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Mini-Review

Infectious Fold and Amyloid Propagation in *Podospora anserina*

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ABSTRACT

Amyloid protein aggregation is involved in serious neurodegenerative disorders such as Alzheimer's disease and transmissible encephalopathies. The concept of an infectious protein (prion) being the scrapie agent was successfully validated for several yeast and fungi proteins. Ure2, Sup35 and Rnq1 in *Saccharomyces cerevisiae* and HET-s in *Podospora anserina* have been genetically and biochemically identified as prion proteins. Studies on these proteins have revealed critical information on the mechanisms of prions appearance and propagation. The prion phenotype correlates with the aggregation state of these particular proteins. In vitro, the recombinant prion proteins form amyloid fibers characterized by rich β sheet content. In a previous work on the HET-s prion protein *Podospora*, we demonstrated the infectivity of HET-s recombinant amyloid aggregates. More recently, the structural analysis of the HET-s prion domain associated with in vivo mutagenesis allowed us to propose a model for the infectious fold of the HET-s prion domain. Further investigations to complete this model are discussed in this review, as are relevant questions about the [Het-s] system of *Podospora anserina*.

THE [Het-s] SYSTEM

In the filamentous fungi a process called vegetative incompatibility induces death of the hyphae in anastomosis when two strains with a different genetic background fuse.[1] The *het-s* locus was classified as one of the loci controlling vegetative incompatibility in *Podospora anserina*[2] because the coexpression of the *het-s* and *het-S* alleles may lead to the death of the hyphae. Expression of *het-s* allele can give rise to two distinct phenotypes: [Het-s] that is incompatible with [Het-S] and [Het-s*] that can fuse with a [Het-S] strain. Figure 1 shows a schematic representation of the Het-s/Het-S system of *Podospora anserina*. The HET-s and HET-S proteins are constituted of 289 amino acids and only differ from each other by 13 amino acids; vegetative incompatibility between the [Het-s] and [Het-S] strains has been attributed to the amino acids difference (D23A, H33P).[3]

George Rizet described the [Het-s] phenotype of *Podospora* as a nonMendelien genetic element in 1952.[2] Janine Besson studied the system in-depth and suspected a protein inheritance by 1962.[4] In 1982 Stanley Prusiner[5] defined prion as the "proteinaceous infectious element" of the scrapie agent and the hypothesis of infectious proteins emerged. Reed Wickner[6] clearly identified protein inheritance in *Saccharomyces cerevisiae* and established the genetic criteria allowing the identification of such phenomena in microorganisms. In 1997, on the basis of these genetic criteria, Joel Bégueret[7] suggested that the [Het-s] element is the prion form of the HET-s protein. The [Het-s] phenotype may appear spontaneously in a [Het-s*] background and then very rapidly propagate to the whole mycelia. [Het-s] is systematically transmitted through hyphae fusion to the [Het-s*] strain and is strictly dependent on the expression of HET-s protein.

THE INFECTIOUS AMYLOID PROTEIN HET-s

Prions propagate by the normal soluble form of the protein converting to an aggregated form in vivo and by having an aggregated amyloid structure in vitro that is resistant to protease degradation.[8-11] We demonstrated that the HET-s protein can exist as two alternative states: a soluble and an aggregated form in vivo.[12] The HET-s-GFP (green fluorescent protein) fusion shows a diffuse fluorescence in the [Het-s*] strain whereas transition to the infectious prion form [Het-s] is associated with the aggregation of the fusion protein. The nonprion HET-S protein is soluble and a double amino acid substitution in HET-s (D23A P33H), which abolishes prion infectivity, suppresses in vivo aggregation

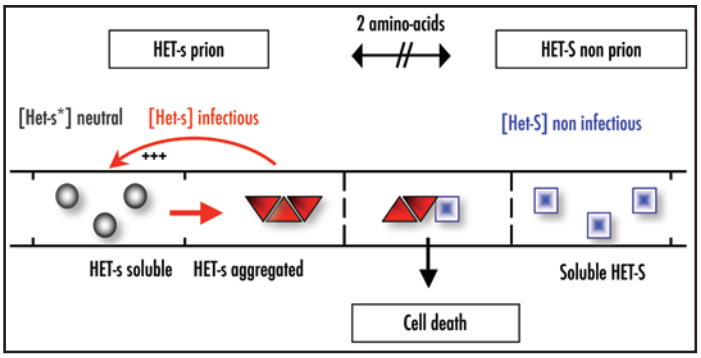

Figure 1. Schematic representation of the Het-s/Het-S system of *Podospora anserina*.

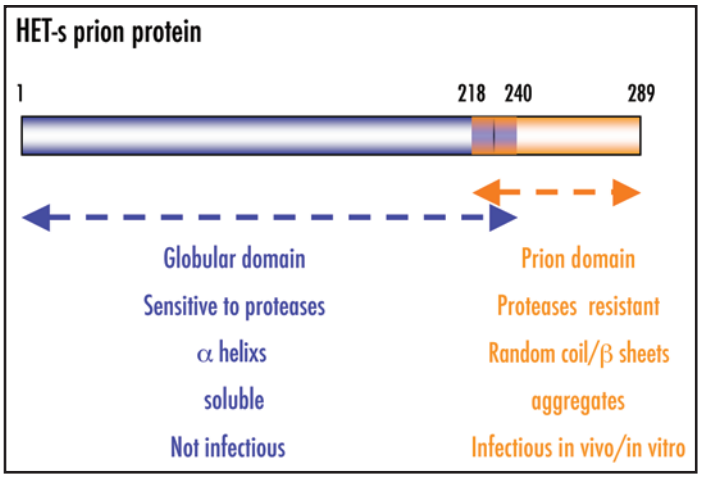

Figure 2. Summary of the main characteristics of the HET-s protein' two domains.

of the GFP fusion (Fig. 2). Structural and biochemical analyses on recombinant HET-s protein have indicated that in vitro aggregation of HET-s recombinant protein has all the characteristics of amyloid proteins.[13] Indeed, the HET-s recombinant protein self-associates into high molecular weight aggregates, and these aggregates greatly accelerate precipitation of the soluble form. HET-s aggregates appear as amyloid-like fibrils in electron microscopy; the transition from soluble to the aggregated state is accompanied by an increase in β-sheet content and the formation of a 7-kDa protease-resistant fragment.

The concept of an infectious protein added a revolutionary step to the point of view of hereditary control.[14] Nonetheless, although several observations had confirmed this concept, the final proof had not been found.[15] In essence, introducing the purified suspected protein in its infectious form to induce the prion phenotype would confirm the infectious protein postulate. J. Weissman was the first to demonstration this with the Sup35 protein in yeast using liposome mediated transfection, but did so without a negative control (the protein in its noninfectious form).[16] The yeast prions exhibited "variants" that differed with respect to the intensity of the altered phenotype,[17,18] and this property demonstrated that prion variants can be generated by introducing distinct amyloid structures in yeast. Associating an amyloid structure with a prion variant phenotype definitively confirmed the prion hypothesis for Sup35, Ure2 and Rnq1 of *S. cerevisiae*.[19-21]

To firmly establish the obvious link between in vitro amyloid aggregation of the HET-s protein and the propagation of the [Het-s] infectious phenotype, we tried diverse methods to introduce the recombinant protein aggregates inside the mycelia of a neutral [Het-s*] strain. The structure of mycelia, which allows cytoplasm mixing between articles, ensures that prion infection will spread rapidly. The biolistic (biological ballistic) method, probably because of breaking the aggregates, was very efficient. The biolistic introduction of amyloid aggregates of the recombinant HET-s protein into fungal cells induces the emergence of the [Het-s] prion with a high frequency, but neither the amorphous aggregates nor the soluble form could do so.[22] Thus, we demonstrated that prion infectivity could be created de novo, in vitro from recombinant protein in this system. Our data designate the HET-s amyloids as the molecular basis of [Het-s] prion propagation. In fact, several methods of fungi classic DNA transformation can be used to introduce protein aggregates in *Podospora*.[23] Using the nonprion form HET-S as a negative control, we were surprised to find that HET-S is able to form amyloid fibers in vitro; these aggregates are infectious when introduced in *Podospora* (Maddelein, unpublished). As discussed below, the folded N-terminal domain is suspected to be responsible for the [Het-S] nonprion phenotype and in vitro conditions may denature this N-terminal domain.

Since the propagation of the infectious form of prion proteins was believed to be a consequence of a conformational transition,[24] the structure of the HET-s recombinant protein was then characterized. This structural analysis showed that the soluble form of the HET-s protein has a modular structure with an N-terminal (1-240), helical, folded domain and a C-terminal flexible domain.[25] Upon HET-s aggregation, the C-terminal part (218-289) forms an amyloid core rich in β-sheet content and resistant to proteolysis degradation. In vivo expression of this C-terminal domain fused to GFP is sufficient for [Het-s] appearance and propagation.[25] Using the biolistic approach, we confirmed that the recombinant peptide HET-s (218-289) aggregated was displaying infectivity.[26] This prion-forming domain, analyzed by high resolution hydrogen/deuterium exchange and mass spectrometry, displays several short (5–10 amino-acid) β sheet segments interrupted by short turns or short loops.[26]

THE INFECTIOUS FOLD OF THE PRION DOMAIN

In order to define the structural organization of the HET-s (218-289) prion domain in its aggregated form, Roland Riek's and Beat Meier's groups first identified the sequence-specific positions of regular secondary structural elements using an improved technique of quenched hydrogen exchange measured by solution NMR.[27] Four segments that displayed slow exchange rates were observed and thus were considered to be involved in hydrogen bonds. To determine which residues were actually involved in the β-sheet secondary structure, high-resolution solid-state NMR spectra of the HET-s (218-289) fibrils on all residues showing slow exchange rate were recorded. Negative deviations of the chemical shifts from random coil values indicated four β-sheet secondary structures. Exceptions were observed at residues 229 and 265.[27,28] The clear connection between the exchange data and the chemical shift data allowed for the confident establishment of the secondary structure in the HET-s (218-289) amyloid fibrils: four β-strands comprising residues 226-234 (β1), 237-245 (β2), 262-270 (β3) and 273-282 (β4) are linked by two short loops and by an unstructured, 15-residue-long segment between β2 and β3 (Fig. 3).[28]

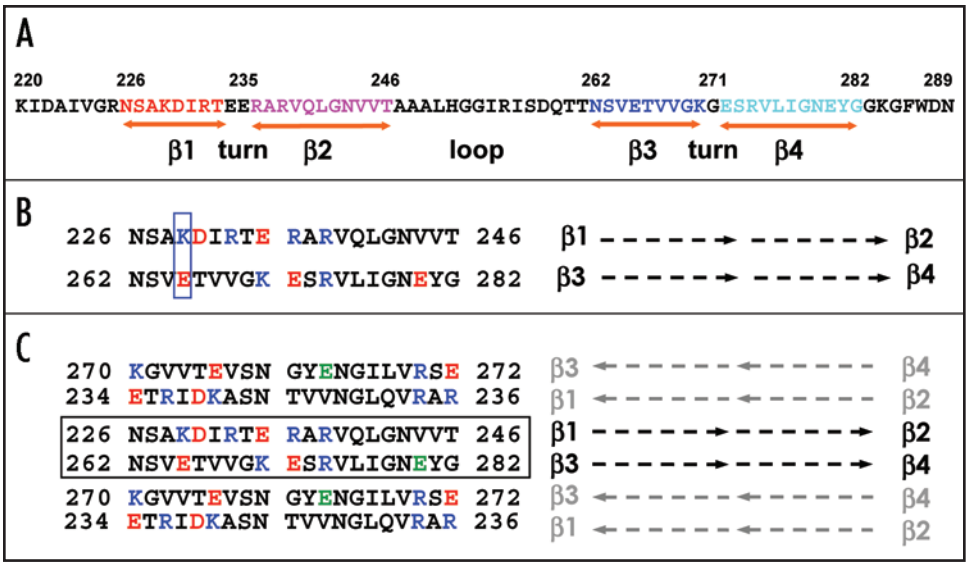

Figure 3. Organization of the structural elements in the HET-s (218-289) fibrils.[28] (A) Two β-strand-turn-β-strand motifs interconnected by a long loop interact through hydrogen bonding between β1 and β3, and β2 and β4. (B) The only charged residues facing towards the inside of the fibril, K229 (β1) and E265 (β3), were proposed to be involved in a salt bridge (blue). (C) An antiparallel intermolecular arrangement may explain the effect of E280K (green) mutation on HET-s infectivity.

To associate the proposed fold of the HET-s (218-289) fibrils with [Het-s] infectivity, a mutagenesis approach was used. Because the fold is based on the β-sheet secondary structure, we introduced proline residues, which are known to act as local β-strand breakers. Interestingly, a mutation previously identified in a random screening for the loss of [Het-s] was creating a proline replacement (T266P).[29] In striking correlation with the structural data, the proline substitutions and the deletions located in the β-strand regions strongly affected [Het-s] infectivity and HET-s-GFP foci formation.[28] In contrast, proline substitutions in the N- or C-terminal flexible tails or in the central loop did not affect [Het-s] infectivity or HET-s-GFP foci formation. These in vivo data correlated strongly with the amyloid conformation of the HET-s prion domain. In taking account of the remarkable sequence identity between β1-β2 and β3-β4 suggesting a repetitive arrangement, we thus propose a likely fold for the HET-s (218-289) fibrils.[28] The two β strand segments interact through hydrogen bonding between β1 and β3 as well as β2 and β4, respectively forming two layers of parallel β sheets. The only charged residues facing towards the inside of the fibril are K229 in β1 and E265 in β3 that are located in adjacent positions that are able to compensate their charges in a salt bridge (Fig. 3). The dimer-like double β-strand-turn-β-strand motif of the HET-s prion domain indicated that a nucleus could be formed by just one molecule. We then proposed that the prion domain may have evolved to optimize the nucleation event by reducing diffusion between the β strands. Consistent with recent findings on the Sup35 prion domain,[30,31] an intermolecular antiparallel interaction between HET-s monomers forming the β sheet was suggested.[32]

Additional experiments were made in order to confirm the existence of the 239-265 salt bridge in vivo. Charge inversion mutations for all charged residues of the prion domain of HET-s were realized but none of them, except for E280K, gave a clear reduction in [Het-s] prion infectivity (Maddelein, unpublished). These results are in agreement with a possible antiparallel organization between HET-s monomers (Fig. 3). This intermolecular arrangement allows a charge inversion in one residue to be compensated by interacting with the charge in the opposite β strand; only the critical mutation E280K cannot be compensated for. Another possibility is that the E280 interacts with the end part of HET-s. Transformants expressing the HET-s deleted for (284-287) have lost the prion phenotype (Maddelein, unpublished) and so, in agreement with structural data,[26,33] the end part of the prion domain seems also to be involved in HET-s aggregation and infectivity.

THE ROLE OF THE N-TERMINAL DOMAIN IN [Het-s] PROPAGATION

Prion propagation has been shown to be dependent on seed generation.[34,35] In yeast, HSP104 is essential for prion maintenance; it was suggested that HSP104 acts by breaking the prion aggregates, allowing efficient transmission of prion seeds.[35-37]

In *Podospora*, [Het-s] propagation does not seem to rely on HSP104 expression (Dos-Reis, Malato, Saupe, unpublished), but this does not exclude the intervention of other cellular factors in prion seed formation, as observed for yeast prions.[38-41] However, if HET-s has evolved to facilitate nucleus formation by the double hairpin organization of its prion domain, one may also suggest that the HET-s prion has evolved to ensure its maintenance in the mycelia. In this view, HET-s has two means to allow seed formation: either by spontaneous breakage of the aggregates, which may be facilitated by the uncompensated charges in the prion domain, and/or by limited prion domain aggregation, which may be due to the adjacent globular domain.

It has been demonstrated that the lack of prion infectivity of the nonprion HET-S is due to the inhibitory effect of the N-terminal, folded domain rather than being due to the polymorphisms in the prion domain.[25] In addition, proline scanning mutagenesis on the HET-S prion domain (Maddelein, unpublished) indicated that the same regions (β1, β2, β3 and β4, Fig. 3) involved in HET-s prion infectivity (HET-s interacting with HET-s)[28] are involved in the barrage reaction (HET-S interacting with HET-s). So the prion domain of HET-S is fully functional, and a conformational variation of the globular domain of HET-S was the best explanation for its nonprion behavior. However, in vitro studies of the globular domains (1-220) did not show a clear difference between the prion HET-s and the nonprion HET-s (23-33).[25] One possibility is that the helical conformation of the N-terminal domain of the nonprion HET-S extends further than residue 220, covering part of the prion domain (Fig. 2). Since at least 4 β strands are needed for nucleus formation, HET-S cannot spontaneously aggregate, but is still be able to interact with a seed formed by the prion HET-s. In this case, the addition of the globular HET-S domain stops HET-s elongation. Indeed, very small aggregates can be observed from the interaction of HET-S with HET-s-GFP during the barrage reaction.[12] The globular domain of the prion HET-s also seems to limit prion domain aggregation. In vivo, full-length HET-s-GFP aggregates by forming dots, but deletion in the N-terminus allows the HET-s (157-289) to form large aggregates[42] displaying paternal inheritance.[43] It is likely

that dots rather than large aggregates may allow easiest transmission of [HET-s] and avoid the requirement of cellular cofactors such as HSP104.

CONCLUDING REMARKS

It is believed that most prions share β-sheet-rich amyloid fibrils as a common structural feature. The proposed model for the infectious fold of the HET-s prion suggests that a nucleus can be formed by just one HET-s molecule.[28] This link between structure and infectivity constitutes a significant step towards the elucidation of the mechanism of prion appearance and propagation. This fascinating HET-s/HET-S system still contains many mysteries. The possibility to create variants, the intervention of cellular factors on [Het-s] propagation, and the molecular species inducing the cell death reaction in Het-s/HET-S interaction are still unresolved questions. We might hope that genetic and structural characterizations of newly described HET-s homologues[44] will bring some answers.

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