is the existence of an equilibrium between PrPSc assemblies and their elementary subunit (suPrP), as previously shown ([*40*](#_ENREF_40)). The existence of this equilibrium makes the size distribution of PrPSc assemblies highly dynamic and dependent on the assemblies’ concentration as shown in figure 1E. Indeed, SV-analysis of 30-fold diluted 127S infected brain in PrP0/0 mouse brain lysate revealed a quaternary structure rearrangement with a shift in lower molecular weight assemblies according to the equilibrium:

where , and are respectively size *i* and *i-1* of PrPSc.

As the existence of suPrP is a generic property of prion strains ([*40*](#_ENREF_40)), the 2nd constraint leads us to assume that A and B assemblies are in equilibrium with their respective suPrP (denoted and ), however with distinct equilibrium constant and . Thus, at any moment of the process of transformation of A to B assemblies the following equilibrium should be respected:

*(1)*

*(2)*

The equilibrium constant and govern then the respective size distribution of A and B assemblies and thus the bimodal aspect of the curve. According to our previous SV calibrations with PrP oligomers and globular mass markers ([*15*](#_ENREF_15)), the size distribution of A and B subassemblies were fixed respectively: *iA*<5 and *iB* centred around 20 PrP-mers. Due to the limited resolution of SV fractionation for small assemblies, we assumed that *Ai* and suPrPB cosediment. The third constraint lays on the fact that the transformation of A to B requires PrPC and that the kinetic is cooperative as shown in figure 1E and 2. This cooperativity implicates that B subassemblies facilitate their own formation according to an autocatalytic process. This can be resumed by the following minimalistic autocatalytic process:

*(3)*

*(4)*

Where C is an active complex reacting with PrPC generating B assemblies. By considering that suPrPB can condensate into B2 (ref) and according to the equilibrium (2) one can establish the reaction model describing the formation of Bi assemblies from the neo-formed *suPrPB*:

*(5)*

*(6)*

Altogether, these six elementary steps constitute the reaction mechanism describing the transformation of A into B subassemblies species. In order to validate the designed mechanism, we translated these elementary reactions into time-dependent differential equations (for more detail see SIXX) and performed simulation using as initial condition the size distribution of PrPSc assemblies immediately after the cyclic amplification (Figure 2A). As shown in figure 4, the size distribution variation as function of time as well as the theoretical size distribution centroid present similar patterns as experimental data (Figure 2D) arguing in the favour of the validity of the autocatalytic pathway (process 3 and 4).

**Discussion**

The mechanisms of prion replication and the dynamics responsible for prion structural diversification in the infected host still remain unclear. In the actual framework of the prion paradigm, the templating process is admitted to occur at the prion assembly interface, leading to an increased size of the complex formed by the template:substrate, out of the fragmentation/depolymerization context. The atypical size distribution observed here at the early stage of the replication process for three distinct prion strains, where accumulation of small size assemblies dominates, contrasts with such canonical templating model and requires additional process considering the dynamic of the replication. Further, the existence of a multi-step conversion process constitutes an unexpected pathway to reconcile the best replicator selection paradigm and diversification process which is inherent to prion adaptation and evolution.

As shown *in vivo* for vCJD, 127S and 139A prion strains, the early stage of the replication process in the brain is dominated by the accumulation of small assemblies whereas higher size subsets are mostly detected at the terminal stage of the pathogenesis. Such quaternary structure diversity, - and beyond the existence of structurally distinct types of assemblies, as defined by their specific infectivity (([*15*](#_ENREF_15)*,* [*20*](#_ENREF_20)) and supplemental figure 1) -, can be exclusively explained by the existence of a balance between at least two kinetic modes taking place at different stages of the pathogenesis. Both could be governed by evolution or fluctuation in the replication micro-environment due to the physio-pathological state of the infected animal and/or to the sequential involvement of specific prion-replicating cell types. However, another possibility could lie in the intrinsic and deterministic properties of the PrP replication process to generate structurally distinct type of assemblies. Discriminating between these two non-mutually exclusive hypotheses is technically difficult to address *in vivo*. The mb-PMCA as a *bona fide* amplification method in a more simplified and kinetically controlled context constitutes a relevant method to investigate the intrinsic propensity of the replication process to generate structurally distinct assemblies. Interestingly and against the common belief, the size distribution of PrPSc assemblies used as seeds was relatively insensitive to mb-PMCA sonication cycles when a simple dilution displaced the assemblies towards smaller size (Figure 1E), as previously reported ([*40*](#_ENREF_40)). These two observations exclude the contribution of fragmentation process during the mb-PMCA sonication cycles to the size distribution pattern of PrPSc assemblies and emphasizes the existence of a constitutional dynamic between PrPSc subpopulation ([*40*](#_ENREF_40)), which should be considered during the replication process. We showed that two sets of PrPSc assemblies A and B were generated during the mb-PMCA reaction. The A and B assemblies constitute two structurally distinct PrPSc subpopulations as supported by their distinct specific infectivity, the bimodal size distribution instead of a continuum, the effect of initial seed concentration on the respective proportions of A and B and the role of PrPC in the transformation of A to B indicating that B assemblies are not resulting from a simple condensation of A assemblies. Therefore, the prion replication process *per se* intrinsically generates structurally diverse PrPSc subassemblies.

According to our SV experiments, small-sized PrPSc assemblies were mainly formed at the early stage of prion replication in the brain and during the mb-PMCA reaction. This was observed with three distinct prion strains (127S, 139A, vCJD) on 3 different PrP genetic backgrounds. Taking into consideration that PrPSc assemblies composing each strain are structurally distinct, one can question how distinct PrPSc assemblies could all generate assemblies A harbouring strain structural information while showing the same quaternary structure (at the SV resolution)? The first explanation could be the existence of common narrow subpopulation of PrPSc (with respect to their quaternary structure) within the three strains serving as best replicator and participating to the formation of A assemblies. However, the PrPSc quaternary structure subset exhibiting the highest specific infectivity *in-vivo* (i.e. the best replicator) can be associated with either small size assemblies (i.e. 127S and 139A, supplemental figure 1 5A and ([*15*](#_ENREF_15)*,* [*20*](#_ENREF_20))) or high molecular weight assemblies (i.e. vCJD, Figure 5B) and is therefore strain-dependent. The existence of a structurally common PrPSc subpopulation is thus unlikely to be at the origin of the generic formation of a small size subset in the brain or A assemblies in the mb-PMCA condition. Intrinsically, the early steps of the replication process favour the emergence of mainly one subspecies A with a highly narrowed size distribution, arguing in favour of a quaternary structure convergence phenomenon during these steps. This structural convergence concerns the PrP domain governing the polymerization (the size of assemblies). However, as the A assemblies harbour the strain structural determinant, one can conclude that A assemblies present certain degree of structural variability allowing the strain structural information encoding (Figure 5A).

All along the quiescent phase and for the three prion strains studied, the A assemblies constitute the precursor species in the formation of B assemblies. Furthermore, the presence of PrPC is required for the evolution of A into B assemblies. This quaternary structure evolution is concerted with a specific infectivity decrease indicative of a structural rearrangement during the transformation of A to B. Even if the first event conducing to the formation of B assemblies remains undetermined, we can assume that A could have the intrinsic propensity to spontaneously evolve into B assemblies in the presence of PrPC (Figure 5B). The cooperative disappearance of A in favour of B assemblies strongly suggests an autocatalytic process (reaction 3 and 4). This last phenomenon evidences the existence of a secondary autocatalytic process until now undescribed in the canonical prion replication process (ref). It could be reasonably envisaged that A could have the intrinsic propensity to generate B assemblies in the presence of PrPC assemblies with a very low efficiency. This parallel pathway to the autocatalytic process could then explain how first set of B assemblies are generated (Figure 6B). The existence of a secondary autocatalytic process could have a crucial importance for the maintaining of PrPSc structural diversity all along the evolution of the pathology. In the absence of this secondary autocatalytic process, e.g. in the absence of PrPC, the system will select the best replicator and the most thermodynamically stable assemblies. Its presence makes the system escaping this rule, allowing the specific accumulation of the autocatalysis product (here the B assemblies) rather than the most thermodynamically stable or high specific infectivity assemblies. This phenomenon could explain why for certain prion strains the most infectious assemblies accumulate as minor population while those with the lowest specific infectivity mostly accumulate ([*15*](#_ENREF_15)*,* [*20*](#_ENREF_20)).

Conclusion

The early step of prion replication for at least three distinct prion strains leads to the formation of small assemblies. The mb-PMCA approach clearly demonstrates the intrinsic properties of the *bona fide* replication process to generate at least two structurally distinct PrPSc subassemblies. The deterministic aspect of the replication process to generate a structurally diverse set of assemblies contrasts with the widespread idea considering the prion diversification process within a given strain (often referred to as the creation of prion quasi-species) as a stochastic event and cogoverned by environmental fluctuations ([*9*](#_ENREF_9)). The secondary autocatalytic pathway leading to the formation of B subassemblies could participate to prion adaptation during transmission events with species barrier. By considering that the transmitted inoculum initially contains A and B assemblies, the autocatalytic conversion process of B could kinetically drive the adjustment and integration of the new-host PrPC in order to generate host adapted B assemblies. This hypothesis is supported by our recent observations where complementation between A and B subassemblies is required to cross existing species barriers (accompanying article).

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**Methods**

Ethics

Animal experiments were conducted in strict accordance with ECC and EU directives 86/009 and 2010/63 and were approved by the local ethics committee of the author’s institution (Comethea; permit numbers 12/034 and 15/045).

Transgenic mouse lines and prion strains

The ovine (tg338 line; Val136-Arg154-Gln171 VRQ allele), human (tg650 line; Met129 allele) and mouse (tga20) PrP transgenic lines have been described previously ([*29*](#_ENREF_29)*,* [*41*](#_ENREF_41)*,* [*42*](#_ENREF_42)). These mouse lines are homozygous and overexpress about 8-, 6-, and 10-fold the heterologous PrPC level on a mouse PrP-null background, respectively. PrP0/0 mice were the so-called Zürich-I mice ([*50*](#_ENREF_50)). Cloned 127S scrapie, human vCJD and mouse 139A prion strains have been serially passaged on tg338, tg650 and tga20 mice, respectively ([*44*](#_ENREF_44)*,* [*45*](#_ENREF_45)). These strains were used as pools of mouse-infected brains, prepared as 20% wt/vol homogenate in 5% Glucose by use of a tissue homogenizer (Precellys 24 Ribolyzer; Ozyme, France).

Time course analysis of prion accumulation

Eight-week-old female tg338, tg650 and tg20 mice were inoculated either intracerebrally in the right cerebral hemisphere with 127S, vCJD and 139A prions (20 μl of a 10% brain homogenate dose). Infected animals were euthanized by cervical column disruption in triplicate at regular time points and at terminal stage of disease. Brains were removed and kept for PrPSc size-fractionation.

Miniaturized bead-PMCA assay

The miniaturized bead-PMCA assay ([*16*](#_ENREF_16)*,* [*40*](#_ENREF_40)*,* [*45*](#_ENREF_45)) was used to amplify prions. Briefly, serial ten-fold dilutions of 127S, vCJD and 139A prions (mouse brain homogenates diluted in PMCA buffer) were extemporarily mixed with brain lysates (10% wt/vol) from healthy tg338, tg650 and tga20 mice as respective substrate and submitted to one round of 96 cycles of 30s-sonication (220-240 Watts) followed by 29.5 min of incubation at 37°C. PMCA was performed in a 96-well microplate format using a Q700 sonicator (QSonica, USA, Delta Labo, Colombelles, France). The amplified products were kept for PrPSc size-fractionation and aliquots were PK-digested (115 μg/ml final concentration, 0.6% SDS, 1h, 37°C) prior to immunoblot analyses, as described below .

Sedimentation velocity fractionation

SV experiments were performed as described previously ([*15*](#_ENREF_15)*,* [*20*](#_ENREF_20)*,* [*40*](#_ENREF_40)). Mouse brain homogenates or PMCA products were solubilized by adding an equal volume of solubilization buffer (50 mM HEPES pH 7.4, 300 mM NaCl, 10 mM EDTA, 2 mM DTT, 4% wt/vol dodecyl- β-D-maltoside (Sigma)) and incubated for 45 min on ice. Sarkosyl (N-lauryl sarcosine; Fluka) was added to a final concentration of 2% wt/vol and the incubation continued for a further 30 min on ice. 150 μl of solubilized samples were loaded atop a 4.8 ml continuous 10–25% iodixanol gradient (Optiprep, Axys-Shield), with a final concentration of 25 mM HEPES pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.5% Sarkosyl. The gradients were centrifuged at 285 000 g for 45 min in a swinging-bucket SW-55 rotor using an Optima LE-80K ultracentrifuge (Beckman Coulter). Gradients were then manually segregated into 30 equal fractions of 165 μl from the bottom using a peristaltic pump and analyzed by immunoblotting or bioassay for PrPSc or infectivity content, respectively. To avoid any cross-contamination, each piece of equipment was thoroughly decontaminated with 5 M NaOH followed by several rinses in deionized water after each gradient collection ([*20*](#_ENREF_20)).

Analysis of PrPSc content by immunoblots

Aliquots of the SV-fractionated PMCA samples were treated with PK (50 μg/ml final concentration, 1h, 37°C) before mixing in Laemmli buffer and denaturation at 100°C for 5 min. The samples were run on 12% Bis-Tris Criterion gels (Bio-Rad, Marne la Vallée, France) and electrotransferred onto nitrocellulose membranes. In some instances, denatured samples were spotted onto nitrocellulose membranes using a dot-blot apparatus (Schleicher & Schuell BioScience (Whatman)). Nitrocellulose membranes were probed for PrP with 0.1 μg/ml biotinylated anti-PrP monoclonal antibody Sha31. Immunoreactivity was visualized by chemiluminescence (GE Healthcare). The protein levels were quantified with the ImageLab software, after acquisition of chemiluminescent signals with a Chemidoc digital imager (Bio-Rad, Marnes-la-Coquette, France). For all SDS-PAGE analyses, a fixed quantity of human recombinant PrP was employed for consistent calibration of the PrP signals in different gels.

Fit of the sedimentograms

Bioassays

Pool of fractions of interest was extemporarily diluted ten-fold in 5% glucose and immediately inoculated by intracerebral route to reporter tg338 mice (20 μl per pool of fraction, n = 5 mice per pool). Mice showing prion-specific neurological signs were euthanized at end stage. To confirm prion disease, brains were removed and analysed for PrPSc content using the Bio-Rad TsSeE detection kit ([*17*](#_ENREF_17)) prior to immunoblotting, as described above. The survival time was defined as the number of days from inoculation to euthanasia. To estimate what the difference in mean survival times means in terms of infectivity, strain-specific curves correlating the relative infectious dose to survival times were used, as previously described ([*15*](#_ENREF_15)) .

Mathematical modeling

Marie / Human

**Legends**

**Figure 1. Size-distibution of PrPSc assemblies from different prion strains at early and late stage of the pathogenesis in vivo and after PMCA reaction**

The size distribution of proteinase K (PK) resistant PrPSc assemblies present in the brain *in vivo* (**A-C**) and in PMCA products (**D-E**) was examined by sedimentation velocity (SV).

(**A-C**) For the *in vivo* sedimentograms, brains from ovine (tg338), murine (tga20) and human (tg650) transgenic mice inoculated with 127S scrapie prions (**A**), 139A mouse prions (**B**) and vCJD human prions (**C**) were collected (as triplicates) at early stage (15, 11 and 120 days post-infection for 127S, 139A and vCJD prions, respectively, blue curves) and end stage of the disease (60, 55 and xxx days for 127S, 139A and vCJD prions, respectively, red curves). The brains were solublized and SV-fractionated. The collected fractions (numbered from top to bottom) were analyzed for PK-resistant PrPSc content by immunoblot.

(**D-E**) For the sedimentograms from PMCA products with PrPC substrate (**D**), the same strains were submitted to a single round of mb-PMCA by using as seed for the reaction 10-5 (139A) or 10-6 (vCJD, 127S) diluted brain homogenates. Thirty minutes after the last sonication, the amplified products were solubilized and SV-fractionated. The mean levels of PK-resistant PrPSc per fraction have been obtained from the immunoblot analysis of *n=4* independent fractionations. The PrPSc assemblies sedimentating in the top and middle fractions were termed A and B, respectively. For the sedimentograms from PMCA products without PrPC substrate (**E**), undiluted 127S infected tg338 brain (20% w/v, red curve) or 1:32 diluted in PMCA buffer (blue curve) were used as seed, mixed with brain homogenate from PrP0/0 mice as substrate and submitted to a single round of mb-PMCA before SV-fractionation (*n=2* independent fractionations).

**Figure 2. Seed concentration and time dependent dynamic evolution of the PMCA-generated PrPSc assemblies**

(**A-B**) SV profile of mb-PMCA products seeded with tenfold dilutions from 127S-infected brain homogenates, as indicated. Thirty minutes after the last sonication, the amplified products were solubilized and SV-fractionated. The mean relative levels of PK-resistant PrPSc per fraction (**A**) were obtained from the immunoblot analysis of *n=4* independent fractionations (representative dot-blot shown). Variation of A and B peak surface area as function of the logarithm of the seed dilution factor (**B**).

(**C**) Processus sequentiel scheme

(**D-E**) PK-resistant PrPSc sedimentograms from PMCA products generated with 127S prions (10-5 dilution) and further incubated at 37°C during the indicated quiescent phase (t), i.e. without sonication. At each time point, the collected products were frozen. All the collected samples were then thawed, fractionated in parallel by SV and analysed by immunoblot (**D**, *n=3* independent experiments, representative dot-blot shown). Evolution of the percentage of A and B populations (A+B=100%) as function of the quiescent phase post-PMCA reaction (**E**).

(**F**) PK-resistant PrPSc sedimentograms from PMCA products generated with 139A and vCJD prions seeds (10-5 dilution) and further incubated for a quescent period of 24h at 37°C.

(**G**) Specific infectivity of the A and B assemblies post-PMCA reaction and after quiescent incubation. Fractions corresponding to the A peak (fractions 1-3) and B peak (fractions 14-16 (days 0 and 2) or 16-18 (day 7)) were pooled and inoculated to groups of reporter tg338 mice at two different dilutions (1:10 and 1:1000) for better accuracy. The specific infectivity of the assemblies was calculated from the mean survival time of the mice by using 127S dose-response curve. \*: incomplete attack rate.

**Figure 3. PrP-dependent generation of B assemblies from A assemblies**

PMCA products from 127S, 139A and vCJD prions (105, 104 and 104 diluted seeds, respectively) were treated with PK (80 μg/ml final concentration, treatment stopped by adding 2mM Pefabloc and 1x EDTA-free Protease inhibitor cocktail) or not to eliminate PrPC, before quiescent incubation at 37°C for 24h, 7 days and 30 days, as indicated. At each time point, the collected products were frozen. All the collected samples were then thawed, SV-fractionated in parallel and analyzed by immunoblot (*n=3* independent experiments).

**Figure 4. Mathematical modeling**

**Figure 5. Quaternary structure convergence and autocatalytic formation of B assemblies**

**(A)** Different prion strains (S1, S2 and S3) give rise to the formation of common oligomeric assemblies termed A with a narrowed size distribution during mb-PMCA reactions. This common quaternary structure convergence at the early stage of the replication process suggests the existence of a common conversion pathway and a common oligomerization domain independent of the strain structural determinant (SSD, represented in red).

(**B**) The A species evolve into B assemblies according to two pathways. The first one (blue arrow) is the spontanous formation of B assemblies in the presence of PrPC. This pathway could explain the initial formation of B assemblies. The second pathway (red arrow) corresponds to the autocatalytical conversion of A assemblies by B in the presence of PrPC je ne comprends pas ce processus??. A and B assemblies are structurally different as jugged bytheir specfic infectivity. De mon point de vue, le schéma n’est pas très explicite. Les seules différences apparentes entre les A et les B c’est la couleur alors qu’on montre bien une différence de taille qui n’est pas évidente pour un lecteur externe. Autant je conçois la voie bleue, j’ai du mal à voir à quoi la voie rouge correspond-elle réellement au niveau mécanistique ?? Je préfère plutôt le modèle en triangle qui illustre mieux les propos du papier, et il intègre même la suPrP; enfin c’est juste ma perception des choses.

**Supplemental figure 1. PK-resistant PrPSc and infectivity sedimentation profile of 139A and vCJD prion strains**

Brain homogenates from tga20 mice infected with 139A prions (**A**) and tg650 mice infected with vCJD prion (**B**) were solubized and SV-fractionated. The collected fraction were analyzed for PK-resistant PrPSc content (black line) and for infectivity (red bars or line) by an incubation time bioassay in reporter tga20 and tg650 mice. The mean survival times values in these mice was reported to standard dose response curves (([*51*](#_ENREF_51)) and unpublished) to determine relative infectious dose values. A relative infectious dose of 0 corresponds to animals inoculated with 2mg of infectious brain tissue.

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