USING STEFIN B AS A MODEL AMYLOIDOGENIC PROTEIN – OVERVIEW

STEFIN B KOT MODEL ZA AMILOIDOGENE PROTEINE – PREGLED

*Eva Žerovnik**

Department of Biochemistry, Molecular and Structural Biology Jožef Stefan Institute, Jamova 39, 1000 Ljubljana, Slovenia

Abstract *Our in vitro studies of human stefin B are described. Human stefin B (cystatin B) gene was reported as the first gene of the two known genes, whose mutations cause progressive myoclonus epilepsy of type 1 (EPM1), also known under the name Unverricht-Lundborg disease. The product of the gene, stefin B, is a globular protein of 98 amino acids and no disulphide bonds. We have characterized this protein thoroughly: from structure, to folding, stability and aggregation. The main focus of this review is on the protein's ability to undergo amyloid fibril formation. This is not something special to stefin B (any protein can under certain circumstances transform into amyloid-fibrils) yet this protein proved as a very suitable model system showing all the characteristics of other more well known systems. It may have some advantages over amyloid-beta or prion peptide studies, for example, it is easier to isolate its oligomers and its aggregates are not infectious. In the Introduction, connection of the process of amyloid-fibril formation to neurodegenerative disease is discussed. Relevance of the molecular, in vitro studies to understand the molecular basis of neurodegenerative pathology is explained. What is known about the structure and function of cystatins and what has been learnt from studies of amyloid-fibril formation of stefin B, is described next. The final chapter is devoted to EPM1. An observation was made that some of the EPM1 mutants have changed aggregation properties, which may have implications for pathology.*

Keywords *stefin B; protein aggregation; amyloid; progressive myoclonus epilepsy; human*

Izvleček *V tem preglednem članku so opisane naše in vitro študije človeškega stefina B. Človeški stefin B (cistatin B) je prvi raziskani gen, izmed dveh znanih, katerega mutacije povzročajo EPM1 (progresivno mioklonsko epilepsijo tipa 1), poznano kot Unverricht-Lundborg bolezen. Produkt gena, stefin B, je globularni protein iz 98 amino kislin in brez disulfidov. Ta protein smo podrobno proučili: od strukture, do zvijanja, stabilnosti in agregacije. Glavni poudarek tega prispevka je na opisu procesa amiloidne fibrilacije. Sicer je znano, da se večina proteinov ob izbranih okoliščinah (T, pH, topilo) lahko pretvori v amiloidne fibrile, a se je stefin B izkazal kot zelo primeren modelni sistem, ki ima vse značilnosti drugih, bolj znanih sistemov. Lahko bi rekli, da ima delo s stefinom B celo nekatere prednosti pred delom s prioni ali amiloidom beta, ker 1) lažje ločujemo posamezne oligomere in, ker 2) mislimo, da agregati stefina B niso infektivni. V Uvodu je predstavljena povezava med procesom nastajanja amiloidnih fibril in nevrodegenerativnimi boleznimi. Razložimo pomen molekularnih, in vitro študij za razumevanje molekularnih osnov nevrodegenerativne patologije. Zatem, kaj je znano o strukturi in funkciji cistatinov in kaj povedo študije amiloidne fibrilacije stefina B. Zadnje poglavje je posvečeno EPM1. Poročali smo, da imajo nekatere EPM1 mutante drugačne lastnosti agregacije, kar bi se morda lahko odražalo v patologiji.*

Ključne besede *stefin B; agregacija proteinov; amiloid; progresivna mioklonska epilepsija; človek*

Correspondence / Dopisovanje:
Eva Žerovnik PhD, e-mail: eva.zerovnik@ijs.si

* The author is a member of the »Proteolysis and Its Regulation« program. The work was supported by grants OB14P04SK and PO-0503 from ARRS.

Introduction

Amyloid[1] plaques or filamentous cellular inclusions are hallmark of neurodegenerative diseases.[2] There is no doubt that aberrant protein folding and aggregation take place in neurodegenerative diseases.[3–6] In Alzheimer's disease (AD) amyloid plaques, made predominantly of the cleavage peptide A-beta, accumulate extracellularly in the brain, whereas the paired helical filaments made of hyperphosphorylated protein Tau accumulate intraneuronally. In Huntington's disease huntingtin's aggregates cause slowing of the axonal transport along microtubules.[7,8] and in Parkinson's disease Lewy bodies are found in the affected neurons. Familial cases and animal models demonstrate, that certain protein mutants, which lead to more heavy aggregation also lead to early outburst of disease,[9,10] therefore, they must be the primary cause. It is not straightforward, whether the inclusions of the aggregated proteins are harmful or beneficial for neurons (at least initially) as they may sequester the toxic protein aggregates.[6] More and more evidence has been gained that smaller, soluble protein oligomers are toxic and not so much the mature fibrils.[11,12]

Even though under stress conditions (such as infection, fever, oxidative damage) some proteins may aggregate in the cell, there are regulatory mechanisms to correct this burden. Under normal conditions, a vital role is played by the two degradation systems of the cell: the ubiquitin proteasome system (UPS) and the autophagy.[13,14] With aging both systems get less efficient and a sporadic neurodegenerative disease may break out. Autophagy has been shown of particular importance in aggregates clearance.[15]

Several attempts to improve prospects for therapy of neurodegenerative diseases are in progress. The pre-clinical and clinical studies comprise stem cells producing nerve growth factors, which fight against neural death,[16] anti-amyloid antibodies, which clear amyloid plaques[17] and so on. Unfortunately, they all face severe side-effects in human trials. One of the most promising trials seems to be gene therapy (at least for Huntington's, familial Parkinson's and Lafora diseases). Therefore, understanding the primary cause of pathology and directing therapies to the route of the problem may be among the most efficient solutions for the future.

In order to arrive at the molecular understanding of the process of protein ordered aggregation, we study amyloid-fibril formation of stefin B – as a model system.

Cystatins and stefins: structure and function

To introduce our model protein, some facts about its structure and function are described. Three-dimensional structures of a number of members of the cystatin family have been determined. Crystal structure of stefin B monomer has been solved in complex with the protease papain.[18] Solution structure of stefin A monomer and dimer[19,20] have been solved by heteronuclear NMR. Structures of cystatin C monomer in solution and that of cystatin C domain-swapped dimer in crystal[21] have also been determined.

The best known function of stefin B is inhibition of the cysteine proteinases.[22,23] Inhibition of cathepsins, in particular, cathepsin B, could partially explain its regulatory role in apoptosis[24,25] and in cancer.[26] However, more and more evidence points to some alternative function of this small protein. Stefin B (sometimes called – cystatin B) has been shown as part of a multi-protein complex specific to the cerebellum.[27] By gene-expression studies it was shown to be overexpressed in amyotrophic lateral sclerosis – ALS[28] and in innate immunity response.[29]

Pathologies observed in stefin B deficient mice are cerebellar apoptosis, ataxia and myoclonus[24] as well as glial cells activation.[30] The protein gets overexpressed post-seizures,[31] implying its neuroprotective role. Cystatin C was similarly found over-expressed in status epilepticus.[32] In double knock-out mice of stefin B and cathepsin B genes[25] apoptosis was reduced maximally by 40 %, whereas ataxia and myoclonus remained, suggesting that stefin B may have other functions than protease inhibition. Of interest is a recent report that tryptophane metabolism (linked to serotonin neurotransmission) is affected in stefin B (cystatin B) deficient mice, making this protein of potential interest in psychiatric diseases.[33] In EPM1, which will be discussed separately, first trials to replace the protein in affected brain regions by the »protein replacement therapy« have been made.[34]

Searching for amyloid pathology, cystatin C is a well known amyloidogenic protein causing cerebral amyloid angiopathy (CAA). A rare hereditary cerebral hemorrhage with amyloid angiopathy of Islandic type (HCHWA-I), also termed hereditary cystatin C amyloid angiopathy (HCCAA), occurs upon L68Q mutation.[35] Cystatin C was found co-precipitated with A-beta in amyloid plaques[36] and inhibited A-beta fibrillation in *in vitro* studies.[37] Stefins A and B (cystatins A and B) together with some cathepsins were found in amyloid plaques of different pathologies,[38] possibly as a means of unsuccessful clearance by autophagy. That ubiquitin proteasome system (UPS) components are often co-precipitated with amyloid plaques is well known.

Amyloid-fibrillation by stefin B

Amyloid fibrils form *in vitro* in a process governed by external and internal forces. Among the external forces are temperature, solvent components, pH, salt and metal ions. Internal factors arise from protein sequence and structure but no final clue has been obtained, which is the main determinant Some predictions of the propensity to form amyloid-fibrils were generated from data on known amyloidogenic proteins,[39,40] which, however, do not hold for all proteins.[41]

The question remains whether hydrophobicity, nonfulfilled H-bonds at the edges of the β-strands, aromatic and charged residues, structurally important residues such as prolines and glycines, which render rigidity or mobility to loops, or a combination of all these contribute? Protein structural class seems im-

portant.[42] It was predicted that proteins of α/β type have to undergo a transition through an α-helical intermediate in the lag phase[43] before they enter the fibril growth. It is possible that amyloid-fibril formation under physiological conditions has been avoided by evolution.[44] This is contradicted by several cases of »functional« amyloids.[45]

Structure and Morphology as a function of solvent and time

Already in 2002, we first reported that this protein forms amyloid-like fibrils *in vitro.*[46, 47] It was shown that the process of fibrillation starts with a lag phase during which granular aggregate accumulates, composed of globular oligomers (Figure 1A). Further characterization of the mature amyloid fibrils formed by stefin B (Figure 1B) and comparison to stefin A, which is less prone to fibrillise, followed.[48] In order to gain insight into differences in sequence, which determine the propensity of stefin B to fibrillise, studies of the mutants, among them the chimeras between the two stefins, were performed.[41]

In the initial stages of amyloid-fibril formation oligomers are often detected. Soluble oligomers, which accumulate in the lag phase, are believed to be more toxic than the mature fibrils.[11, 12] In order to design oligomerization inhibitors, which could prevent toxicity, it would be of utmost importance to solve three-dimensional structure of such a pre-fibrillar oligomer.

Stefin B forms well defined oligomers, which appear upon several freeze-thaw cycles, already at neutral pH and can be isolated by gel-filtration. By solving three-dimensional structure of stefin B tetramer[49] it was shown that oligomerization is strongly coupled to cis-trans proline isomerization. It was revealed that stefin B tetramer is made from two domain-swapped dimers, which swap loops.[49] Thus, domain swapping has an important role in amyloid-fibril formation of cystatins and, likely, of a number of other proteins.

Morphology and size of the oligomeric particles during the lag phase and subsequent growth phases can be followed using time-resolved transmission electron microscopy (TEM), atomic force microscopy (AFM) and dynamic light scattering (DLS), (Čeru et al., 2008). With such studies we hope to decipher the mechanism of fibrillation. However, the sequence of events is not always clear-cut. There may be parallel routes, and the mechanism depends on the mutant studied and the conditions chosen. To see the influence of various solvent conditions, we have recorded morphology of amyloid-fibrils formed by stefin B at pH 3 and 5, with and without the organic solvent TFE.[50]

Kinetics as a function of protein concentration and temperature

To come closer to the mechanism we decided to study the effects of protein concentration and temperature, respectively, on the fibrillation rates. We then used a set of differential mathematical equations to fit the data (Škerget et al., 2008, submitted). To fit the kinetics a model was proposed, taking into account previous studies (both, structural & morphological). The model, which fits kinetic data rather well, predicts a nucleus, in which a number N_I = 64 of, presumably, domain swapped dimers accumulate and undergo a slow conformational change before the fibril growth continues. It also predicts that a side-pathway of trapped oligomers becomes important at higher temperatures than 35 ˚C and at higher protein concentrations.

From temperature dependence 2 high enthalpic barriers were calculated, consistent with findings in some other amylodogenic proteins, including A-beta.

Morphology and size of the prefibrillar aggregates and fibrils

The prefibrillar aggregates accumulate in the lag phase of fibril formation, which is either 48 hours at pH 3.3 (high ionic strength) or 170 hours at pH 4.8, 10 % TFE, both at room temperature. Fibrils eventually grow even at pH 4.8, with no TFE added.[46] The size of the globular oligomers (Figure 1A) is around 12 nm in

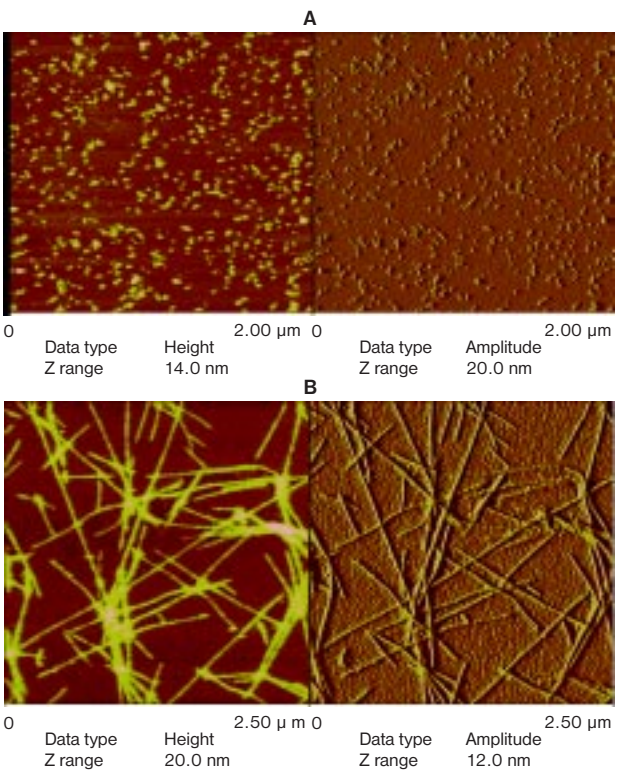

Figure 1. *Atomic force microscopy images. Images were obtained in tapping mode and they represent height variation (left) and the amplitude of the tip (right). We thank Miha Škarabot (IJS, Dept. Condensed Matter Physics), who contributed these data. (A) Globular oligomers making the prefibrillar aggregate. The sample of stefin B aggregates was taken within the lag time of fibrillation and was absorbed on a mica surface for at least 30 s, after which the solvent was blown away by dry air. (B) Mature fibrils. A sample of stefin B fibrils was taken at the end of the fibrillation reaction.*

diameter, which also is the width of the fibrils (Figure 1B). Height of the oligomers and of the fibrils is 3 nm. There also are fibrils with a double height, of around 6 nm.[48]

Interaction of the aggregates with membranes and their cytotoxicity

We have shown that stefin B prefibrillar aggregates composed of globular oligomers (such as those in Figure 1A) interact with predominantly acidic phospholipid membranes and exert cytotoxicity.[51] The aggregates were introduced in the medium in which the cells were incubated over-night. To measure toxicity and co-localization of the aggregates when protein will be expressed in cells, is our next challenge.

EPM1 mutant studies

The majority of idiopathic epilepsies are caused by mutations in genes that code for ion channels (channelopathies). Among the idiopathic epilepsies very rare are purely monogenic, rather they are polygenic. In addition to the idiopathic epilepsies, about 200 single gene disorders are known, in which epilepsy is an important part of the phenotype; among them are the progressive myoclonus epilepsies.

Progressive myoclonus epilepsy of type 1 – EPM1, also known as Unverricht Lundborg disease,[52] is a serious condition, which, however, does not deteriorate that fast as for example EPM2, also known as Lafora disease. The Unverricht-Lundborg disease occurs mainly in Baltic and Mediterranean regions. It is a progressive myoclonus epilepsy with autosomal recessive inheritance. The onset of disorder is between 6 and 18 years of age, the course is characterized by progressive myoclonic jerks and generalized tonic-clonic seizures. In later stages, the disorder is accompanied by mental deterioration, dysarthria and ataxia. Pathology has shown a marked loss in Purkinje cells in the cerebellum, neuronal loss in the spinal cord and medial thalamus and a proliferation of Bergmann glia.[53]

Genetic studies have led to discovery of stefin B (cystatin B) as the responsible gene.[54, 55] Most common change reported is the dodecamer repeats expansion in the promoter region of the cystatin B gene (CSTB),[55] which leads to reduced mRNA and protein levels. New advances in studies of the responsible gene have been made.[56] There often is the case that one patient is heterozygous, possessing one mutant and one dodecamer repeats gene. In such cases 50–60 % expression of stefin B mutated protein still takes place.[57]

After Suzuki et al., 2004:[58] »An emerging role appears for several epilepsy genes whose role is in the maintenance of normal neuronal structures, through cell-survival and cell-death pathways. A common epilepsy mechanism may involve abnormalities in functional connectivity that are due to small structural changes in the brain. Subtle increases in specific populations of neurons may be associated with juvenile myoclonus epilepsies (JME), whereas extensive cell death is associated with more severe, progressive myoclonic epilepsies.«

We have determined stability and fibrillation rates of the G4R mutant and the fragment of stefin B to residue 68,[59] both occurring in some patients with EPM1. First of all, it should be understood that both mutated proteins lack activity as the protease inhibitors (loss of function). In the aggregation study[59] it was found that the G4R accumulated in a prefibrillar, aggregated state, whereas the fragment was immediately transformed into fibrils. Cellular consequences of the aggregation behavior of the G4R mutant (i. e., accumulation of the toxic prefibrillar aggregates) remains to be seen. Our hypothesis[60] is that those mutants, which accumulate in the form of prefibrillar aggregates, gain in toxic function, in addition to loss of normal function. In such a case, neurodegenerative changes of EPM1 could perhaps be halted by the same therapeutical approaches that are being introduced in AD or Parkinson's disease, where neuronal renewal, fighting oxidative stress, chelating toxic metals, clearance of cellular inclusions and replacement of damaged genes could be applicable one day.

The group of Melli and co-workers have shown that cystatin B, including the exonic EPM1 mutants, aggregates in the cells, especially under conditions of overexpression. They have observed oligomers in the cell and discovered a polymerizing factor, which promotes oligomer formation. Their thesis is that the oligomers might be functional.[61]

Acknowledgements

The author thanks all the co-workers (whose names can be read from common publications), but in particular the young researchers, who have finished or are still working on their PhD: Manca Kenig, Sabina Rabzelj, Slavko Čeru and Katja Škerget. Special thanks go to Luise Kroon-Žitko for skillful technical assistance.

References

1. Sipe JD, Cohen AS Review: History of the amyloid fibril. J Struct Biol 2000; 130: 88–98.
2. Goedert M, Spillantini MG, Davies SW. Filamentous nerve cell inclusions in neurodegenerative diseases. Curr Opin Neurobiol 1998; 8: 619–32.
3. Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. Nat Med 2004; 10: S10–S17.
4. Lansbury PT, Lashuel HA. A century-old debate on protein aggregation and neurodegeneration enters the clinic. Nature 2006; 443: 774–9.
5. Žerovnik E. Amyloid-fibril formation; Proposed mechanisms and relevance to conformational disease. Eur J Biochem 2002; 269: 3362–71.
6. Howlett DR. Protein misfolding in disease: cause or response? Curr Med Chem – Immunol Endoc & Metab Agents 2003; 3: 371–83.
7. Rubinsztein DC. The molecular pathology of huntington's disease (HD) Curr Med Chem – Immunol Endoc & Metab Agents 2003; 3: 329–40.
8. Roy S, Zhang B, Lee VMY, Trojanowski JQ Axonal transport defects: a common theme in neurodegenerative diseases Acta Neuropathologica 2005; 109: 5–13.

9. McCutchen SL, Lai Z, Miroy GJ, Kelly JW, Colon W. Comparison of lethal and nonlethal transthyretin variants and their relationship to amyloid disease biochemistry 1995; 34: 13527–36.
10. Conway KA, Harper JD, Lansbury PT Jr. Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early onset Parkinson disease. Nat Med 1998; 4: 1318–20.
11. Haass C, Selkoe DJ. Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Mol Cell 2007; 2: 101–12.
12. Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, et al. Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation in vivo. Nature 2002; 416: 535–9.
13. Cuervo AM. Autophagy: many paths to the same end. Mol. Cell Biochem 2004; 263: 55–72.
14. Ding WX, Ni HM, Gao W, Yoshimori T, Stolz DB, Ron D, Yin XM. Linking autophagy to ubiquitin-proteasome system is important for the regulation of endoplasmatic reticulum stress and cell viability. Am J Pathol 2007; 171: 513–24.
15. Yamamoto A, Cremona ML, Rothman JE. Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway. J Cell Biol 2006; 172: 719–31.
16. Tuszynski MH, Thal L, U H-S, Pay MM, Blesch A, Conner J, Vahlsing HL. Nerve growth factor gene therapy for Alzheimer's disease. J Mol Neurosc 2002; 19: 207–8.
17. Tampellini D, Magrané J, Takahashi RH, Li F, Lin MT, Almeida CG, Gouras GK. Internalized antibodies to the Abeta domain of APP reduce neuronal Abeta and protect against synaptic alterations. J Biol Chem 2007; 282: 18895–906.
18. Stubbs MT, Laber B, Bode W, Huber R, Jerala R, Lenarcic B, Turk V. The refined 2.4 $Å Angstrom$ X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. EMBO J 1990; 9: 1939–47.
19. Martin JR, Craven CJ, Jerala R, Kroon-Žitko L, Žerovnik E, Turk V, Waltho JP. The three-dimensional solution structure of human stefin A. J Mol Biol 1995; 246: 331–43.
20. Staniforth RA, Giannini S, Higgins LD, Conroy MJ, Hounslow AM, Jerala R, et al. Three-dimensional domain swapping in the folded and molten-globule states of cystatins, an amyloid-forming structural superfamily. EMBO J 2001; 20: 4774–81.
21. Janowski R, Kozak M, Jankowska E, Grzonka Z, Grubb A, Abrahamson A, Jaskolski M. Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping. Nat Struct Biol 2001; 8: 316–20.
22. Turk, V. and Bode, W. (1991). The cystatins: protein inhibitors of cysteine proteinases. FEBS Lett., 285: 213–9.
23. Turk V, Turk B, Turk D. Lysosomal cysteine proteinases: facts and opportunities. EMBO J 2001; 20: 4629–33.
24. Pennacchio LA, Bouley DM, Higgins KM, Scott MP, Noebels JL, Myers RM. Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. Nature Genetics 1998; 20: 251–8.
25. Houseweart MK, Pennacchio LA, Vilaythong A, Peters C, Noebels JL, Myers RM. Cathepsin B but not cathepsins L or S contributes to the pathogenesis of Unverricht-Lundborg progressive myoclonus epilepsy (EPM1). J Neurobiol 2003; 56: 315–27.
26. Kos J, Krasovec M, Cimerman N, Nielsen HJ, Christensen IJ, Brunner N. Cysteine proteinase inhibitors stefin A, stefin B, and cystatin C in sera from patients with colorectal cancer: relation to prognosis. Clin Cancer Res 2000; 6: 505–11.
27. Di Giamo R, Riccio M, Santi S, Galeotti C, Ambrosetti DC, Melli M. New insights into the molecular basis of progressive myoclonus epilepsy: a multiprotein complex with cystatin B. Hum Mol Genet 2002; 11: 2941–50.
28. Malaspina A, Kaushik N, de Belleroche J. Differential expression of 14 genes in amyotrophic lateral sclerosis spinal cord detected using gridded cDNA arrays, J Neurochem 2001; 77: 132–45.
29. Lefebvre C, Cocquerelle C, Vandenbulcke F, Hot D, Huot L, Lemoine Y, Salzet M. Transcriptomic analysis in the leech Theromyzon tessulatum: involvement of cystatin B in innate immunity. Biochem J 2004; 380: 617–25.
30. Lieuallen K, Pennacchio LA, Park M, Myers RM, Lennon GG. Cystatin B-deficient mice have increased expression of apoptosis and glial activation genes. Hum Mol Genet 2001; 10: 1867–71.
31. D'Amato E, Kokaia Z, Nanobashvili A, Reeben M, Lehesjoki AE, Saarma M, Lindvall O. Seizures induce widespread upregulation of cystatin B, the gene mutated in progressive myoclonus epilepsy, in rat forebrain neurons. Eur J Neurosci 2000; 12: 1687–95.
32. Pirttila TJ, Lukasiuk K, Hakansson K, Grubb A, Abrahamson M, Pitkanen A. Cystatin C modulates neurodegeneration and neurogenesis following status epilepticus in mouse. Neurobiol Dis 2005; 20: 241–53.
33. Vaarmann A, Kaasik A, Zharkovsky A. Altered tryptophan metabolism in the brain of cystatin B-deficient mice: a model system for progressive myoclonus epilepsy. Epilepsia 2006; 47: 1650–4.
34. Andrade DM, Scherer SW, Minassian BA. Protein therapy for Unverricht-Lundborg disease using cystatin B transduction by TAT-PTD. Is it that simple? Epilepsy Res 2006; 72: 75–9.
35. Jensson O, Palsdottir A, Thorsteinsson L, Arnason A, Abrahamson M, Olafsson I, and Grubb A. Cystatin C mutation causing amyloid angiopathy and brain hemorrhage. Biol Chem Hoppe Seyler 1990; 371: Suppl: 229–32.
36. Maruyama K, Kametani F, Ikeda S, Ishihara T, Yanagisawa N. Characterization of amyloid fibril protein from a case of cerebral amyloid angiopathy showing immunohistochemical reactivity for both beta protein and cystatin C. Neurosci Lett 1992; 144: 38–42.
37. Sastre M, Calero M, Pawlik M, Mathews PM, Kumar A, Danilov V, et al. Binding of cystatin C to Alzheimer's amyloid beta inhibits in vitro amyloid fibril formation. Neurobiol Aging 2004; 25: 1033–43.
38. Ii K, Ito H, Kominami E, Hirano A. Abnormal distribution of cathepsin proteinases and endogenous inhibitors (cystatins) in the hippocampus of patients with Alzheimer's disease, parkinsonism-dementia complex on Guam, and senile dementia and in the aged. Virchows Archiv A Pathol Anat 1993; 423: 185–94.
39. Chiti F, Taddei N, Bucciantini M, White P, Ramponi G, and Dobson CM. Mutational analysis of the propensity for amyloid formation by a globular protein. EMBO J 2000; 19: 1441–9.
40. Lopez de la Paz M, Serrano L. Sequence determinants of amyloid fibril formation. Proc Natl Acad Sci USA 2004; 101: 87–92.
41. Kenig M, Jenko-Kokalj S, Tusek-ŽnidariC M, Pompe-Novak M, Guncar G, Turk D, et al. Folding and amyloid-fibril formation for a series of human stefins' chimeras: any correlation? Proteins: Structure, Function & Bioinformatics 2006; 62: 918–27.
42. Pellarin R, Caflish A. Interpreting the Aggregation Kinetics of Amyloid peptides. J Mol Biol 2006; 360: 882–92.
43. Daggett V. α-sheet: the toxic conformer in amyloid diseases? Acc Chem Res 2006; 39: 594–602.
44. Stefani M, Dobson CM. Protein aggregation and aggregate toxicity: new insights into protein folding, misfolding diseases and biological evolution. J Mol Med 2003; 81: 678–99.
45. Fowler DM, Koulov AV, Alory-Jost C, Marks MS, Balch WE, et al. Functional amyloid formation within mammalian tissue. PLoS Biology Vol. 4, No. 1, 2005.
46. Žerovnik E, Pompe-Novak M, Škarabot M, Ravnikar M, Muševic I, Turk V. Human stefin B readily forms amyloid fibrils in vitro. Biochim Biophys Acta 2002; 1594: 1–5.
47. Žerovnik E, Zavašnik-Bergant V, Kopitar-Jerala N, Pompe-Novak M, Škarabot M, Goldie K, et al. Amyloid fibril formation by human stefin B in vitro: immunogold labelling and comparison to stefin A Biol Chem 2002; 383: 859–63.
48. Jenko S, Škarabot M, Kenig M, Guncar G, Muševic I, Turk D and Žerovnik E. Different propensity to form amyloid fibrils by two homologous proteins – Human stefins A and B: Searching for an explanation. Proteins: Structure, Function and Bioinformatics 2004; 55: 417–25.
49. Jenko Kokalj S, Guncar G, Stern I, Morgan G, Rabzelj S, Kenig M, et al. Essential role of proline isomerization in stefin B tetramer formation. J Mol Biol 2007; 366: 1569–79.
50. Žerovnik E, Škarabot M, Škerget K, Giannini S, Stoka V, Jenko Kokalj S, Staniforth RA. Amyloid-fibril formation by human stefin B: influence of pH and TFE on fibril growth and morphology. Amyloid 2007; 14: 237–47.
51. Anderluh G, Gutierrez-Aguirre I, Rabzelj S, Ceru S, Kopitar-Jerala N, Macek P, et al. Interaction of human stefin B in the prefibrillar oligomeric form with membranes – correlation with cellular toxicity. FEBS J 2005; 272: 3042–51.

52. Kagitani-Shimono K, Imai K, Okamoto N, Ono J, Okada S. Unverricht-Lundborg disease with cystatin B gene abnormalities. Pediatr Neurol 2002; 26: 55–60.
53. Haltia M, Kristensson K, Sourander P. Neuropathological studies in three Scandinavian cases of progressive myoclonus epilepsy. Acta Neurol Scan 1969; 45: 63–77.
54. Pennacchio LA, Lehesjoki AL, Stone NE, Willour VL, Virtaneva K, Maio J, et al. Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). Science 1996; 271: 1731–4.
55. Lalioti MD, Scott HS, Buresi C, Rossier C, Bottani A, Morris MA, Malafosse A, et al. Dodecamer repeat expansion in cystatin B gene in progressive myoclonus epilepsy. Nature 1997; 386: 847–51.
56. Joensuu T, Kuronen M, Alakurtti K, Tegelberg S, Hakala P, Aalto A, et al. Cystatin B: mutation detection, alternative splicing and expression in progressive myclonus epilepsy of Unverricht-Lundborg type (EPM1) patients. 2007; 15: 185–93.
57. Kaasik A, Kuum M, Aonurm A, Kalda A, Vaarmann A, Zharkovsky A. Seizures, ataxia, and neuronal loss in cystatin B heterozygous mice. Epilepsia 2007; 48: 752–7.
58. Suzuki T, Delgado-Escueta AV, Aguan K, Alonso ME, et al. Mutations in EFHC1 cause juvenile myoclonic epilepsy. Nat Genet 2004; 36: 842–9.
59. Rabzelj S, Turk V, Žerovnik E. In vitro study of stability and amyloid-fibril formation of two mutants of human stefin B (cystatin B) occurring in patients with EPM1. Protein Science 2005; 14: 2713–22.
60. Ceru S, Rabzelj S, Kopitar Jerala N, Turk V, Žerovnik E. Protein aggregation as a possible cause for pathology in a subset of familial Unverricht-Lundborg disease. Med Hypotheses 2005; 64: 955–9.
61. Cipollini E, Riccio M, Di Giaimo R, Dal Piaz F, Pulice G, Catania S, et al. Cystatin B and its EPM1 mutants are polymeric and aggregate prone in vivo. Biochim Biophys Acta 2008; 1783: 312–22.

Accepted 2008-02-16