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D159 and S167 are protective residues in the prion protein from dog and horse, two prion-resistant animals

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ABSTRACT

Prion diseases are fatal neurodegenerative diseases caused by misfolding of the prion protein (PrP). These conditions affect humans and animals, including endemic forms in sheep and deer. Bovine, rodents, and many zoo mammals also developed prion diseases during the "mad-cow" epidemic in the 1980's. Interestingly, rabbits, horses, and dogs show unusual resistance to prion diseases, suggesting that specific sequence changes in the corresponding endogenous PrP prevents the accumulation of pathogenic conformations. *In vitro* misfolding assays and structural studies have identified S174, S167, and D159 as the key residues mediating the stability of rabbit, horse, and dog PrP, respectively. Here, we expressed the WT forms of rabbit, horse, and dog PrP in transgenic *Drosophila* and found that none of them is toxic. Replacing these key residues with the corresponding amino acids in hamster PrP showed that mutant horse (S167D) and dog (D159N) PrP are highly toxic, whereas mutant rabbit (S174 N) PrP is not. These results confirm the impact of S167 and D159 in local and long-range structural features in the globular domain of PrP that increase its stability, while suggesting the role of additional residues in the stability of rabbit PrP. Identifying these protective amino acids and the structural features that stabilize PrP can contribute to advance the field towards the development of therapies that halt or reverse the devastating effects of prion diseases.

1. Introduction

Prion diseases are a diverse group of neurodegenerative conditions affecting humans and other mammals (Colby and Prusiner, 2011; Ironside et al., 2017; Prusiner, 1998). Sporadic Creutzfeldt-Jacob disease is the most common prion disease in humans and is characterized mainly by aggressive dementia (Knight, 2017). Other sporadic, inherited, and transmitted prion diseases can present with motor deficits or sleep perturbations (Knight, 2017). Prion diseases are the only human neurodegenerative disorders with true correlates in mammals (Zlotnik and Rennie, 1965), making rodents ideal models to understand the mechanisms of disease transmission and pathogenesis. Scrapie, bovine spongiform encephalopathy (BSE), and chronic wasting disease (CWD) are prion diseases described in sheep and goats, bovine, and cervids, respectively (Mathiason, 2017). One common link to all these conditions is the characteristic spongiform brain pathology. The second main feature is the brain deposition of misfolded, insoluble conformations of the prion protein (PrP) resistant to proteases termed scrapie PrP (PrP^{Sc}) (Prusiner, 1998). Prion transmission requires the catalytic conversion of cellular PrP (PrP^{C}) by PrP^{Sc}, but this interaction may be hindered by sequence differences, an observation that underlies the "species barrier" phenomenon (Telling et al., 1996; Telling et al., 1995). It is well known that the C-terminal globular domain, which contains three α-helices and two small β-pleated domains, plays a key role in PrP pathogenesis (Donne et al., 1997; Inouye and Kirschner, 1998; James et al., 1997). An increase in β-sheet content is proposed to alter PrP solubility, aggregation, and neurotoxicity, three new features of PrP during pathogenesis (Aguzzi et al., 2008; Inouye and Kirschner, 1998; Prusiner, 1998). Despite major efforts to understand the mechanisms regulating PrP conversion, transmission, and pathogenesis, there are currently no treatments to stop or reverse these diseases.

Over 50 pathogenic mutations and several artificial mutations have provided important but limited clues about how the amino acid sequence modulates PrP conformational dynamics and pathogenesis (Lloyd et al., 2011). In addition, nature provides an understudied source of sequence variants: the PrP zoo. Most amino acid changes

Abbreviations: α3, (helix 3); Ca, (Canis, dog PrP); EqPrP, (Equus, horse PrP); PrP, (Prion protein); RaPrP, (rabbit PrP); sodium dodecyl sulfate-polyacrylamide gel electrophoresis, (SDS-PAGE); WT, (wild type)

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accumulate during evolution by neutral drift, although some variants may be subject to selection if they alter critical PrP functions. The challenge to identify key residues mediating PrP conversion from these natural variants is that several changes accumulate even in closely related species. For instance, mice and hamsters are rodents sensitive to prion diseases that accumulate 13 amino acid differences in the 202 amino acids of mature PrP (amino acids 29–231), four of them in the globular domain. Although many of these substitutions are conservative (similar properties), their contribution to PrP conformation cannot be dismissed. In fact, many pathogenic mutations responsible for dominantly inherited prion diseases in humans are conservative too, particularly in hydrophobic residues (e.g., V180I, V203I, V210I) (Lloyd et al., 2011). PrP from a few animals known to be resistant to prion diseases – rabbits, horses, and dogs – are an underutilized resource that can teach us about the intrinsic determinants of PrP conformational changes and toxicity. Although PrP from these three animals also show several amino acid changes compared to hamster PrP, focusing on unique changes in the globular domain can identify the critical residues that confer structural stability to PrP and prevent pathogenesis.

Scrapie is an endemic disease described in sheep and goat > 250 years ago. Although scrapie is not transmissible to humans, it can be transmitted experimentally to rodents (mouse, rat, hamster, bank vole) (Chandler, 1971; Chandler and Fisher, 1963; Zlotnik and Rennie, 1963; 1965), but not to rabbits (Gibbs Jr. and Gajdusek, 1973) even after passage through mice (Barlow and Rennie, 1976). In contrast to sheep and goats, there is no endemic prion disease in bovine. Unfortunately, the human-mediated transmission of scrapie to bovine resulted in the mad-cow epidemics in the mid-80's (Wells et al., 1987). From that initial event, bovine prions spread to humans as a new disease called variant Creutzfeldt-Jakob diseas (Will et al., 1996). Bovine prions also spread to several domestic and zoo animals, including felines, ferrets, and others (Kirkwood and Cunningham, 1994), demonstrating the susceptibility of many mammals with no known endemic prion disease. Horses and dogs were also exposed to prions during the mad-cow epidemics through bovine-contaminated feed. Despite this exposure, not a single case of prion disease has been described among horses and dogs, so far (Kirkwood and Cunningham, 1994). These are domestic animals with good veterinarian care and prion diseases are easily diagnosed in any animal due to the typical spongiform degeneration of the brain followed by confirmatory molecular tests. Two hypotheses have been proposed to explain the resistance of a few animals to prion diseases: intrinsic factors (differences in PrP sequence) or extrinsic factors (cellular factors). In this regard, rabbit cells can convert transgenic PrP from susceptible animals (Courageot et al., 2008) while fragments of rabbit PrP (RaPrP) are sufficient to prevent conversion of chimeric rabbit-mouse PrP (Gibbs Jr. and Gajdusek, 1973). These results argue for a normal cellular environment in rabbit cells, suggesting that the inefficient conversion of RaPrP is due to protective substitutions in its endogenous sequence. Thus, it is likely that prion-resistant mammals carry "protective" amino acid substitutions that stabilize PrP^C conformations and hinder conversion into pathogenic conformations. Identifying the key amino acid changes responsible for stabilizing the tertiary structure of rabbit, horse, and dog PrP can reveal key insight to understand PrP misfolding and pathogenesis.

In addition to these limited experimental manipulations and natural history of prion diseases, NMR structure is available for the globular domain of PrP from rabbits, horses, and dogs (Lysek et al., 2005; Perez et al., 2010; Wen et al., 2010b), while high-resolution X-ray crystallography is only available for (RaPrP) (Khan et al., 2010). In RaPrP, serine (Ser, S) at 174 (human numbering, see Fig. 1A) (Vorberg et al., 2003) is proposed to form a helix-capping motif that stabilizes the β2-α2 loop and distal helix 3 (Khan et al., 2010). Introducing an asparagine (Asn, N) found in most animals at 174 (S174 N) increases RaPrP conformational instability *in vitro* (Khan et al., 2010), supporting the protective role of S174. We recently confirmed the conformational stability and lack of toxicity of RaPrP expressed in *Drosophila*, which is in sharp contrast to the high toxicity of hamster PrP (Fernandez-Funez et al., 2010; 2011). Recent studies challenged the traditional idea that rabbit is resistant to prions, although only 20% of animals inoculated with rabbit-adapted prions developed disease after long incubations on second passage (Chianini et al., 2012). Furthermore, structural studies also identified candidate protective residues in horse and dog PrP, including S167 in horse PrP (*Equus,* EqPrP) and aspartic acid (Asp, D) at 159 in dog PrP (*Canis*, CaPrP) (Lysek et al., 2005; Perez et al., 2010). *In vitro* conversion studies have shown that both rabbit and dog PrP can be converted into infectious isoforms that induce disease in mice expressing bovine and human PrP (Vidal et al., 2013). But these laboratory conditions do not indicate that rabbits and dogs can be infected with prions under natural conditions. We recently demonstrated that the N159D substitution increases the conformational stability of mouse PrP and reduces its toxicity in *Drosophila*, supporting the protective role of D159 (Sanchez-Garcia et al., 2016). Overall, *Drosophila* has shown to be a model highly sensitive to the expression of PrP from different species (hamster, mouse, ovine, rabbit, human), making this a powerful model to study PrP folding and toxicity (Fernandez-Funez et al., 2017; 2011; Thackray et al., 2012).

Here, we show that transgenic flies expressing WT PrP from rabbit, horse, and dog demonstrate no toxicity. We also examined the *in vivo* consequences of mutating the suspected protective residues in their respective backbones to the corresponding residues in hamster PrP (HaPrP): RaPrP-S174 N, EqPrP-S167D, and CaPrP-D159N. Despite previous *in vitro* evidence (Khan et al., 2010), we found that RaPrP-S174 N was not toxic in transgenic flies. In contrast, replacing single amino acids in horse and dog PrP resulted in dramatic locomotor dysfunction and shortened lifespan. Interestingly, EqPrP-S167D and CaPrP-D159N induced different phenotypes in degenerating brain neurons, suggesting the accumulation of different pathogenic conformers that perturb distinct cellular pathways. These studies demonstrate the role of the primary sequence of rabbit, horse, and dog PrP in conferring structural stability, which explains the resistance of these animals to prion diseases under natural conditions. Also, S167 and D159 seem to introduce local and global changes in the PrP globular domain that result in decreased β-sheet content and increased conformational stability. Overall, this knowledge can be leveraged to search for genetic or pharmacologic agents that can stabilize these domains and prevent PrP conversion, hence protecting against prion diseases.

2. Results

2.1. Identification of protective residues in the globular domain of PrP

To identify key residues mediating resistance to PrP misfolding and disease, we aligned the sequence of the globular domains of PrP from susceptible and resistant mammals, including human (Hu), Syrian hamster (Ha), mouse (Mo), rabbit (Ra), horse (Eq), and dog (Ca) PrP. These sequences are highly conserved, with most changes due to conservative substitutions in hydrophobic residues (Fig. 1A). Since we use human PrP (HuPrP) as the reference for the alignment, all PrP sequences are numbered according to HuPrP throughout this paper to avoid confusion. The alignment shows that most of the variability accumulates around the loops (except the α2-α3 loop) and the distal end of helix 3. Interestingly, the β2-α2 loop or "rigid loop" is physically close to in the distal α3 in the 3D structure of the folded protein (Fig. 1B). This 3D domain is proposed to be key for regulating PrP stability and the interaction site with a yet unknown chaperone termed protein-X (Kaneko et al., 1997). Since there are no common substitutions in the three prion-resistant animals, we focused on the residues identified in the literature as key regulators of the stability of rabbit (S174), horse (S167), and dog (D159) PrP (Khan et al., 2010; Lysek et al., 2005; Perez et al., 2010; Vorberg et al., 2003).

For experimental purposes, we used HaPrP structure as control because we have shown that it is more neurotoxic and conformationally