On the evolutionary trajectories of signal-transducing amyloids in fungi and beyond

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ABSTRACT. In the last decade, multiple reports have established that amyloids can bear important functional roles in a variety of biological processes and in distant taxonomic clades. In filamentous fungi, amyloids are involved in a signal transducing mechanism in which a group of NOD-like receptors (NLRs) controls downstream effector proteins to induce a programmed cell death reaction. A structurally characterized example of fungal signal-transducing amyloid is the prion-forming domain (PFD) of the HET-S toxin from Podospora anserina. Amyloid-mediated programmed cell death is equally reported in metazoans in the context of innate immunity and antiviral response. The cell death reaction, described as programmed necrosis, is dependent on an amyloid-forming RHIM motif (RIP homotypic interaction motif). An evolutionary link between the RHIM and the PFD signaling amyloids has been previously reported. Our recent study ties further the signaling amyloids in fungi and metazoans, reporting a fungal signal-transducing domain with amyloid and prion-like properties, which shows significant sequence similarity to the metazoan RHIM motif. Here, I discuss the expanding class of the signal-transducing amyloids and reflect on the possible evolutionary scenarios of their diversification.

KEYWORDS. functional amyloid, HET-S, NLR, prion, programmed cell death, RHIM, signal-transduction

“Having a protein that can exist in an on- and off state where the on-state is infectious is a wonderful way to transmit information. Nature would be very stupid if it didn't utilize this system in order to solve specific problems during evolution.”

Adriano Aguzzi, 2009

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INTRODUCTION

Extensively investigated in the context of human health as pathological agents causing fatal neurodegeneration, amyloids have also important and diverse biological roles. Functional amyloids, unlike those resulting from protein misfolding, are under selective pressure to maintain and adapt the amyloid fold and are integrated in complex, well-regulated molecular processes. In prokaryotes like bacteria, functional amyloids are a major constituent of biofilms, regulate morphological differentiation or serve as virulence factors. In mammals, amyloids are involved in the storage and release of hormones and in scaffolding skin pigmentation processes. Evolution seems to exploit 2 main properties in amyloids – the robustness and stability of the β-sheet-rich folds and the inherent prion-like features of their polymeric, self-templating structures. In microbial eukaryotes like Saccharomyces cerevisiae, amyloid-based prions may function as evolutionary facilitators and are shown to modulate phenotypic flexibility and inheritance. Another fungal prion under selective pressure is the [Het-s] prion in the filamentous ascomycete Podospora anserina. [Het-s] controls a self/non-self recognition mechanism that limits the horizontal transmission of senescence plasmids in P. anserina. As might be expected for a functional prion, [Het-s] is extremely abundant in wild populations of P. anserina. It thus appears that functional amyloids not only carry a variety of molecular functions, but are also found in particularly distant taxa. In this context, recent reports expose a new functional role for amyloids as signal-transducing domains controlling programmed cell death. Signal-transducing amyloids can be defined as motifs or domains adopting an amyloid fold exclusively in response to precise endogenous or exogenous signals and functioning as structural template or scaffold to bind and/or activate downstream proteins. Despite the uncertain phylogenetic relations between the amyloids involved in signal transducing, these domains represent the first described class of functional amyloids to be conserved in different kingdoms of life. This remarkable observation suggests that amyloid signaling may be of an old origin and, as a corollary, prompts the idea of its universality throughout the tree of life.

Amyloid Signal-Transduction in Fungi and Metazoans

The discovery of amyloid-mediated signal transduction mechanisms in fungi was greatly facilitated by the available structural information on the [Het-s] prion. The high resolution NMR structure of the prion domain of the HET-s protein, has led to the establishment of a model system for studying the prion properties of amyloids and the underlying structural features. The prion-forming domain (PFD) of HET-s (HET-s (218-289)), situated at the extreme C-terminus of the protein, contains two 21 amino acid repeats (R1 and R2). The two repeats present a sequence of alternating hydrophobic and polar/charged residues and 2 strongly conserved glycines and are separated by a poorly conserved 16 amino acid flexible loop. Each repeat forms 4 β-strands and is alternatively stacked in the β-solenoid arrangement of the amyloid fibers. The HET-s PFD is disordered in the monomeric form of the protein and is appended to a second N-terminal α-helical domain, termed HeLo. The HeLo domain is shown to have cytotoxic activity, although in the case of HET-s, a loss-of-function mutation has occurred in it. However, an allelic variant – HET-S (large S) – sharing the domain organization of HET-s has a functional, cytotoxic HeLo domain. HET-S is a pore-forming toxin that exerts membrane-disrupting activity through the HeLo domain when its PFD adopts the β-solenoid amyloid fold. The PFD of HET-S can be templated to adopt the β-solenoid fold either by the [Het-s] prion – a reaction occurring during the non-self recognition process – or by a NOD-like receptor (NLR) termed NWD2. NLRs are intracellular molecular receptors involved in innate immunity in metazoans and are proposed to carry the same function in fungi. NWD2 is encoded by the gene adjacent to het-S in the genome of P. anserina and this genomic clustering is conserved in a large number of ascomycetes. A 21 amino acids motif (R0), homologous to R1/R2 motifs found in the PFD of HET-S, is situated at
the extreme N-terminus of NWD2. The R0 motif shares the same pattern of hydrophobic/polar residues and the conserved glycines as the repeats of the PFD and is equally able to adopt a HET-S-like amyloid fold. The R0 motif is essential for signal transducing by NWD2. In the current model, when the NOD-like receptor is activated by the detection of a specific molecular signal, it oligomerizes bringing together the R0 motifs, which cooperatively adopt the β-solenoid fold. The molecular complex of activated NWD2 molecules then templates the unstructured PFD of HET-S to induce cell death. Essentially, the signal transduction mechanism is based on the prion properties of the HET-S/s amyloid fold.

In metazoans, prion-like polymerization and formation of higher order complexes are also main features of signal transduction in innate immune response and antiviral defense. These prion-like mechanisms may or may not be based on propagation of amyloid folds. In this context, a short amyloid motif termed RHIM (RIP homotypic interaction motif) is controlling a necrotic programmed cell death pathway which molecular hallmarks include organelle swelling and cell membrane rupture. The RHIM motif mediates, through homotypic interactions, the formation of amyloid signaling complex of RIP1 and RIP3 (Receptor-interacting serine/threonine-protein) kinases. A functional RHIM motif can be as short as 19 residues and is characterized by a conserved I(V)QI(V)G amino acids sequence. Mutational analyses have revealed that this short amyloidogenic stretch is critical for in vivo amyloid signaling by RIP1 and RIP3. While there is no precise structural information regarding the amyloid form of the RHIM motif, a recent study suggest that it might form a HET-S/S-like amyloid fold and that an evolutionary relation, based on sequence alignments and HMMs (Hidden Markov Model) comparisons, exists between the 2 amyloid domains.

The RHIM motif, unlike the 2 repeats of the HET-S/s PFD, is found in only one copy on RIP1 and RIP3 kinases. In this regard, it is more similar to the R0 motif, found in only one copy on the NWD2 NLR-like receptor in *P. anserina*. Yet, another human protein ZBP1 (Z-DNA binding protein) - an innate immunity sensor for foreign DNA – is shown to have 2 or 3 successive RHIM motifs. Furthermore, proteins from *Branchiostoma*, have also been reported to bear 2 repeats of the RHIM motif, disposed next to domains otherwise involved in metazoan innate immunity. The evolutionary constraints defining the number of amyloidogenic repetitions found in different proteins are not well understood today. It is possible that the nature of the motif itself, the spacing between the repetitions of the motif (if it exists in repeated form), and/or the mode of nucleation, may all influence to some extent the copy number distribution of the amyloidogenic motifs. Precise structural information on the amyloid fold of the RHIM motif could be of great value in establishing an unambiguous structural relation to the HET-S/S amyloid fold and would offer the possibility to compare the molecular features of amyloid folding and stability of 2 distantly related signaling amyloids. Additional information on the protein complexes within which the amyloid structures are formed, would be invaluable in the pursuit of better understanding of the nucleation events – a defining characteristic of the amyloid signaling.

**Extensive Diversification of Signal-Transducing Amyloids in Fungi**

We have recently demonstrated that a protein motif showing high sequence similarity to the metazoan RHIM, termed PP (pseudo-palindromic), acts as a signal-transducing amyloid domain in NLR-mediated cell death signaling, in fungi. The PP amyloid motif was identified in a previous *in silico* search for putative signal transducing amyloids. PP and RHIM have in common a pseudo-palindromic amino acids sequence (G-φ-Q-φ-G), shown to be part of the amyloid core for both motifs. Two relatively well-conserved asparagine residues...
extend this sequence homology further, each flanking the pseudo-palindrome. The reported similarities relate even more the metazoan and fungal amyloid transducing domains. It is conceivable, despite the short length of these motifs, to propose a common evolutionary origin for PP and RHIM, especially considering that the 2 motifs execute a similar function in related biological processes.

The PP motif (~17 amino acids residues) is functionally equivalent to the PFD of HET-S and is found in proteins with the same domain architecture, at the place of the PFD. There are gene clusters, similar to het-S/nwd2 cluster from *P. anserina*, in which one of the genes is encoding a HeLo-PP protein and the other a PP-containing NLR-like protein. However, there are at least 2 notable differences between the PFD and the PP-motif that could be underlined. First, the PP motif seems to be controlling several different effector domains, a situation not described for the HET-s PFD. Indeed, a protein called SBP (SESB-PP), bearing a putative lipase (SESB) as an effector domain at its N-terminus, contains a functional PP motif. The SBP encoding gene is a part of a 3 genes cluster in the genome of *Chaetomium globosum*, a species in the same order as *P. anserina*. The two other genes of the cluster encode a protein with a HeLo-like membrane-targeting, cytotoxic domain fused to a PP motif and a PP-containing NLR protein. The exclusive association of some effector domains with the PP motif may suggest a functional difference between PP and HET-s PFD. Different properties of the amyloid structures formed by the PP motif and the HET-s PFD, combined with constraints of the activity of the effector itself, could explain the observed domain assortments. The second notable difference between the 2 motifs is that the PP motif lacks a repeated organization similar to the PFD (with R1 and R2) and is present in only one copy on all currently identified PP-containing proteins. Here, one might speculate, considering the reported evolutionary relation between the metazoan RHIM motif – similar to PP – and the HET-s PFD, that a repeated version of a PP or PP-like motif, might have given birth to an ancestral, PFD-like domain. In this highly speculative scenario, specific advantages related to the repetitiveness of the amyloid motifs, would have been selected and would have impacted their distribution in the ascomycetes. Functional and structural analysis would define to what extent this fundamental difference (state of repetitiveness) has played a role in the evolution of the signaling amyloids.

The apparent success of the amyloid-based signaling in fungi might have been another contributing factor for the diversification of the amyloid motifs. The motifs have diversified to keep the signal-transducing specificity between the NLR protein and its respective downstream effector and reduce the chances of interference between different functional units. In support of this hypothesis, we did not find interactions in vivo between the PFD of HET-s and a PP amyloid motif, despite the fact that both motifs were able to propagate as prions in *P. anserina*. This would suggest that there is signal specificity for each of the 2 amyloid folds, possibly based on structural differences between them.

The diversity of signal-transducing amyloid motifs in fungi appears to be greater than the functionally characterized HET-s PFD and PP motifs. We have recently reported the identification of a group of HET-s-related amyloid motifs (HRAMs), largely distributed in filamentous ascomycetes. HRAMs, like the PFD, have 2 repeats (R1/R2) and are associated to HeLo and HeLo-like effector domains controlled by NLR receptors. The HRAMs have diversified in at least 5 discrete phylogenetic classes, although they still present the same pattern of hydrophobic/polar residues and one strongly conserved glycine as the repeats of the PFD. Sequence co-variance analyses suggest that the HRAMs adopt an amyloid fold similar to HET-S/s β-solenoid. Thus, if the driving evolutionary force behind the diversification of the HRAMs is to generate new signaling specificities, so that different signaling pathways do not interfere, this class of amyloids represents the perfect system to decipher the relations between structure and prion properties. What determinants, in structurally related amyloids, would specify and assure precise transmission of the signal is a question that may be possible
to answer with further functional and structural studies on the HRAMs. Intriguingly, phylogenetic diversification is observed, at present, only for the group of more complex amyloids like the HRAMs. One might suggest that the higher complexity and cooperativity of these folds offer more opportunities for subtle changes that impact the signaling specificity of the folds rather than shorter motifs like the PP. Yet, it is unclear to what extent our inventory of the signal transducing amyloids is exhaustive. It is possible that the phylogenetic divergence of shorter motifs would make them more difficult to recognize by sequence homology and would eventually lead to underestimation of their abundance. Currently, only one other putative signaling amyloid motif of similar size to PP, has been identified in fungi. The σ motif, named after an infectious element from Nectria haematococca, consists of 2 conserved sub-motifs (A and B) of 9 and 8 amino acids respectively, repeated 3 times in different patterns (AAA, ABA, ABB). Both sub-motifs expose a well-conserved glutamine and glycine residues in a sequence (G-x-Q) resembling partially to the central part of a PP (G-w-Q-G) motif. Phylogenetic relation between these 2 motifs seems unlikely, nevertheless one could imagine an evolutionary scenario in which one or several short amyloidogenic stretches have become more complex, in order to acquire different signaling specificity, giving birth to motifs like PP and σ. Those ‘proto signaling motifs’ with amyloidogenic propensity, could have shared similarities with a different class of ungapped short motifs, mediating protein-protein interactions, termed SLiMs (Short linear Motifs). SLiMs are extremely short in size (between 3 and 12 amino acids) and found mostly in disordered regions – characteristics associated with some of the amyloid signaling motifs (ex. PP and σ). Nonetheless, beside those similarities, evolution seems to have selected different features in both classes of motifs. For instance, SLiMs are involved in transient and low-affinity interactions dependent on a highly limited number of ‘hotspot residues’, shaping dynamic signaling networks. This seems to be a crucial difference when one considers the robustness and stability associated with amyloid folds. In addition, the identified SLiMs far exceed in number the identified signaling amyloids and can be subject of frequent convergent evolution. The short size of the amyloid motifs involved in signal transduction, prompts us also to consider the scenario of convergent evolution for this class of functional motifs. This alternate evolutionary scenario seems no less interesting. It implies that discrete structural elements have been chosen repeatedly and recruited for signaling, thus stressing the exclusive importance of such short sequence motifs in attaining a functional signaling amyloid.

**CONCLUSION**

Signal-transducing amyloids have become the first class of functional amyloids to span 2 kingdoms of life. Their diversity, outreach and the similar biological process, in which they play a role, suggest that amyloid signaling has emerged in early evolutionary times. This hypothesis puts forward 2 important questions: to what extent have we uncovered the diversity of the signaling amyloids and how widespread is their distribution through the tree of life? The information at our disposal on this class of amyloids and some of their identified common traits, could be used as a starting point to new in silico approaches on larger datasets in search of additional signal-transducing amyloids. However, the short size of these motifs and their unbiased sequences – as opposed to the N/Q-rich prion domains in *S. cerevisiae* – could make such analyses particularly complicated and lead to a high number of false positives. As for the outreach of the amyloid signaling and the possibility to find signaling amyloids in
other organisms than fungi and metazoans, one might find an inspirational suggestion in recently documented study on the herpes simplex virus (HSV). Two viral RHIM-containing proteins, ICP6 and ICP10 (from HSV1 and HSV2 respectively), act as inhibitors of necroptosis by disrupting the RHIM-dependent interactions between RIP1/RIP3 kinases. Thus, some viral pathogens possess signal-transducing amyloids in order to adapt in an arms race with their host for survival. It may be possible that other organisms such as bacteria use this strategy to circumvent the fungal immune system where amyloid signaling is proposed to play a role. Beyond the 2 above-mentioned questions, further investigations on the signal-transducing amyloids should aim to expand our current knowledge on the fundamental properties of amyloid and prion biology.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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