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LL-37 and CsgC exemplify the crosstalk between anti-amyloid, antimicrobial, and anti-biofilm protein activities

Jaime Santos, Salvador Ventura*, Irantzu Pallarès*

Protein misfolding and aggregation into amyloid fibrils is the main pathological hallmark of neurodegenerative diseases, including Alzheimer's, Parkinson's, Huntington's, and prion diseases (Chiti and Dobson, 2017). These insoluble fibrillar deposits possess a common structure characterized by a cross-β-sheet conformation in which β-strands run transversely to the fiber axis and form an intermolecular network of hydrogen bonds. However, amyloid formation is not only found in disease; the unique properties of this protein fold are also exploited by nature to perform a growing list of relevant and highly conserved cellular functions (Otzen and Riek, 2019). Pathogenic and functional amyloid formation needs to be regulated to sustain organism fitness, and a wide range of strategies have evolved to prevent uncontrolled aggregation. Importantly, we are not only exposed to our endogen amyloidogenic proteins, but we also face the threat of food and bacterial amyloids. For instance, many bacterial species in the gut microbiome can form an amyloid scaffolded biofilm, which facilitates bacterial proliferation, promotes the synergy between the host and the microbiome, and may eventually play a role in the pathogenesis of different diseases. It is then plausible to speculate that our own systemic defense against endogenous amyloids can work to fight this exogenic risk. Indeed, given the common structural properties shared by unrelated amyloids, it could be expected that the same cellular agents would mediate the response to human amyloids and those from other sources. In this perspective, we provide context for this idea by exploring the overlap between anti-microbial, anti-biofilm, and anti-amyloid activities, defining a framework for developing novel therapies for neurodegenerative diseases.

LL-37, an antimicrobial peptide with anti-amyloid and anti-biofilm activities: Found in all kingdoms of life, antimicrobial peptides are short, cationic, and amphipathic sequences that act as the primary innate immune response against a broad spectrum of pathogens. In a recent study, we demonstrated that the prototypical α-helical cathelicidin, LL-37; the active C-terminal component of the human cationic antimicrobial protein hCAP18, can bind to α-synuclein oligomers and fibrils, the molecular culprits of neurodegeneration in Parkinson's disease, with nanomolar affinity and without interfering with the functional form of the protein (Santos et al., 2021). This high-affinity interaction stalls aggregation and abrogates α-synuclein oligomers' toxicity. Notably, this is not the first evidence of LL-37 working as an anti-amyloid molecule. Previous reports indicate that it inhibits amyloid β peptide and islet amyloid polypeptide self-assembly, which have been shown to be associated with the loss of cortical neurons, a critical step towards Alzheimer's disease pathogenesis, and pancreatic β-cell degeneration in type 2 diabetes, respectively; even if, in these cases, LL-37 also interacts with the monomeric form of the peptides, acting in a less specific way (Armiento et al., 2020).

Strikingly, LL-37 is a potent antibiofilm agent, interfering with bacterial amyloid polymerization (Kai-Larsen et al., 2010). In many enteric bacteria like *Escherichia coli* and Salmonella, the curli system promotes the formation of extracellular amyloid fibers that entangle into a biofilm matrix. CsgA, the precursor of curli fibers, is transported to the cell surface as an unfolded polypeptide; there, the interaction with CsgB triggers amyloid formation. LL-37 has been shown to inhibit curli fibrillogenesis and biofilm formation by precluding CsgA polymerization at substoichiometric concentrations (Kai-Larsen et al., 2010).

Thus, LL-37 is an outstanding example of a molecule that intertwines different anti-amyloid activities. One could speculate that the original function of LL-37 as an antimicrobial peptide was interfering with bacterial amyloid polymerization. Eventually, the molecular determinants for this original anti-amyloid function, also encode for a side activity as an α-synuclein chaperone. Noteworthy, LL-37 coexists with α-synuclein in disease-relevant tissues, like the brain or the gut, suggesting that LL-37-mediated α-synuclein protection occurs naturally.

Supporting this hypothesis, CsgA and α-synuclein inhibition by LL-37 share some common mechanistic traits. In α-synuclein, the interaction occurs between the opposed hydrophobic and positively charged surfaces at the antimicrobial peptide amphipathic helix and the complementary hydrophobic exposed regions adjacent to the negatively-charged and disordered C-terminal tails in the aggregates. The surface complementary rather than specific intermolecular residue-to-residue contacts drive selective binding, as demonstrated (Santos et al., 2021). Notably, Brauner and co-workers concluded that the mechanism underpinning the CsgA inhibitory activity of LL-37 is similar to that we proposed for α-synuclein (Kai-Larsen et al., 2010), with electrostatic encounters between the two molecules occurring independently of the primary sequence, and playing a crucial role in both the specificity of CsgA recognition and the blockage of amyloidogenic regions. The observed inhibitory potency at substoichiometric concentrations suggests that LL-37 binds to soluble oligomeric species of CsgA, preventing their conversion to a fibrillar structure, as it does for α-synuclein.

CsgC, the other side of the coin: The above-described LL-37 activities match those of CsgC, a protein component of the curli system. The biogenesis of extracellular amyloids requires exquisite control to prevent premature amyloidogenesis within the cell. CsgC is a member of the curli system that acts as a periplasmatic chaperone that inhibits intracellular CsgA aggregation at substoichiometric concentrations (Evans et al., 2015). CsgC is a monomeric protein of 110 residues arranged in an immunoglobulin-like β fold, with seven strands forming two sheets. A dramatic loss of the inhibitory potency was observed when basic side chain residues located in a CsgC exposed and positively charged patch were mutated. In contrast, CsgC mutations that do not involve charge changes have little effect. Interestingly, the CsgC chaperone anti-amyloid mechanism of action exploits the same biophysical traits in its protein target as LL-37 and, not surprisingly, the positive charge pattern on the CsgC beta-sheet surface is highly conserved among homologues (Taylor et al., 2016). Indeed, CsgC structural homologues displaying low sequence identity but holding the electrostatic surface retain the inhibitory potential, while those with a high sequence similarity but a less pronounced basic patch are ineffective in blocking CsgA aggregation.

Closing the circle, the data reported by Evans et al. (2015) evidence that CsgC also inhibits α-synuclein amyloid formation. CsgA and α-synuclein sequence alignment detects relevant sequence similarity between the CsgA imperfect repeat R3 and the α-synuclein C-terminal domain, thus explaining how molecules targeting these charged recognition motives with electrostatic complementary could bind transiently to the soluble prefibrillar oligomers and stall the transition into fibrillar species of two proteins that could not be less related evolutionarily.

More than a casual encounter: LL-37 and CsgC activities define a two-way road, a human molecule that inhibits human and bacterial amyloids and a bacterial protein that blocks human and bacterial fibrillation. They are likely two of many molecules that exhibit moonlighting anti-amyloid functions. In support of this idea, our results suggest that the ability of LL-37 to inhibit α-synuclein aggregation may be common to other antimicrobial peptides that share the same physicochemical properties, such as the phenol-soluble modulin α3 (Santos et al., 2021) or the LL-III peptide (Oliva et al., 2021).

The anti-microbial anti-amyloid conundrum has been described in other structural folds, such as two antimicrobial α-defensins that exhibit multi-target inhibitory activities against amyloid formation (Zhang et al., 2021). A final intriguing case is transthyretin, one of the proteins responsible for the transport and delivery of the thyroid hormone thyroxine and retinol to cells in humans, which combines amyloidogenic and antimicrobial properties with inhibitory and anti-biofilm activities (Jain et al., 2017).

Taken together, the above-discussed examples are in support of the emerging perspective that anti-amyloid, antimicrobial, and anti-biofilm crosstalk can be molecularly encoded in unrelated protein structures (**Figure 1A**). If true, there might be a repertoire of therapeutically relevant molecules awaiting discovery at the intersection of these worlds.

The microbiome, ally or foe? To add a new layer of complexity to this scenario, recent studies support that amyloid formation can be enhanced through heterologous seeding between human and nonhuman amyloidogenic proteins, bypassing

the species barrier. This is relevant because the human organism is more than 99% microbial, in terms of genes. Indeed, the gut microbiome alone encodes many more amyloidogenic proteins than those observed so far in human tissues. With a growing body of evidence suggesting that initial amyloid formation of neuronal proteins may originate in the gut and then be transmitted to the brain through the enteric nervous system (Kim et al., 2019), understanding interspecies amyloid cross-reactivity is becoming increasingly necessary. In a recent study, Wang et al. (2021) identified 38 genes in the bacterial genome as host neurodegeneration promoting agents, including those related to curli formation. It was subsequently demonstrated that bacteria-derived CsgA cross-seeds and colocalizes with α-synuclein in human neuroblastoma cells and a *Caenorhabditis elegans* (*C. elegans*) Parkinson's disease model. This process is bidirectional and seems widespread, since CsgA has also been observed to promote fibrillation of Aβ, SOD1, and polyQ-expanded huntingtin in *C. elegans* models of Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease, respectively (Wang et al., 2021).

In addition to its role in unwanted cross-seeding, functional microbial amyloids are recognized by the host's innate immune system as displaying a pathogen-associated molecular signature recognized by toll-like receptor 2 and the inflammasome, eliciting a proinflammatory response. This constitutes an evolutionary selected strategy to fight exogenous amyloids. However, in turn, it compromises homeostasis, inducing the production of proinflammatory chemokines in microglia, which ultimately can lead to pathogenic protein aggregation of neuronal proteins in the brain.

In this framework, one last reckless question remains. May microbiota also plays a protective role? As well as microbiota amyloids may trigger disease, to which extent anti-amyloid bacterial proteins, like CsgC, might counterbalance them? May they already play a role in preventing the endogenous aggregation of human proteins in the enteric nervous system?

All in all, it is now clear that amyloid formation and amyloid inhibition are connected phenomena that transcend individual species. Exploring this crosstalk appears as a promising strategy for understanding the etiology of human amyloid diseases, but also to fight them. Bacterial amyloids are suspicious of being pathogenesis triggers, especially at the brain-gut axis, and targeting this initial propagation needs to be explored as a therapeutic alternative (**Figure 1B**). In turn, bacteria and hosts possess a repertoire of structurally diverse molecules with the ability to control endogenous and exogenous amyloidogenesis. These molecules may hold biomedical value themselves or set the bases for discovering structurally related therapeutic entities.

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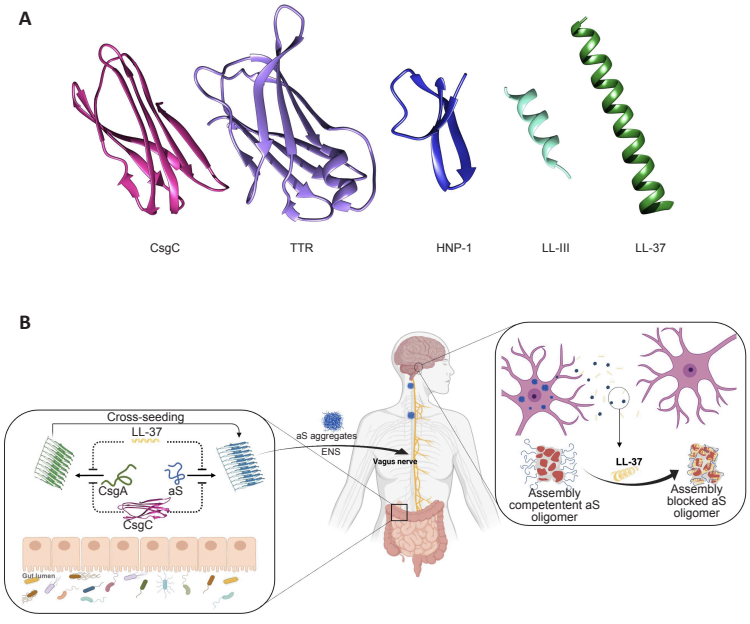

Figure 1 | Structures of selected peptides with anti-amyloid, antimicrobial and anti-biofilm activities and visual overview of the gut-brain axis.
(A) Left to right CsgC (2xsk), monomeric transthyretin (TTR) (4D7B), α-defensin HNP-1 (3HJD); LL-III peptide (structure generated using AlphaFold) and human cathelicidin LL-37 peptide (2K6O). The structures were visualized in Chimera (UCSF). (B) Overview of the proposed LL-37 and CsgC anti-amyloid, antimicrobial, and anti-biofilm crosstalk in the microbiome-gut-brain axis in Parkinson's disease. aS: α-Synuclein; ENS: enteric nervous system; HNP: human neutrophil peptides 1. Created with BioRender.com.

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