secHsp70 as a tool to approach amyloid-β42 and other extracellular amyloids

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ABSTRACT
Self-association of amyloidogenic proteins is the main pathological trigger in a wide variety of neurodegenerative disorders. These aggregates are deposited inside or outside the cell due to hereditary mutations, environmental exposures or even normal aging. Cumulative evidence indicates that the heat shock chaperone Hsp70 possesses robust neuroprotection against various intracellular amyloids in Drosophila and mouse models. However, its protective role against extracellular amyloids was largely unknown as its presence outside the cells is very limited. Our recent manuscript in PNAS revealed that an engineered form of secreted Hsp70 (secHsp70) is highly protective against toxicity induced by extracellular deposition of the amyloid-β42 (Aβ42) peptide. In this Extra View article, we extend our analysis to other members of the heat shock protein family. We created PhiC31-based transgenic lines for human Hsp27, Hsp40, Hsp60 and Hsp70 and compared their activities in parallel against extracellular Aβ42. Strikingly, only secreted Hsp70 exhibits robust protection against Aβ42-triggered toxicity in the extracellular milieu. These observations indicate that the ability of secHsp70 to suppress Aβ42 insults is quite unique and suggest that targeted secretion of Hsp70 may represent a new therapeutic approach against Aβ42 and other extracellular amyloids. The potential applications of this engineered chaperone are discussed.

KEYWORDS
Amyloid β, Alzheimer disease; ataxin 3; Drosophila; Hsp27; Hsp40; Hsp60; Hsp70; neurodegeneration; protein misfolding

Introduction

Alzheimer disease (AD) is a progressive, incurable neurologic disorder characterized by memory loss, cognitive decline and degeneration of brain neurons. It is the most prevalent neurodegenerative disease and the leading cause of dementia among older people. A prominent pathological feature in the AD brain is the abnormal, extracellular deposition of the amyloid-β42 peptide (Aβ42). This peptide has an extraordinary ability to undergo conformational changes and is highly amyloidogenic. Interestingly, the heat shock chaperone Hsp70 has been found associated with extracellular deposits in AD. Since Hsp70 is a cytosolic protein, it has been suggested that such association may be a consequence of release due to non-specific processes, such as cell death. Alternatively, it has been proposed that Hsp70 may go out of the cells through exosomes to stop the accumulation of proteotoxic assemblies, which agrees with the increased levels of Hsp70 seen in AD.

Whatever the case, if the interaction with Aβ42 assemblies outside the cell is too extensive the extracellular levels of Hsp70 would be severely affected. In this situation, an imbalance between neuronal Hsp70 function and the toxic accumulation of Aβ42 may be a major trigger for the neuronal death.

In this regard, we recently hypothesized that the rational delivery of Hsp70 to the extracellular space would be an effective approach to prevent formation of toxic assemblies of Aβ42 and subsequent neurodegeneration. This hypothesis was supported by previous studies showing that Hsp70 has the ability to alleviate the aggregation of Aβ42 in several experimental models. For instance, in vitro studies in a cell-free system

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indicate that Hsp70 inhibits early stages of Aβ42 aggregation. This inhibitory effect causes dissociation of preformed oligomers but not fibrils, suggesting that this chaperone targets oligomeric intermediates on the Aβ42 aggregation pathway. Also, Hsp70 demonstrated neuroprotective activity against intracellular Aβ42 in primary culture, while downregulation of Hsp70 led to increased protein aggregation in transgenic worms expressing intracellular Aβ42. Taken together, these studies suggested that if Hsp70 were present in the same cellular compartment in which Aβ42 is produced, it would suppress the early aggregation of Aβ42. Thus, we reasoned that the thoughtful enhancement of Hsp70 in the extracellular milieu would prevent or delay pathologies associated with extracellular deposition of Aβ42.

**secHsp70: A robust blocker of Aβ42-induced toxicity**

To test the aforesaid hypothesis, we created transgenic flies expressing human Hsp70 fused to a signal peptide for secretion (secHsp70). We found that secHsp70 robustly suppresses a variety of Aβ42 phenotypes including the glassy eye, locomotor dysfunction, shortened lifespan, premature cell death, and neurodegeneration of brain neurons. We also found that secHsp70 exerts neuroprotection without obvious changes in Aβ42 steady-state levels or aggregation. Interestingly, this protective effect does not require the foldase activity of secHsp70. Instead, neuroprotection is mediated by the holdase activity as evidenced through mutations of the substrate-binding domain. We concluded that secHsp70 neutralizes Aβ42 without the assistance of factors involved in protein folding or degradation and that the holdase activity of secHsp70 is essential to mask neurotoxic Aβ42 epitopes. Thus, we strongly believe that secHsp70 blocks Aβ42 neurotoxicity by inducing the accumulation of nontoxic aggregates and/or preventing pathological interactions with cellular substrates. Further experiments are required to define the precise mechanisms of secHsp70 neuroprotection.

**Are other heat shock chaperones effective against extracellular Aβ42?**

To address this question we tested additional heat shock protein family members that possess different roles and distributions. These include Hsp27, a small chaperone carrying extra antioxidant and antiapoptotic roles; Hsp40, a DNAJ domain chaperone with essential or accessory functions in a variety of processes including nascent chain folding, transport and degradation of proteins; and Hsp60, a nuclear-encoded mitochondrial chaperone that is also present in the cytosol, extracellular space and on the cell membrane. To facilitate comparison between these chaperones, we targeted the insertion of the transgenes to the same chromosomal location to achieve similar expression levels. Thus, we created PhiC31-based UAS lines carrying human Hsp27, Hsp40, Hsp60 and Hsp70 with and without signal peptide for secretion. We first compared the ability of the normally expressed chaperones (cytosolic) against the toxicity induced by mutant Ataxin3-Q78 (Atx3-Q78) in the fly eye. Since Atx3-Q78 is an intracellular amyloid with well-characterized phenotypes, this experiment served as control to functionally assess the strength of the new PhiC31-based transgenes. As expected, only Hsp40 and Hsp70 rescued the Atx3-Q78 phenotype (Fig. 1A), suggesting that the expression levels elicited by the site-specific integration of the transgenes are sufficiently high to achieve neuroprotection. However, when the same chaperones were engineered for secretion and tested against extracellular Aβ42, only secHsp70 displayed robust protection of the Aβ42-induced eye phenotype (Fig. 1B). This result highlights the remarkable ability of secHsp70 to suppress Aβ42 insults. Thus, in the following sections, we discuss potential uses and applications of this engineered chaperone.

**Future directions**

**Impact on other extracellular amyloids**

After confirming that the extracellular delivery of Hsp70 prevents Aβ42-related phenotypes, the next logical step will be to expand its uses to other extracellular amyloids. Interestingly, several transgenic fly strains that accumulate extracellular amyloidogenic proteins are already available in different laboratories. These include flies expressing Aβri and ADan peptides (familial British and Danish dementia), mutant transthyretin (familial amyloidotic polyneuropathy), PrP (prion disorders), mutant lysozyme (hereditary lysozyme amyloidosis), and amylin (type 2 diabetes) to name a few. All these strains exhibit amyloid-related phenotypes in the eye or CNS and, thus, are ideally suited to investigate the potential protective effect of secHsp70 against each of these extracellular amyloids (Fig. 2).
Organelle-specific targeting
Converging data indicate that Aβ42 can be internalized from the extracellular space via endocytic and non-endocytic pathways (see below) leading to organelle dysfunction and neuronal death. Of note, confocal and biochemical studies have shown that Aβ42 can penetrate into mitochondria and interact with several mitochondrial components, including complex II of the respiratory chain. These interactions result in severe mitochondrial dysfunction, a key pathological event in AD. Therefore, it will be interesting to fuse the same Hsp70 isoform used above to a mitochondrial targeting signal to induce its deliberate deployment into this organelle (mitHsp70, Fig. 2). This study would reveal whether the “masking” ability of this chaperone can also protect against Aβ42-induced mitochondrial toxicity. If this is the case, a combinatorial approach co-expressing secHsp70 and mitHsp70 may potentiate the neuroprotection against Aβ42-related pathologies. On the other hand, the same rationale can be applied to target other pathological protein aggregates that accumulate in different organelles. Among these, the nuclear accumulation of C9orf72-derived dipeptide repeats linked to ALS/FTD will be a relevant target. Thus, the engineering of a nuclearly targeted Hsp70 version (nucHsp70, Fig. 2) may have extensive applications in this regard.
Spreading of amyloidosis

Several studies indicate that, despite their different origins, misfolded proteins can exit affected cells and behave as amyloid seeds in the extracellular milieu.\textsuperscript{20} Of note, these seeds can penetrate other cells to propagate formation of toxic assemblies and subsequent neurotoxicity. In this regard, extracellular A\textsubscript{β42} can be internalized by both active and passive mechanisms, which results in cell-to-cell propagation of toxic oligomers.\textsuperscript{21,22} Interestingly, human tau can be also released to the extracellular milieu and internalized into neighboring cells through endocytic mechanisms.\textsuperscript{23} In addition, recent evidence indicates that α-synuclein, SOD1 and huntingtin amyloids are also associated with transcellular propagation.\textsuperscript{24} Therefore, it will be important to determine whether the deliberate deployment of Hsp70 in the extracellular milieu would target toxic amyloid seeds to prevent or minimize the spreading of amyloidosis (Fig. 2).

Learning and memory studies

Extracellular deposition of A\textsubscript{β42} in Drosophila leads to age-dependent learning defects.\textsuperscript{25} Thus, another logical step of our work will be to define whether secHsp70 can suppress these behavioral deficits (Fig. 2). On the other hand, several transgenic mouse models have been instrumental in studying A\textsubscript{β42} accumulation and memory decline.\textsuperscript{26} However, the potential protective role of Hsp70 upon engineered secretion has not been investigated yet in any mouse model of AD. This could be easily achieved by global expression of secHsp70 in the mouse brain through somatic brain transgenesis.\textsuperscript{27} Therefore, it will be important to determine whether the extracellularly targeted Hsp70 has the ability to stop or delay the A\textsubscript{β42}-associated memory decline. If so, the results of these studies will have profound implications for future design of therapeutic strategies.

Concluding remarks

Although Hsp70 is one of the most potent suppressors of protein misfolding and neurodegeneration, this is the first time that a secreted form that expands its range of action to the secretory pathway and extracellular space has been engineered and tested against A\textsubscript{β42}.\textsuperscript{8} In our opinion, adding this new tool to prevent or delay the formation of toxic extracellular amyloids will result in additional knowledge about the abnormal biology of A\textsubscript{β42} in AD. In addition, it may expand the spectrum of...
therapeutic options for other neurodegenerative diseases, particularly those involving intercellular propagation of misfolded proteins. We anticipate that our work will stimulate research in the areas described above and that the following years will shed light onto the therapeutic potential of secHsp70 and other derived modifications.

Materials and methods

Drosophila stocks and genetics

The Aβ42 flies carry 2 tandem copies of Aβ42 fused to the Argos signal peptide with their own UAS regulatory sequence. These flies imitate the duplication of the APP gene associated with familial AD in humans.28 Flies expressing the Atx3-Q78 transgene were kindly provided by N. Bonini.29 For expression in the eye, the UAS-Aβ42 and UAS-Atx3-Q78 transgenes were first recombined with the gmr-Gal4 driver to generate w; gmr-Gal4, UAS-Aβ42(2X)/CyO and w; gmr-Gal4, UAS-Atx-Q78/CyO, respectively, and then crossed with the chaperone-related UAS lines. The cDNAs encoding for human Hsp27 (a gift from H. Kampinga),30 Hsp40 (Addgene #19468), Hsp60 (Ori-gene SC111640) and Hsp70 (a gift from N. Bonini) were isolated from their respective plasmids and subcloned with and without signal peptide into the injection vector pJFRC-MUH (Addgene #26213). The resulting constructs were verified by sequencing and targeted to the attP2 landing site (3rd chromosome) by PhiC31-mediated integration. Details of cloning are available upon request.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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