

University of Groningen

Genetic factors and analysis of protein misfolding in vivo

Holmberg, Mats

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2015

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Holmberg, M. (2015). *Genetic factors and analysis of protein misfolding in vivo*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Chapter 5: General discussion

5.1 Summary

MOAG-4 and its human orthologues SERF1A and SERF2 appear to be small, intrinsically unfolded proteins that promote protein aggregation. Their function is conserved between *Caenorhabditis elegans* and humans, as is the amino acid sequence and overall positive charge, with only smaller changes throughout eukaryote. Differences in sequence or context have not revealed any significant differences in function between Moag-4, SERF1 and SERF2, although a difference in subcellular distribution has been found for SERF1 and SERF2. It is reasonable to assume that some differences in function will eventually be found.

SERF1 has been shown to bind aSyn early in the aggregation process and promote the formation of so-called on-pathway oligomers that can form amyloids through fibrillisation [1]. However, SERF1 is not bound to the final aggregate. Similarly, the interaction between MOAG-4 and polyQ also seems to be lost at later stages of the aggregation process, since MOAG-4 has been shown to be excluded from aggregates formed in polyQ aggregation models. The fact that direct interactions of MOAG-4 or SERFs with aggregation-prone polyQ peptides in worms and tissue culture cells could not be detected suggests either low-affinity binding or an indirect interaction in cells (unpublished observations, Eva Teuling). This stands in contrast with the interactions between SERF1 and aSyn which are in the order of 10 μM [1]. Nevertheless, the above experimental conditions varied strongly and a difference in interaction strength cannot be considered established. The promotion of aggregation by this family of proteins seems to be specific for amyloidogenic proteins and not to extend to non-amyloid protein aggregation. MOAG-4 and SERF1 have been shown *in vitro* and *in vivo* to catalyse A β , aSyn and polyQ aggregation (inside and outside of a Huntingtin exon 1 context). *In vitro*, SERF1 has also been shown to enhance amyloid formation of the mouse prion protein, but not the aggregation of citrate synthase and insulin which are non-amyloidogenic proteins [2, 3].

Although little is known about the pathway in which MOAG-4 and the SERF proteins act, the protective effect of its removal from *C. elegans* appears to be independent of the heat shock response and of protein degradation machineries (Chapter 2). This is evidenced by the fact that in the absence of MOAG-4, the significant increase in protein aggregation normally observed upon the removal of heat shock transcription factor 1 (HSF-1), molecular

chaperones or factors required for protein degradation is no longer seen. Conversely, MOAG-4 does not seem to be part of the heat shock response, as we could not observe any changes in expression of MOAG-4 upon heat shock, despite the presence of several Hsp1 binding sites within 5000 base pairs upstream of the *moag-4* gene (Rogier Burggraaff and Mats Holmberg, unpublished observations). As SERF and MOAG are also sufficient to promote amyloid-specific aggregation *in vitro* without the need for other factors, these observations suggest that MOAG-4 and the SERF proteins act downstream of these protein quality-control systems to drive aggregation-prone proteins into aggregates. In *C. elegans*, the strong dependence of protein aggregation on MOAG-4 indicates a lack of alternative pathways for protein aggregation. One possibility is that MOAG-4 acts as the only significant gateway to active amyloid protein sequestration. The lack of effect of the components of the heat shock system in MOAG-4-deficient worms suggests that any potential MOAG-4-independent effects on protein aggregation will be small relative to passive, non-catalysed aggregation. The extent to which this is also true for mammalian systems that express SERF orthologues from several chromosomal loci is still unknown. The strength of the gateway effect of MOAG-4 might also be due to the short timeframes of *C. elegans* protein aggregation models. In this scenario, slower aggregation pathways might be less dependent on MOAG-4.

Chapter 4 of this thesis contains the description of a method developed for the native screening of fluorescently labelled aggregation-prone proteins. The method is based on the separation of fluorescently labelled proteins within biological samples through an agarose gel followed by analysis using a fluorometric scanner. The chapter also describes how to combine this method with others to characterise protein species of interest. The method was developed to analyse the effect of modifiers of aggregation on the aggregation process, but would also be suitable for monitoring protein modifications, cleavage products or oligomerisation. The main strengths of the method are the speed and low workload of the initial step which allows for quick screening of experimental conditions and reliable monitoring of any changes in the distribution of protein species. In the initial electrophoresis step in a 1% agarose gel, microscopically visible protein aggregates are consistently retained in the loading wells. This is evidenced by the fact that no significant alteration in the ratio of retained fluorescent signal is detected when aggregates are pre-separated by centrifugation. The results obtained also correlate with those of aggregates quantified *in vivo*. Further advantages of this method are that it makes a separate dot-blot for aggregate retention unnecessary and there are no

problems with signal linearity detection as is common with other techniques, *e.g.* horseradish peroxidase [4], although some loss is unavoidable due to fluorescence quenching.

5.2 Role of MOAG-4 and SERF proteins in biology

5.2.1 Potential limitation of model organisms for neurodegenerative diseases

In the creation of models of human disease, the biological relevance of the chosen cell type or organism should always be taken into consideration. Model organisms are typically chosen to mimic a specific phenotype in a relatively short period of time. In models of age-associated NDDs, these models are typically chosen to mimic specific phenotypes in reasonable timeframes for experiments, to overexpress toxic proteins or to select aggressively aggregating protein isoforms [5].

However, choosing a model organism – no matter how closely it reproduces features seen in patients – brings about problems. The time components (*e.g.* short life span and accelerated disease progression) can be of particular concern, especially when working with amyloid disorders, which are stochastically sensitive and have an exponential growth phase. The amplification of disease severity and progression seen in model organisms can also increase the likelihood of findings not being similar to those found in patients. These undesired effects are still a possible cause for the observed effect of SERF as there is a lack of data regarding the influence of SERF-1/2 in patients suffering from amyloid NDDs. Whether or not SERF loci susceptibilities to NDDs are found, targeting or mimicking the aggregation promoting effect of SERF can still be a strong therapeutic strategy. Before the robustness of the MOAG-4 and SERF phenotypes can be properly evaluated, the link between protein and disease has to be further investigated.

5.2.1 Other functions of MOAG-4 and SERF

The possibly mild effects of MOAG-4/SERF1 in humans and the lack of identified interaction partners in the amyloid promoting activity, lead us to speculate that the evolutionary conservation of MOAG-4 and SERF could be due to functions unrelated to the formation of amyloids. In support of this theory, genetic studies have linked the human SERF-1 locus to spinal muscular atrophy (SMA) [6]. SMA is a motor neuron disorder which causes progressive degeneration of motor neurons in the anterior horn of the spinal cord, leading to

muscle weakness and atrophy. However, it was shown that the association with the SERF-1 locus was more likely dependent on the neighbouring SMN (survival motor neuron) gene. Whether or not SERF-1 or SERF-2 have an effect on the development of NDDs, they could still be targets for therapy.

5.2.3 Cellular protection strategies

In the promotion of fibrillisation, SERF-1 seems to work purely as a catalyst, accelerating the formation of amyloids, while not having any ability to induce amyloid conformations in proteins that do not form them spontaneously. This is illustrated by amyloid kinetics experiments with SERF-1 and the fusion protein GST-httQ53ex1 where SERF-1 was only able to promote aggregation after proteolytic removal of the GST moiety [1].

A physiological role for fibrillisation is supported by the fact that many disease-associated amyloid plaques form in non-neuronal tissues. The benefit of stowing away unwanted proteins that the cell is otherwise poorly equipped to deal with is most obvious in somatic cells that are constantly replaced. These cells have a limited lifespan and their function need only be maintained to avoid the cost of replacing them. This argument can also be extended to cell types that cannot be replaced as easily, such as those in the central nervous system. In other words, it may well be the case that fibrillisation in neurons works together with neuronal plasticity to potentially stave off detrimental effects until after reproduction and, until recently, well beyond the average human life span. The demonstration that SERF-1 specifically shifts misfolded aSyn into 'on-pathway' oligomers and hence reduces the amount of soluble oligomers in cells would fit well with a survival strategy of multicellular, mortal organisms.

5.3 Possible mechanism

There are several possible ways for MOAG-4 and SERF to induce aggregation and convey the amyloid specificity. The overall positive charge is a striking feature of MOAG-4 and SERF and is necessary for the high affinity binding to the C-terminal domain of aSyn [1]. It is possible that the MOAG-4/SERF-induced increase in the local concentration of positive charge around the amyloidogenic protein acts similarly to the high concentration of hydrogen ions in an acidic environment: low pH seems to enhance the aggregation of several disease-

causing amyloidogenic proteins. It has been shown by sedimentation and electron microscopy that A β ₄₂ incubated at pH 5.8 forms fibrils faster than the same material incubated at pH 7.4. Interestingly, A β ₄₂ produced at the lower pH is more harmful to PC12 rat adrenal cells [7, 8]. An increased aggregation rate in acidic environments has also been observed in aSyn aggregation [9, 10]. However, although not very common, there still exist many positively charged proteins and protein domains, and if a local unspecific distribution of cations were sufficient to promote fibrillisation, this correlation would have been identified in other screens. To formally rule out this possibility, binding and fibrillisation assays could be carried out on a sample of small, preferentially flexible proteins that have a pI of approximately 10.

If charge is insufficient to account for the activity of MOAG-4 and SERF, it is reasonable to assume a contribution from the sequence and structure of the protein. One possibility is that MOAG-4 and SERF act as a primary template for amyloidogenesis. This, however, is difficult to reconcile with the known binding of SERF-1a to residues 111 to 140 in the C-terminus of aSyn instead of to the amyloid-prone and hydrophobic residues in the centre of the protein (61 to 95). In the case of aSyn, this binding instead suggests that MOAG-4 and SERF can expose amyloid-prone sequences by binding to and removing sequences that would otherwise prevent fibrillisation. This type of perturbation has previously been associated with increased amyloid formation [11-13].

A third possible explanation of phenotypes caused by MOAG-4 and SERF is the difference between reversible and irreversible kinetics. Although SERF does not induce non-amyloid aggregation, there is no data indicating the extent to which binding is limited to amyloidogenic peptides. In the case of aSyn, where binding is salt-dependent, the link still has to be made between C-terminal binding and fibril formation, and we lack data pointing to binding specificity. The case can be made that even with binding being indiscriminate between amyloidogenic and non-amyloidogenic proteins, the binding kinetics may differ: in the case of an amyloid protein a structural change might be irreversible after initial oligomerisation due to the cross- β hydrogen bond strength. On the other hand a similar change might be insufficient to nucleate a stable aggregate in a protein not prone to form amyloids. Although this model is hard to prove experimentally, the first steps would be to measure binding to non-acidic amyloid protein and similarly charged non-amyloid proteins. After this, proteins can be designed for experiments in which NMR chemical shifts or the

environment sensitivity of tryptophan fluorescence can reveal whether or not MOAG-4/SERF is still bound when the substrate protein forms an oligomer.

5.4 Further perspectives

In general, there are two main topics that require investigation in order to understand the function of MOAG-4 and SERF. The first one is its role in disease development which is critical to understand the relevance of MOAG-4 and SERF as a factor in human disease. The most straightforward method would be to correlate SERF-1 and SERF-2 protein as well as mRNA levels with the onset of aggregation or cell death in post-mortem human brain tissue. As SERF-1 and SERF-2 are heterogeneously expressed throughout the brain and since the stochastic nature of amyloid formation can induce neighbouring cells to undergo very different fates, these measurements should be made on small sections or, if possible, at the level of individual cells. This is possible when looking at aggregates, but not when analysing cell death, since cell death in itself precludes these measurements. Instead, analysis would have to be done on larger tissue sections to obtain a median value correlating the amount of cell death with the expression levels in remaining cells. Elevated SERF levels would be expected to correlate with increased fibrillation of amyloidogenic proteins, which can be readily assayed by filter retardation assays or native agarose gel electrophoresis. If, under the conditions studied, aggregates reduce the exposure to toxic protein species, high post-mortem SERF-1 and SERF-2 levels would relate to a high amyloid load, but a low or late onset of neuronal cell death.

The second main topic is the creation of a consistent model that explains the mechanism of MOAG-4 and SERF. The most critical experiments to help us understand the function of MOAG-4 and SERF would be those that allow the study of MOAG-4/SERF binding to amyloidogenic proteins other than aSyn. The binding to a specific charged region in aSyn which are missing from many other amyloidogenic proteins is hard to reconcile with the general amyloid promoting effect of MOAG-4 and SERF-1. Little can be said about the general mechanism before binding has been analysed for more substrates. A suitable candidate substrate protein would be A β ₄₀. Like aSyn, this protein has been intensively studied by NMR, and unlike polyQ allows for residue assignment. A β ₄₀ also has a slow fibrillation rate, which should allow binding studies of isotopically enriched samples, as well as an estimated pI of 5.6, with 5 acidic and 6 basic residues distributed relatively evenly over the first 28 residues.

A β_{40} thus combine feasibility of the analysis of a known substrate shown to work *in vitro* with a clear lack of the binding motif found in aSyn. This would be expected to result in a strongly reduced binding of MOAG-4 and SERF. It would be interesting to see if this results in a lower level of activity and to identify those residues of MOAG-4/SERF and the substrate protein that participate in the recognition.

Another mechanistic question that remains to be answered is which residues of MOAG-4 and SERF participate in substrate recognition and catalysis. Since MOAG-4 and SERF are small and disordered proteins, NMR is a suitable method for investigating these proteins structurally. As no orthologue of MOAG-4 and SERF has previously been investigated in this way, a resonance assignment will be required to allow verification of the predicted secondary structure as well as mobility within the protein. After this, purified, isotopically unlabelled aSyn can be used to find the residues involved in recognition after which it might be possible to build a credible mechanistic model. Towards the end of the studies that led to this thesis, steps were taken towards the isolation of ^{13}C ^{15}N enriched MOAG-4 for NMR measurements and resonance assignments. However, due to problems in purifying an intact protein, these experiments were not yet completed at the time of writing.

Reference list

1. Falsone SF, Meyer NH, Schrank E, Leitinger G, Pham CL, Fodero-Tavoletti MT, Holmberg M, Dulle M, Scicluna B, Gesslbauer B, Rückert HM, Wagner GE, Merle DA, Nollen EA, Kungl AJ, Hill AF, Cappai R, Zangger K. SERF protein is a direct modifier of amyloid fiber assembly. *Cell Rep.* 2012 (2), p. 358-71.
2. Buchner J, Grallert H, Jakob U. Analysis of chaperone function using citrate synthase as nonnative substrate protein. *Methods Enzymol.* 1998(290), p. 323-38.
3. Scheibel T, Weikl T, Buchner J. Two chaperone sites in Hsp90 differing in substrate specificity and ATP dependence. *Proc Natl Acad Sci U S A.* 1998 (4), p. 1495-9.
4. Heinicke E, Kumar U, Munoz DG. Quantitative dot-blot assay for proteins using enhanced chemiluminescence. *J Immunol Methods.* 1992 (2), p. 227-36.
5. Chin J. Selecting a Mouse Model of Alzheimer's Disease. In: Erik D. Robertson (eds) *Methods in Molecular Biology 670* 2011, ISBN 978-1-60761-743-3, Springer New York Dordrecht Heidelberg London, p. 169-90.
6. Scharf JM, Endrizzi MG, Wetter A, Huang S, Thompson TG, Zerres K, Dietrich WF, Wirth B, Kunkel LM. Identification of a candidate modifying gene for spinal muscular atrophy by comparative genomics. *Nat Genet.* 1998 (1), p. 83-6.
7. Su Y1, Chang PT. Acidic pH promotes the formation of toxic fibrils from beta-amyloid peptide. *Brain Res.* 2001 (1-2), p. 287-91.
8. Wood SJ, Maleeff B, Hart T, Wetzel R. Physical, morphological and functional differences between pH 5.8 and 7.4 aggregates of the Alzheimer's amyloid peptide A β . *J Mol Biol.* 1996 (5):870-7.
9. Uversky VN, Li J, Fink AL. Evidence for a partially folded intermediate in α -synuclein fibril formation. *J Biol Chem.* 2001(276), p. 10737-44.
10. Morris AM, Finke RG. α -Synuclein aggregation variable temperature and variable pH kinetic data: A re-analysis using the Finke-Watzky 2-step model of nucleation and autocatalytic growth. *Biophys Chem.* 2009 (1-3), p. 9-15.
11. Murray, IV, Giasson, BI, Quinn, SM, Koppaka, V, Axelsen, PH, Ischiropoulos, H, Trojanowski, JQ, Lee, VM. Role of alpha-synuclein carboxy-terminus on fibril formation in vitro. *Biochemistry.* 2003(42), p. 8530-40.
12. Levitan, K, Chereau, D, Cohen, SI, Knowles, TP, Dobson, CM, Fink, AL, Anderson, JP, Goldstein, JM, Millhauser, GL. Conserved C-terminal charge exerts a profound influence on the aggregation rate of α -synuclein. *J. Mol. Biol.* 2011 (411), p. 329-333.
13. Hoyer, W, Cherny, D, Subramaniam, V, Jovin, TM. Impact of the acidic C-terminal region comprising amino acids 109-140 on alpha-synuclein aggregation in vitro. *Biochemistry.* 2004(43), p. 16233-42.