Folia Neuropathologica

Thoughts on mammalian prion strains

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Folia Neuropathol 2009; 47 (2): 104-113

Abstract

A plethora of prion strains can be propagated indefinitely in hosts homozygous for the PrP gene. Within the framework of the "protein-only" hypothesis, the strain-specific properties are enciphered in the conformation of the strain-associated PrP^{Sc}. Are these conformations codetermined by additional components, whose presence or absence within an infected cell could define the cell's competence to replicate a particular strain? Which cellular components, if any, contribute to the PrP^{C}-to-PrP^{Sc} conversion in the cell? Many questions still remain to be answered in the field launched and nurtured by Carlton Gajdusek, to whom this essay is dedicated.

Key words: *CDI, conformation-dependent immunoassay, conformation templating, conformational population distribution, conversion, PrP^{C}-to-PrP^{Sc}, CPA, cell panel assay, Gajdusek, glycoform, PMCA, protein misfolding cyclic amplification, protein-only, SSCA, standard scrapie cell assay, strains, TSE, transmissible spongiform encephalopathy.*

Carlton Gajdusek was one of the most brilliant and entertaining individuals I have encountered. Given a keyword, he would enlarge upon it, drawing on his encyclopaedic knowledge, illustrate it with personal anecdotes that would lead to a new theme, in a monologue that could last for hours and would be continued from a distance with long, handwritten letters and volumes of his memoirs. It is with pleasure that I dedicate this review to the memory of the extraordinary scientist who laid the foundations of prion research (albeit not in these terms).

The nature of the agent causing transmissible spongiform encephalopathies (TSEs), the prion, has been debated since the mid-1960s. The concept of a "slow" or "unconventional" virus [32] as well as that of a "virino", an infectious agent proposed to consist of an agent-specific nucleic acid enveloped in a host-specified protein [45], lost support as intense efforts in many laboratories failed to identify a TSE-specific nucleic acid or even a nucleic acid long enough to encode a small protein [82,85]. On the other hand, the biochemical linkage of infectivity and PrP^{Sc}, an abnormal form of the normal host protein PrP^{C}, as well as the linkage between the PrP gene and both familial prion diseases [40,65] and susceptibility to prions [78,88], provided support for an updated version of the "protein only" hypothesis [37], namely that the infectious agent is an abnormal conformer of PrP, named PrP^{Sc}, that "multiplies" by catalyzing the conversion of PrP^{C} into a likeness of itself [76]. The finding that PrP knockout mice were resistant to scrapie [13] fulfilled a central prediction of the "protein-only" hypothesis and significantly promoted its acceptance, without however proving it.

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The discovery that different strains of prions can be propagated in inbred mouse strains expressing but a single PrP sequence, and the finding that prion strains were selective in regard to the cells in which they can replicate, raised two distinct questions: (1) How is strain specificity encoded by the prion; and (2) how can cells distinguish between different prion strains, as reflected by their ability to propagate them? Whereas strain-specific properties are associated with distinct conformations of PrP [17,19,76], these conformations may be co-determined by other factors, such as the N-linked glycans [17,19] or by cell-derived components, whose presence or absence within an infected cell might co-determine its competence to replicate a particular strain [101].

Normal and abnormal forms of PrP

Mature murine PrP^{c} consists of 254 amino acids, is encoded within one exon of a single-copy gene and is expressed in almost all tissues, albeit preferentially in the brain [6]. PrP^{c} is highly susceptible to proteinase K (PK) digestion, is attached to the outer surface of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor and can be readily cleaved off with phosphatidylinositol-specific phospholipase C (PIPLC). PrP^{c} may carry two, one or no asparagine-linked glycans, of which there are 52 or more variants [28,83, 84]. Prion-infected brain or cell cultures contain conformers of PrP that differ from PrP^{c} and are collectively designated as PrP^{Sc}. One form of PrP^{Sc}, designated $rPrP^{Sc}$ (or PrPres), is rich in beta-sheet structure and partially resistant to PK, in contrast to PrP^{c}, which is rich in alpha-helical structure [50,81], and sensitive to PK. Infectivity is invariably associated with multimeric forms of PrP [91] and is irreversibly lost after treatment of infectious preparations with denaturants [77]. Beside $rPrP^{Sc}$ there are also disease-related PrP species that are susceptible to standard PK digestion at 37°C, termed $sPrP^{Sc}$ ("protease-sensitive PrP^{Sc}") [73,74,85-87,95]. $sPrP^{Sc}$ differs from PrP^{c} in that it is precipitable by antibodies reacting with $rPrP^{Sc}$ but not with PrP^{c}, such as 15B3 [52,70]; moreover, certain epitopes (90-125 in mouse PrP) that are exposed in PrP^{c} are occluded and only revealed after denaturation, as measured by the conformation-dependent immunoassay (CDI) [87]. It has not been established whether $sPrP^{Sc}$ is infectious. The three-dimensional structure of $rPrP^{Sc}$ has not been determined but models based on various physical measurements have been proposed [36].

Replication of prions

The protein-only hypothesis proposes that prions consist of a conformational isomer of PrP^{c}, and that their replication comes about by an autocatalytic conversion of PrP^{c} to the pathogenic isoform PrP^{Sc}. The "nucleation model" predicates that PrP^{c} is in equilibrium with PrP^{Sc} (or a precursor thereof), with the equilibrium strongly favoring PrP^{c}, and that PrP^{Sc} is stabilized only when it adds to a crystal-like seed of PrP^{Sc} [33,41]. Cleavage of aggregates is postulated to explain the exponential increase of PrP^{Sc} during infection [72,92]. The nucleation model is most convincingly supported by the protein misfolding cyclic amplification (PMCA) reaction, which converts PrP^{c} into infectious PrP^{Sc} by multiple cycles of sonication and incubation [15,31], but also by studies of "yeast prions" [92,96,103]. PMCA allows extensive replication of infectious prions in a cell-free system containing highly purified (but not completely pure) PrP^{c} and poly(A) or poly(dT) [24]. These experiments should definitively lay to rest the perennial proposal that the infectious agent is a virus [64], even though prions may be secreted from cells in particulate form, as exosomes [1,30,98].

De novo generation of prions

The occurrence of sporadic CJD in humans has been attributed to a rare spontaneous conversion of PrP^{c} to PrP^{Sc}, which then becomes self-propagating and transmissible. Similarly, apparently spontaneous rare events in sheep and cattle may give rise to "atypical" forms of scrapie and bovine spongiform encephalopathy, respectively [2,9]. Spontaneous generation of prions occurs in transgenic mice bearing a mutated prion gene [89]. Legname et al. reported that injection of a beta-sheet-rich fibrillar preparation derived from recPrP into mice overexpressing PrP resulted in transmissible neurological disease [54,55]. Finally, it has been reported that prions can be generated in unprimed PMCA experiments [3,24]. These very different lines of evidence argue quite persuasively that prions can be generated *de novo*.

Prion species

A prion species is defined by the amino acid sequence of the donor's PrP. Transmission of prions between different animal species frequently results in low attack rates and long incubation times, which are abrogated upon repeated transmission to the

recipient species. The so-called "species barrier" is ascribed to differences in the PrP sequences between prion donor and recipient that hinder the accretion of host PrP^{C} to the incoming $rPrP^{Sc}$ seed [20]. In the case of trans-species prion transfer to mice, the barrier is often, but not always, overcome by replacing the murine PrP genes by their counterpart from the donor, for example Syrian hamster [78], cattle [51], human [52], or cervids [11]. Replacing the murine PrP gene by its counterpart from another species does not recreate the environment of the donor species; many genes other than PrP play a role in susceptibility to prions [56,57,66,68], and Prusiner and colleagues have proposed a critical role for an as yet hypothetical host-derived "protein X" in prion replication, which could be a chaperone facilitating conversion [43,94, 105]. A "species barrier" may also come about within the same animal species; for example, there are two distinct PrP alleles in different mouse lines, the *Prnp*^{a} (108L, 189T) and the *Prnp*^{b} allele (108F, 189V), and transfer of prions between mice with differing PrP alleles is subject to a similar barrier as is transfer between different animal species [67].

Prion strains

Prion populations differing in phenotypic properties, such as pathogenic potential or physicochemical characteristics, but associated with PrP^{Sc} having the same amino acid sequence, constitute distinct strains. A large number of different prion strains, originally characterized by the incubation time and the neuropathology they elicit, can be propagated indefinitely in hosts homozygous for the PrP gene (*Prnp*) [12]. Many of the "classical" prion strains currently propagated in mice and hamsters originated from scrapie-infected sheep or goats [26], but distinct strains occur also in man [21] and cattle [7]. The "protein-only" hypothesis proposes that the strain-specific properties of the prion are enciphered exclusively in the conformation of the cognate PrP^{Sc} [76]. Indeed, in many instances different strains are associated with PrP^{Sc} species differing in physicochemical properties such as susceptibility to PK digestion [53], electrophoretic mobility following PK treatment (reflecting different cleavage sites in the amino proximal region) [8,21,93], stability towards denaturation agents [74] or the ratio of di, mono and unglycosylated forms [21]. The conformation-dependent immunoassay (CDI) provides a sensitive tool for differentiating between different conformations of PrP associated with distinct prion strains [5,86,87]. The protein-only hypothesis thus postulates that each prion strain is associated with a different isoform of PrP that can convert PrP^{C} to a likeness of itself. The idea that a protein may assume dozens of different stable conformations appears bizarre if one thinks in terms of functional enzymes, but less so if one considers stable polymers or crystals [29]. The concept of "conformation templating" at the protein level is supported by the cell-free conversion experiments of Caughey and his colleagues [16,58], the seeding experiments with PrP^{C} [42,97] and most convincingly by the PMCA-mediated faithful propagation of prion strains [14].

Experiments with "yeast prion" strains have shown that conformational specificity can be propagated in vitro by pure, unglycosylated proteins [48,92]. Nonetheless, in view of the great multiplicity of mammalian prion strains and the apparent propensity of strains to multiply more efficiently in some cell types than in others, it is conceivable that post-translational modifications of PrP, such as glycosylation, sulfoxidation [22], proline cis-trans isomerism [18] or, more likely, association with some cellular components [101] might favor certain PrP conformations and hence the preferential propagation of particular strains.

When a prion strain is transferred from one host species to another and subsequently returned to and propagated for several passages in the original host species, it may in some instances change, or "mutate". For example, mouse-derived 139A passaged through hamster and subsequently propagated repeatedly in mouse resulted in a new strain, 139A-H2M; however, Me7 subjected to the same procedure remained apparently unchanged [46,47]. Legname et al. reported that a plethora of distinct prion strains, as characterized by incubation time and stability toward guanidinium chloride, were generated by passaging prions generated in mice inoculated with polymerized recombinant PrP to mice overexpressing wild type PrP [55].

Propagation of prions in cultured cells

In vivo, prions and PrP^{Sc} are accumulated mainly in neurons, follicular dendritic cells of the spleen and glia; in cell culture, susceptibility to persistent infection has been described not only for cells of neuronal and glial origin, but also for fibroblastic and epithelial cells, inasmuch as they express the PrP sequence corresponding to the prion donor (*84, 85*) [4].

Cell lines exposed to prions can become acutely infected, as judged by the production of $rPrP^{Sc}$ during a few days, but persistent propagation of infectivity and $rPrP^{Sc}$ occurs only in a subset of these lines [99].

N2a neuroblastoma cell populations are susceptible to persistent infection by mouse prions (Chandler strain), albeit with low efficiency [71], which means that they have to be exposed to high concentrations of prions and that only a small fraction of the population becomes persistently infected [79,80]. It is however possible to select from N2a populations subclones that are susceptible to infection at much lower prion concentrations and that yield a higher proportion of infected cells [10,27,49,71]. Although Nishida et al. selected their clone from transgenic N2a cells that overexpressed PrP, this feature is not required for enhanced susceptibility [27,49,100].

Prion strain typing by the cell panel assay (CPA)

Classically, the distinction between mouse-adapted prion strains requires determination of incubation times in at least two mouse lines, that extend over 6-10 months. We have developed a cell-based assay, the cell panel assay (CPA), which can distinguish between various murine prion strains within less than 2 weeks [62].

The CPA is based on the standard scrapie cell assay (SSCA), a method for the rapid and sensitive quantification of prions in vitro. In a typical SSCA, 5000 cells are exposed to a serial dilution of a prion preparation for 3 days and after reaching confluence, propagated for 3 splits. After reaching confluence 20 000 cells are filtered onto the membranes of 96-well filter plates. The dried cells are lysed, PK-digested, denatured with guanidinium thiocyanate and PrP^{Sc} is visualized by ELISA. $rPrP^{Sc}$-positive cells present as discrete spots and are counted using the Zeiss KS Elispot system. The proportion of $rPrP^{Sc}$-positive cells ("spots/20 000 cells") is plotted against the logarithm of the brain homogenate dilutions (Fig. 1) [49,63]. The "response index" (RI) of a cell line to a particular prion strain is the reciprocal of the dilution that gives 300 "spots" (or 1.5% of plated cells, a value lying in the linear part of the response curve) under standard assay conditions. RI values can be converted to LD_{50} units by comparison with the SSCA of a mouse-titered prion preparation run in parallel. The partially automated SSCA is at least as sensitive as the mouse bioassay, allowing quantitation of a 10^{-7} dilution of RML-infected brain homogenate (about 30 LD_{50} units/ml), more precise (standard deviation 15-25%) and at least 10 times faster (10 days). The use of semi-automated equipment allows the simultaneous processing of 500 samples or more in quadruplicate by one operator.

The cell panel assay (CPA) is based on the SSCA and is carried out on a set of four cell lines, PK1, R33, CAD5 and LD9, that show different responses to various prion strains [62]. In a typical CPA, the cells of the 4 lines are exposed to serial dilutions of mouse brain homogenates infected with prion strains RML, Me7, 301C or 22L and subjected to the SSCA. Fig. 1A shows that the RML dilutions required to give 300 "spots") were 5×10^{-7} on CAD5 cells, 8.3×10^{-7} on PK1 cells, and 5×10^{-6} on LD9 cells, from which the cognate RI vales can be calculated (Table I). In contrast, R33 cells failed to yield 300 spots even at 10^{-3}, the lowest dilution of RML used. Figs. 1B-D show the plots for 22L-, Me7- and 301C-infected brain homogenates, respectively. Because distinct prion preparations may contain different levels of prions, the RI must be referred to some other infectivity-related parameter. We arbitrarily chose as reference value the RI obtained on LD9 cells. By the criterion of the RI ratio, CAD5 cells are 9.9, PK1 cells 6.4 and R33 cells < 0.005 times as responsive to RML as LD9 cells (Table I). The CPA as described allows the distinction of RML, 22L, 301C and Me7 prion strains.

Cell tropism of strains

Different prion strains cause different location of lesions and PrP^{Sc} deposition in the brain [23] and, as described above, may exhibit different tropism for cell lines (Table I). The uptake of PrP^{Sc} by cultured cells appears to be a non-specific process [61] and therefore discrimination between strains probably reflects the capacity of the cell to replicate prions at an appropriate rate. The idea that strain recognition is mediated by the nature of the glycans carried by PrP^{Sc} [38] is not supported by the finding that two distinct prion strains could be propagated by PMCA using unglycosylated PrP^{C} [75].

The mechanism underlying specificity for brain areas and for cell lines need not be the same. Persistent infection requires that the rate of PrP^{Sc} synthesis be at least equal to the rate of PrP^{Sc} depletion [102]. In cell culture, depletion of PrP^{Sc} comes about by degradation, secretion and cell division, whereas in brain, where PrP^{Sc}

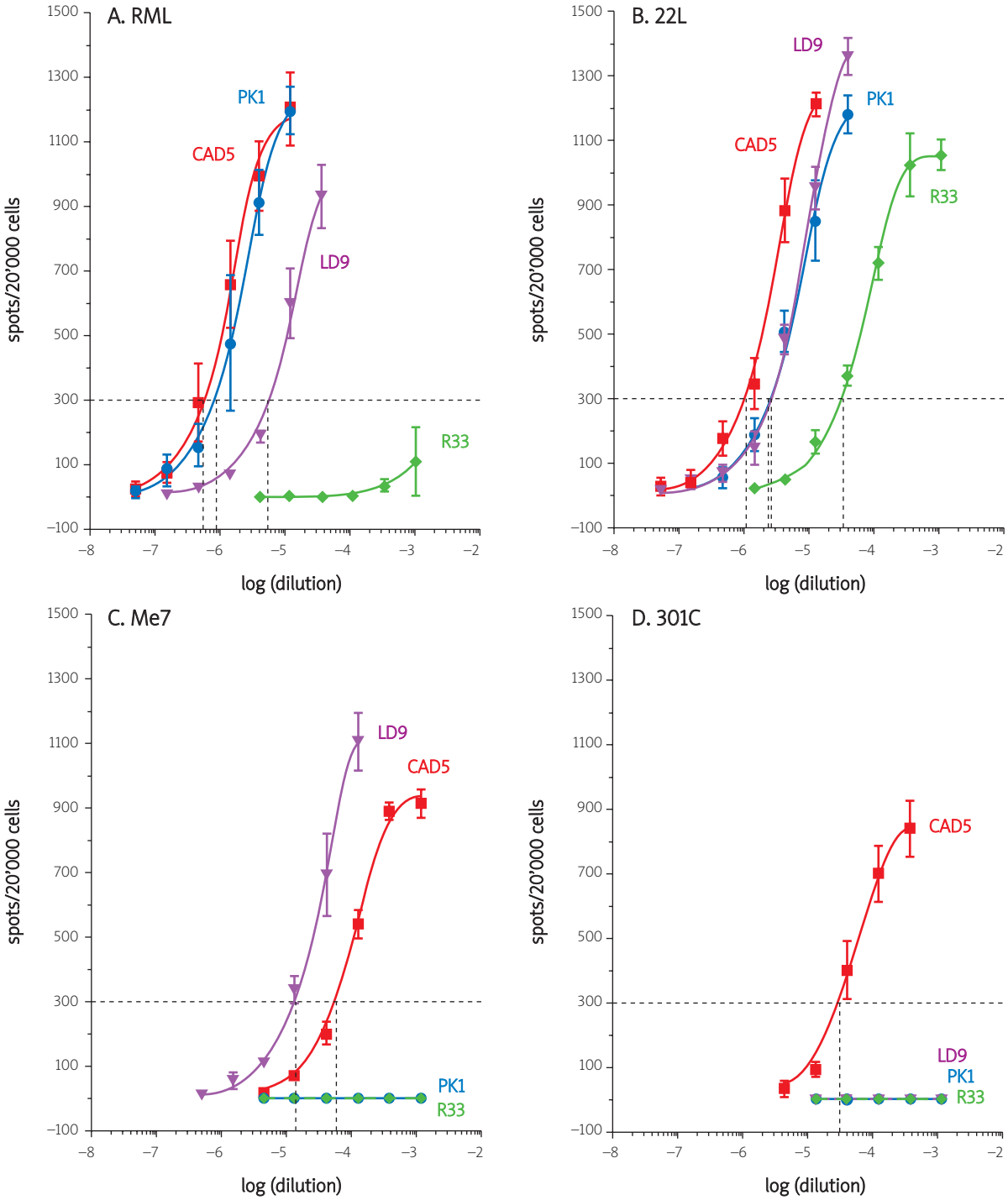

Fig. 1A-D. Response of PK1, CAD5, LD9 and R33 cells to various prion strains. Cells (PK1, blue; CAD5, red; LD9, violet; R33, green) were exposed to serial 1:3 dilutions of 0.1% homogenates of brains infected with (A) RML (B) 22L (C) Me7, (D) 301C and subjected to the standard scrapie cell assay. The number of PrP^{Sc}-positive cells is plotted against log [dilution] of the brain homogenates. Response index (RI) of a cell line to a prion strain is defined as the reciprocal of the dilution yielding 300 scrapie-positive cells per 20 000 cells under the conditions of the assay. The characteristic metric we currently use for strain identification is the ratio of the RI of a cell line relative to that of LD9 cells and is shown in Table I. Reproduced from ref. [89].

Table I. Response index (RI)* of cell lines for various prion strains

Prion	RI ([1/dilution] × 10^{-5})				RI ratio		
	LD9	CAD5	PK1	R33	CAD5/LD9	PK1/LD9	R33/LD9
RML_{BRAIN}	1.9	19	12	< 0.01	9.9	6.4	< 0.005
$Me7_{BRAIN}$	0.9	0.2	<< 0.01	<< 0.01	0.2	<<0.01	<< 0.01
$22L_{BRAIN}$	4.0	10	4.1	0.33	2.5	1.0	0.08
$301C_{BRAIN}$	<< 0.01	0.36	<< 0.01	<< 0.01	>> 36	na	na

** The response index is defined as the reciprocal of the dilution of the prion that results in 300 spots under the standard conditions of SSCA. Because the RI depends on the prion concentration in the sample, this variable is eliminated by expressing the ratio of RI relative to that given by one cell line, arbitrarily chosen to be LD9. The RI ratios differ characteristically for the four prion strains.*

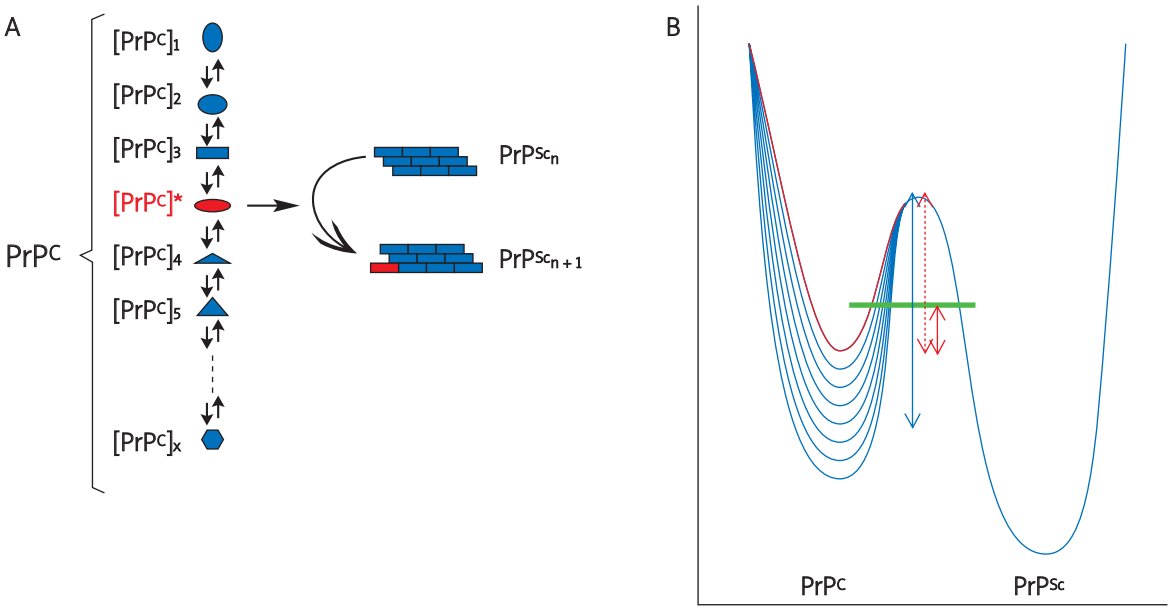

Fig. 2A-B. Hypothetical energy diagram for PrP^{C}-to-PrP^{Sc} conversion. (A) It is assumed that PrP^{C} can assume a variety of conformations and that the various conformers are in dynamic equilibrium and present at different steady state levels. (B) The conformer designated $[PrP^{C}]$* (red curve) requires the lowest activation energy (red dotted double-headed arrow) for conversion to PrP^{Sc} of a particular strain. The green horizontal line indicates the lowering of activation energy resulting from the action of a hypothetical cell component, such as a chaperone, that would facilitate PrP^{C}-to-PrP^{Sc} conversion (red solid double-headed arrow). The blue vertical arrow indicates the activation energy of the average PrP^{C} population.

accumulates predominantly in neurons, there is no depletion by cell division. Thus, slowing down cell division of cultured cells not only increases the accumulation of PrP^{Sc} [34] but may allow cells to become chronically infected by strains to which they are resistant under normal growth conditions (E. Smith and C.W.). The fact that many drugs that "cure" chronically infected cell lines are largely ineffective in abrogating prion disease *in vivo* [51] reflects at least in part the fact that there is no PrP^{Sc} depletion by cell division in the brain. PrP^{Sc} degradation has been attributed to the action of cysteine proteases [59,60]; PrP^{Sc} of different strains might show different sensitivity to degradation which could contribute to or determine

susceptibility to chronic infection. The other side of the equation, the rate of PrP^{Sc} synthesis, reflects the activation energy required for the conversion process and thus is a function of both the structure of the PrP^{Sc} multimer, which is believed to be strain-dependent, and of the conformational population distribution [104] of the PrP^{C} serving as substrate (Fig. 2). The finding that the rate of PrP^{Sc} amplification by PMCA varies considerably for different murine strains (P. Saá and C.I. Lasmezas, personal communication) supports the view that PrP^{Sc} structure might be rate-determining also *in vivo*. The conformational population distribution may depend on post-translational modifications of PrP such as glycosylation or others mentioned above, but perhaps also on association with cellular components [101] which, by favoring certain PrP conformations, could promote preferential propagation of particular strains. The remarkable affinity of PrP^{C} for RNA [35,69, 90] and the requirement for polyanions in the PMCA reaction using purified PrP^{C} as substrate [24,25] support the view that cell components other than PrP^{C} may play an important role in prion propagation [101]. As suggested by Prusiner and his colleagues [43,94,105], the conversion process may be catalyzed by a chaperone; indeed, different strains might require different chaperones and maybe the chaperone content of a particular cell type may contribute to its capacity for propagating a particular strain.

Outlook

Many basic questions regarding prion strains remain to be answered. Although there is convincing evidence that the PrP^{Sc} conformation of distinct strains is different, it is not known to which extent the conformation may depend on features other than the amino acid sequence of the PrP, for example the nature of the glycans or additional, cell-derived components. It would be desirable to obtain large quantities of highly purified PrP^{Sc} from a single cell line, infected separately with several different prion strains, determine the glycans carried by each strain-associated PrP^{Sc} and search for associated molecules, such as small RNAs or other cell components. The deepest insight will of course be gained once the 3-dimensional structure of PrP^{Sc} can be determined at an appropriate resolution, currently still a formidable task. As regards the cell specificity of prions strains, the question to be answered is whether one or more cell components participate in catalyzing the PrP^{C}-to-PrP^{Sc} conversion, and if so, whether such components are specific for different strains or sets of strains. The good news is that there are still many opportunities for making interesting discoveries; the sad news, that Carlton is not here to enjoy them.

Acknowledgements

I thank C.A. Baker and S.P. Mahal for critically reading the manuscript.

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