

INTERACTION BETWEEN OLIGOMERS OF STEFIN B AND AMYLOID-BETA IN VITRO AND IN CELLS

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Running head: Stefin B oligomers inhibit A β fibril formation

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In order to contribute to the question of putative role of cystatins in Alzheimer's disease and in neuroprotection in general, we studied the interaction between human stefin B (cystatin B) and amyloid- β (1-40) peptide (A β). Using surface plasmon resonance (SPR) and electrospray mass spectrometry (ESI MS) we were able to show a direct interaction between the two proteins. As an interesting new fact, we show that stefin B binding to A β is oligomer specific. The dimers and tetramers of stefin B, which bind A β , are domain-swapped as judged from structural studies. Consistent with the binding results, the same oligomers of stefin B inhibit A β fibril formation. When expressed in cultured cells, stefin B co-localises with A β intracellular inclusions. It also co-immunoprecipitates with APP fragment containing the A β epitope. Thus, stefin B is another APP / A β binding protein *in vitro* and likely in cells.

Neurodegenerative diseases present a huge burden in developed world's aging population. They are all in one way or another connected to aberrant protein folding and aggregation of the proteins involved (1). Various protein conformational disorders of the central and peripheral nervous system are known, which often appear sporadically but also run in families. These are among others: Parkinson's and Alzheimer's, disease, dementia with Lewy bodies,

vascular and fronto-temporal dementia and

alotrophic lateral sclerosis. A β peptide implicated in Alzheimer's disease pathology is a cleavage product of the membrane A β precursor protein (APP). It is the main constituent of extracellular amyloid plaques, however, together with its oligomers, it also resides intracellularly (2). It has been shown that A β oligomers prepared *in vitro* and those extracted from living cells exert cytotoxicity and cause symptoms of reversible memory loss in animal models (3).

Amyloid proteins oligomers have special structural properties, which are reflected in a common anti-oligomer antibody (4). This antibody does not only bind the oligomers against which it was raised but it binds also chaperones and some other proteins involved in dis-aggregating protein aggregates in cells (5). A β -binding proteins, the so called "amateur chaperones", were suggested to have a potential in Alzheimer's disease (AD) therapy (6,7).

It has been shown before that human cystatin C is an A β -binding protein (8). Cystatins are single chain proteins that inhibit cysteine cathepsins (9). Human stefin B (also known as cystatin B) is a member of sub-family A of cystatins, classified as family I25 in the MEROPS scheme (10). Stefin B, a protein of 98 amino acid residues and 1 Cys residue, is predominantly intracellular, whereas cystatin C, a protein of 120 residues and 2 disulphide bonds, is a

secretory protein. 3D structures of stefins and cystatin C have been determined, among others, the solution structure of stefin A (11) and cystatin C (12,13).

Human cystatin C has been found as constituent of senile plaques of AD patients (14) and stefins A and B have also been reported to localise to amyloid plaques of various origin (15, 16). It has been suggested that cystatins play a role in Alzheimer's disease (17,18).

Stefin B has been used as a model protein to study amyloid fibril formation, often in comparison to the more stable stefin A (19-21). Stefin B has a much higher tendency than stefin A to form dimers, higher oligomers and amyloid fibrils, nevertheless, stefin A domain-swapped dimer could be prepared under more extreme solution condition and its structure was determined by NMR (22, 23). The model for the mechanism of stefin B fibrillization involves off-pathway lower oligomer formation (probably involving domain-swapping, due to a high energetic barrier) and a larger nucleus in the order of 30 dimers, explaining both unusual behaviour at higher protein concentrations and a relatively long lag phase (21).

Similarly to some other amyloid proteins, it has been demonstrated that stefin B interacts predominantly with acidic phospholipids and that higher oligomers, distinct from monomers, dimers and tetramers, are toxic to cells (24, 25). Moreover, studies on the pore forming characteristics of this protein have confirmed the suggestion that the toxicity of the oligomers is related to membrane perforation (26).

As already said, A β peptide interacts with quite a number of amyloid proteins (6,7). To mention just a few: gelsolin, α 2-macroglobulin, crystalin- α B. In this work we probed the interaction of A β with human stefin B. We have chosen conditions under which A β fibrillises and stefin B does not. The interaction of A β with stefin B has been confirmed directly by using surface plasmon resonance (SPR) where A β was bound to the sensor chip and samples of separated monomers, dimers, tetramers and higher oligomers of the wild type E31 stefin B or the dimeric Y31 stefin B were injected across. Interaction of the Y31 stefin B dimers with A β was additionally confirmed by electrospray ionization

mass spectrometry (ESI MS). As a further proof of the interaction, the fibrillization of A β was completely inhibited by exactly those oligomers, which showed binding by SPR. Co-localization and co-immunoprecipitation cellular experiments affirm the possibility that the two proteins interact in the cell.

EXPERIMENTAL PROCEDURES

Preparation and isolation of the recombinant human stefin B

Recombinant human stefin B was expressed in *E. coli* and purified by affinity and gel chromatography (27). On the size exclusion chromatography using Superdex 75 column, the wild-type, E31 stefin B, eluted as a set of well defined oligomers (28), allowing isolation of monomers, dimers, tetramers and higher oligomers. Y31 stefin B eluted from the Superdex 75 column predominantly as a dimer. The recombinant proteins have Ser at position 3 instead of Cys to prevent covalent disulfide bond formation.

Thioflavin T (ThT) fluorescence measurements

A stock solution of A β peptide in distilled water was prepared at 476 μ M concentration and kept on ice. An aliquot was added to the reaction mixture of stefin B in PBS, pH 7.3. The order of adding the proteins is very important, as A β starts to fibrillise immediately at pH 7.3 (PBS) and at 40 °C, with only a short lag phase. The fibrillization reaction took place at 40 °C and at different molar ratios of the two proteins, stefin B to A β (1:1 to 1:8). The starting concentration of stefin B (of any oligomeric form) was 17 μ M, to which A β was added to final concentrations of 17 μ M, 34 μ M, 68 μ M and 136 μ M. During the reaction, at several time points, an aliquot was taken from the fibrillization mixture and added to a 0.025 M phosphate buffer pH 7.5 with ThT dye dissolved to A416=0.66. ThT fluorescence spectra were measured from 455 to 550 nm (λ_{ex} = 440 nm) with a luminescence spectrometer LS 50B, Perkin Elmer and the intensity at 482 nm was read. To probe the status of A β before the CM5 chip preparation, continuous time course of ThT

fluorescence was measured at 482 nm ($\lambda_{ex} = 440$ nm) for 7 minutes, after A β was incubated for a minute in the buffer of pH 4.0 (as for coupling) with ThT dye dissolved.

Transmission electron microscopy – TEM

Protein samples were taken at certain time points of A β fibrillization and at the plateau of the reaction. 15 μ l of a 17 - 34 μ M solution, diluted 10 to 50 times as appropriate, were applied on a Formvar and carbon coated grid. If not indicated otherwise, after 5 minutes the sample was soaked away and the grid was stained with 1 % uranyl acetate. To check the initial status of A β on the sensor chip, a sample of A β (after 1 min of incubation at pH 4.0) was put on the grid (with 5 more min left on the grid surface) and TEM was recorded.

Philips CM 100 transmission electron microscope was used and at the applied voltage of 80 kV, magnifications were from 10000 x to 130000 x. Images were recorded by a Bioscan CCD camera Gatan, using Digital Micrograph software.

Surface plasmon resonance – SPR

SPR experiments were performed using a Biacore X biosensor instrument. A β peptide was covalently bound to the sensor chip CM5. A β peptide was coupled to the surface of the sensor flow cell activated with 0.4 M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and 0.1 M N-hydroxysuccinimide. The buffer used for coupling was 10 mM acetate buffer pH 4.0. The excess of reactive groups was blocked with ethanolamine. Monomers, dimers, tetramers and higher oligomers of the wild-type E31 stefin B and dimeric iso-form Y31 stefin B were used as analyte. Kinetic measurements were performed at 25 °C at a flow rate of 5 μ l/min in PBS buffer of pH 7.3. To obtain the concentration range where stefin B protein interacts with A β peptide bound on the chip, concentrations of Y31-variant (0.5 – 200 μ M) and E31-variant (20 – 200 μ M) were applied. The SPR response was compared with a control sensor flow cell on the same chip, which was activated and blocked in the same way as flow cell with ligand, omitting the injection of A β peptide. Regeneration of the sensor surface was performed with 3 μ l of 10

mM glycine pH 3.0 in the case of Y31-variant or 5 μ l of 2M NaCl in the case of E31-variant.

Preparation of samples for ESI MS

Stefin B (Y31-variant, which is predominantly dimeric) was purified by SEC on SuperdexTM 75 GL column (10x300 mm) (GE Healthcare, Giles, United Kingdom) connected to an ÄKTA Purifier system (GE Healthcare, Giles, United Kingdom). Protein was eluted with 20 mM ammonium acetate (pH 7.5) at a flow rate of 1 ml/min. 0.2 ml fractions were collected. UV detection at 230, 280 and 360 nm was used to monitor elution.

ESI MS studies of stefin B – A β interaction

SEC purified sample of stefin B (the dimer of the Y31 variant) was diluted with 20 mM ammonium acetate (pH 7.5) to 2 μ M and injected at the flow rate of 6 μ l/min into the ESI-Q-TOF mass spectrometer (QSTAR Elite, AB Sciex instruments). MS spectra were recorded in the m/z region from 500 – 5000 Da using the following instrument parameters: ion spray voltage 4500 V; source gas 25 l/min; curtain gas 20 l/min; declustering potential 60V; focusing potential 320 V; detector voltage 2450V. Similar conditions were used for ESI-TOF MS analysis of 2 μ M A- β (1-40) and the mixture of 2 μ M stefin B with 2 μ M A β (1-40) in 20 mM ammonium acetate.

Cell lines and transfection experiments

Chinese hamster ovary (CHO-K1) cells expressing wild type APP (APP-wt) or APP with the codon 670/671 “Swedish” mutation (APP-Sw), as described (29), were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and selected in 200 mg/ml G418. All reagents were from Invitrogen, UK. Life Technologies, Inc.

To induce expression of stefin B, cells stably expressing APP were seeded in 6-well plates and incubated overnight to reach 70-80% confluence and 10 μ g of plasmid (the multiple cloning site (MCS) pcDNA3 vector (Invitrogen)) were transfected with LipofectamineTM 2000 according to the manufacturer’s instructions.

Assessment of levels of full length APP, APP metabolites in CHO cells

Cells were lysed in lysis buffer (1% TX-100, 150 mM NaCl, Tris pH 7.4) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics). For Western blotting, equal amounts of protein were loaded onto 4-12% Tris-glycine gradient gels, transferred onto nitrocellulose membranes, probed with the antibodies described below and analyzed using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NB, USA)

For APP and its metabolites detection, polyclonal antiserum CT15, which recognizes the C terminus of APP, was used (30).

A β quantitation by ELISA

A β sandwich ELISAs were carried out using a human A β (1-40) kit (Invitrogen) following manufacturer's instructions. Experiments were performed in triplicate and repeated three times. Statistical analysis was carried out in SPSS v16.0. Data are expressed as the mean \pm SEM and $p < 0.05$ was considered statistically significant

Immunostaining

CHO-K1 cell line expressing APP-Sw, grown on 12 mm coverslips, was transiently transfected with wild type stefin B (pcDNA3-stBwt) using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were then washed with PBS and fixed in 4% paraformaldehyde. Paraformaldehyde was quenched with 150 mM glycine. Cells were permeabilized with 0.2 % (v/v) Triton X-100 in PBS. After quenching and permeabilization, coverslips were washed with PBS. Coverslips were then blocked in Ab buffer (PBS, with BSA 1% w/v and fish skin gelatin 1% w/v). Rabbit polyclonal anti-stefin B (31), and mouse monoclonal anti-A β [DE2B4] from Abcam were used for co-localisation studies. The concentrations of the primary antibodies were 1 mg/ml. They were diluted in Ab buffer as follows: DE2B4 mouse monoclonal anti-A β 1:500 and rabbit polyclonal anti-stefin B 1:800. All secondary antibodies were diluted 1:500 in Ab buffer. Incubation was either overnight at 4°C for primary antibodies or at room temperature for 2 hours for secondary antibodies. After incubation

coverslips were once again washed as described above and mounted on slides in Prolong antifade reagent. All AlexaFluor-conjugated secondary antibodies and Prolong Antifade reagent were from Molecular Probes (Invitrogen).

Immunoprecipitation and Western blotting

Cells co-expressing APP-wt or APP-Sw and stefin B were lysed in RIPA buffer. The lysates were applied to the immuno-affinity Sepharose-protein A resin prepared with polyclonal anti-rabbit stefin B antibodies, after which 15% SDS gels and Western blotting with anti-A β antibodies [DE2B4] were performed. To detect bound proteins the same antibodies were used as described above, only, dilution was 1:400 for the primary anti-A β antibodies.

Confocal Microscopy

Confocal images were taken with a Carl Zeiss LSM510 Axio observer microscope equipped with Polychrome V monochromator using an Imago Type QE CCD camera at the Reference Center for Confocal Microscopy (LN-MCP, Institute of Pathophysiology, School of Medicine, Ljubljana, Slovenia). Sequential acquisition was used to minimize cross-talk between red and green channels. The hardware was configured with two control samples, one with single labelled/expressing green cells and the other with single labelled/expressing red cells. The background of collected images was corrected by ImageJ rolling ball algorithm plug in and quantitative co-localisation analysis was performed by JACoP (32), an ImageJ co-localization plug in.

RESULTS

Initial conformation and oligomeric state of stefin B variants

Two variants (iso-forms) of wild-type human stefin B were reported thus far. Y31 stefin B, as derived from protein sequencing (33) was later cloned (27) and its 3D structure in complex with papain was

determined (34). Y31 variant is predominantly dimeric in solution while the more common wild-type as reported in the gene bank, E31 stefin B, exists as a mixture of oligomers (28,25).

The two variants differ in stability; the Y31 variant proved as a more labile protein (by 11 kJ/mol) as shown by urea denaturation (35) and, it transformed into a molten globule conformation, either by reducing pH (36) or by mutation of P74S (37).

The P79S mutant of the Y31-variant forms a tetramer. The crystal structure and the NMR structure in solution show that two domain-swapped dimers “intercalate” by exchanging loops to form the tetramer (37). The peptide bond preceding Pro 74 is *cis* in the tetramer, while apparently not in the monomeric stefin B in complex with papain (34).

Separate oligomers of the wild type E31 stefin B were isolated (Fig. 1) by the size exclusion chromatography (SEC). The elution diagrams of monomers, dimers and higher oligomers are shown in Fig. 1A. After the SPR experiment, after 14 days at 4°C, we checked again if there was some equilibration of the oligomeric species (Fig. 1B). As elutions did not change much, one can judge that oligomers are rather stable. From the initial samples as applied to SEC and SPR, one can say that in monomer’s sample there is < 10% dimers; in dimer’s sample there are < 5% monomers and < 10% tetramers; in the tetramer’s sample there are < 5% dimers and < 20% higher oligomers; in the higher oligomers there are < 10 % of other species (> 5% tetramers). Oligomers, which are surprisingly stable, were additionally checked by ESI MS (not shown), which confirmed their molecular weight.

Stefin B inhibits A β fibril growth in an oligomer specific manner

Conditions were established under which A β fibrillises regularly and stefin B does not. The mixture undergoing fibrillization was left unperturbed between measurements of ThT fluorescence. Amyloid-fibril formation by A β , alone and with stefin B in a 1:1 molar ratio, are shown in Figure 2. Dimeric Y31 stefin B completely inhibits A β fibril growth (Fig. 2A), which was also confirmed by transmission electron microscopy (TEM) (Fig. 3A). Wild-type E31 stefin B, which is composed from monomers and different oligomers,

shows no comparable inhibitory effect (Fig. 2A, Fig. 3B).

To see the effect of individual oligomers of the wild-type E31 stefin B on fibril growth of A β , monomers, dimers, tetramers and higher oligomers of stefin B were isolated by SEC (Fig. 1A, B). Each was then separately mixed with A β . It was observed that only the tetramers inhibited the A β fibril growth completely (Fig. 2B).

Stefin B interacts with A β depending on the oligomeric state of the former

SPR measurements

Oligomer specific binding between stefin B and A β was demonstrated by using surface plasmon resonance (SPR). A β peptide was immobilised on the surface of a sensor chip CM5 and samples of the two stefin B variants and isolated oligomers were injected across. By several control experiments we made sure that A β bound to the chip was not considerably aggregated or fibrillar. We judge so from low ThT fluorescence of the A β sample in the buffer used for coupling before application to the chip and the TEM taken at the same time (Suppl, Fig.1). Even though some fibrils have formed in the first 5 minutes of incubation at pH 4 buffer for coupling, the majority of the peptide (70 %) does not bind ThT and is thus not in a fibrillar form. Furthermore, after immobilization of A β , the first injection of 25 mM NaOH desorb a significant amount of the peptide, which possibly represented noncovalently associated A β into oligomers or fibrils. We cannot exclude that minor amount of A β is in the aggregated or fibrillar form and thus the exact determination of rate constants or equilibrium affinity constant of the interaction was not feasible. The sensorgrams, however, showed clear interaction as depicted below and were reproducible.

Dimeric Y31 stefin B reacted with A β in a concentration dependent manner (Fig. 4A). To verify the interaction, we also immobilised Y31 stefin B and were able to observe the binding of A β (Fig. 4B). Tetramers of the wild-type E31 stefin B bound strongly to A β , but not so monomers, dimers and higher oligomers (Fig. 4C), thus, explaining the specific inhibitory effect of the tetramers on A β fibril growth (Fig. 2B). This interaction of the wild-

type tetramers with A β was also concentration dependent as shown in Fig. 4D.

ESI MS measurements

Formation of the complex between stefin B and A β was monitored directly in an electrospray ionization mass spectrometry (ESI MS) experiment. The ESI MS spectrum of A β (1–40) displayed 3 major peaks (with charges +5, +4 and +3), which correspond to a molecular mass species of 4329.91 Da (theoretical mass 4329.89 Da). The isolated Y31 stefin B dimer displayed 2 major peaks corresponding to a molecular mass of 22,314.2 Da (theoretical Mw 22,301.4 Da). In the spectrum of stefin B and A β (1–40) mixture, 3 new peaks with molecular mass of 26,644.4 (charges +11, +10 and +9, respectively) were observed, which corresponds to the theoretical mass of the complex formed between one Y31 stefin B dimer and one molecule of A β (1–40).

The ESI MS results (Fig.5) clearly show the formation of a complex between Y31 stefin B dimer and A β (1–40). It coexists together with uncomplexed components and therefore it can be considered as weak. A similar complex between wild-type E31 stefin B tetramer and A β could not be observed, due to the lower intensity of MS peaks corresponding to higher oligomers.

Effect of stefin B on APP metabolism and A β generation in cells

In order to explore a possible functional link between stefin B and A β *in vivo*, we studied the effect of co-expressing stefin B in cell culture expressing APP.

Transfection of stefin B into CHO cells expressing either wild-type or Swedish mutant APP had no impact on cell toxicity, as determined by LDH release levels (not shown). Further it was shown (Fig. 6) that the presence of stefin B did not modify levels of wild-type or Swedish full length APP. Similarly, stefin B did not affect A β (1–40) secretion levels. In the absence of stefin B we observed a ~6-fold increase in A β levels in cells bearing the Swedish mutation (29), a difference that did not change in the presence of stefin B (Fig. 6).

Nevertheless, the presence of stefin B did have an impact on APP metabolism. Specifically, in cells expressing wild type APP, it led to an increase in the levels of C-terminal fragments of APP cleaved by β -secretase (bCTF) but not those derived from α -secretase (aCTF). By contrast, in cells expressing APP-Sw, stefin B reduced the level of aCTF without affecting bCTF (Fig. 6).

Co-immunoprecipitation experiment

In order to examine if APP / A β and stefin B associate in cells, we have transfected cDNA for stefin B into CHO cells expressing either wild-type or Swedish mutant APP. 24 hours after transfection the cells were lysed and immunoprecipitated with polyclonal antibodies against stefin B. Fraction bound to the anti - stefin B antibodies and the lysate were then run on 15% SDS gels followed by Western blotting with antibodies against A β [DE2B4] (Fig. 7) and stefin B (not shown).

As Figure 7 shows, a complex between stefin B and approximately 15 kDa C-terminal fragment of APP, comprising A β sequence, was detected in the APP-wt cells but not so in the cells expressing APP-Sw.

Co-localization of stefin B and A β aggregates in cells

The possibility that A β aggregates and stefin B co-localise intracellularly was explored next. CHO-K1 cell line expressing APP-Sw, with the level of A β is ~6-fold increased (29), was transiently transfected with stefin B. Staining with mouse anti-A β antibodies and rabbit polyclonal anti-stefin B antibodies showed that stefin B and A β peptide associate with A β inclusions (Fig. 8). Visual inspection of the staining pairs suggested co-localization of the two proteins in A β inclusions, possibly A β aggresomes (38). The regions of interest (ROIs) were defined by a quality co-localization analysis. We used public domain tool JACoP for quantitative co-localization (Table 1). Dispersed scatter plots, the ICA plots, (Fig.8, graphs A,B,C) where most of the pixels are found on the right side of the plot, indicate partial co-localization of A β and stefin B.

DISCUSSION:

Conclusions from the *in vitro* studies

The first indication for the *in vitro* interaction between A β and stefin B has been obtained by ThT fluorescence (Figs. 2A,B) and TEM data (Fig. 3A), which show complete inhibition of A β fibril growth by the wild-type E31 stefin B tetramers and dimeric Y31-variant. In accordance, SPR measurements show binding of exactly these two oligomeric forms of stefin B to A β (Fig 4A-D). This contrasts with monomers, dimers and the higher oligomers of wild-type E31 stefin B, which do not show considerable binding by SPR (Fig. 4C) and no inhibition of A β fibrillization. Small inhibition observed by the samples of higher oligomers (Fig. 2B) can be ascribed to presence of small amounts of the tetramer in the sample (Fig. 1A). A complete separation of the higher oligomers from monomers, dimers and tetramers is not possible (there are always about 5% tetramers present) yet transitions are slow and have always been checked before and after the measurements.

The fact that dimers of Y31 stefin B behave differently from those of E31 stefin B could be due to lower stability of the Y31 variant (35) and its tendency to form a molten globule (36). Thermal denaturation experiments (E.Ž. unpublished) indicate that Y31 stefin B dimers could be domain-swapped, similarly to stefin A dimers (22,23) and cystatin C dimers (39).

Conclusions from the cellular studies

Our studies using cultured cells (Fig. 6) show that the presence of stefin B does not change the steady-state levels of full length wild-type or Swedish mutant APP, in line with a previous report showing that cystatin C had no effect on APP levels or processing (40).

There are some interesting observations of the influence of stefin B co-expression on the ratio of the APP C-terminal fragments, which seems worthwhile to explore in any future studies. Namely, the a-CTF (C-terminal fragment obtained upon α -secretase cleavage) gets reduced in the APP-Sw case and the b-CTF increased in the APP-wt expressing cells). Furthermore, co-immunoprecipitation experiment on the lysates of

CHO-K1 cells expressing APP-wt or APP-Sw (Fig. 7) demonstrates a complex between stefin B and APP fragment of around Mr 15 kDa in the wild type APP expressing cells. This kind of fragment was reported some time ago by Greengard in 1993 (41). The 15 kDa C-terminal fragment is the major membrane-bound APP fragment and is most likely non-amyloidogenic (41). Obviously, the monoclonal mouse anti-A β antibody DE2B4 recognises only soluble peptides comprising the A β segment. This also would explain, why there is no soluble fraction observed in the A β rich APP-Sw cells (lanes 3 and 4 in Fig. 7). The co-localization experiments in CHO-K1 cell line expressing APP-Sw, supported by quantitative correlation analysis (Fig. 8 and Table 1), shows that stefin B partially co-localises with A β intracellular inclusions, which represent aggregated A β .

Together, these results support the possibility that stefin B interacts with A β and with APP C-terminal fragments *in vivo*

On the possible chaperone function of stefin B domain-swapped oligomers

There is some evidence, currently under debate, that stefin B (cystatin B) might have additional function(s) other than cysteine protease inhibition. The protein was found as a component of a multi-protein complex specific to the cerebellum, together with a number of cytoskeleton proteins (42). Oligomerization of stefin B in cells was reported recently by Melli and co-workers (43) and the same phenomenon has been observed also *in vitro* (28,25). Stefins B oligomers and aggregates in cells (43), which were confirmed and characterised further by our group (Čeru et al, paper in preparation), have no known function in the cell. On the basis of the *in vitro* and the cell culture results presented in this work we propose that stefin B tetramers (and likely domain-swapped dimers) may bind to A β *in vivo* in such a way as to prevent its fibril formation. This so called “amateur” chaperone action was recently ascribed to some other A β -binding proteins (7). This hypothesis seems supported by TEM data (Fig. 3A-C), which show longer and more regular fibrils of A β in presence of the wild type E31 stefin B (Fig. 3B); in comparison to those of A β fibrils alone (Fig. 3C). A

complete inhibition of fibril growth at the stage of granular aggregates happens in presence of the dimeric Y31-variant (Fig. 3A). The tetramers of E31 stefin B, which also completely inhibit ThT fluorescence (Fig. 2B) and bind strongly to A β (Fig. 4C,D) are expected to block fibrillization at the same stage.

From the 3D structures of stefin B monomer (34), tetramer (37) and protection pattern of the fibrils (44) it should be possible to derive possible A β binding site. The binding surface is not present in the higher oligomers, which showed no binding by SPR. Their 3D structure has not been solved as yet. In the future, as crystallization of the complex between stefin B and A β is a difficult task, maybe interaction could be observed by NMR.

Binding of A β to the β sheet edge was proposed for other A β binding proteins (5). Such a surface in stefin B could represent unpaired strand 5 or, alternatively, exposed surface formed by strands 2 and 3 by domain-swapping.

The feature common to chaperones is their transient interaction with non-native conformations of other proteins, which is followed either by preventing aggregation, correct folding or unfolding and, subsequent targeting to proteasomal or lysosomal degradation (45). Small heat shock proteins (Hsps) are made of typically 12-24 repeats of α -crystallin domains, which form dimers. The small heat shock proteins, α B-crystallins, bind to and inhibit amyloid fibril formation of A β , β 2-microglobulin (46) and of α -synuclein (47). In all these cases the

interaction between the interacting proteins was weak.

In conclusion:

Mutations in stefin B (cystatin B) gene cause EPM1 (48). Mostly they lead to reduced protein expression, however, some are functional and lead to changed proteins, with different stability and aggregation properties (28). Lack of stefin B causes knock-out mice to undergo extensive loss of neurons in the granule layer of the cerebellum (49). They also show signs of ataxia and myoclonus. Stefina B gets overexpressed in many conditions of neurodegeneration, such as ALS and AD but as shown recently also in conditions of oxidative stress (50). The loss of inhibitory function might be related to mis-regulation of stefin B – cathepsin B axis of proteolysis and cell death. However, as the results presented here seem to suggest, aberrant protein folding of stefin B or, aggregation of other proteins – stefin B acting as a chaperone, might also be an explanation for increased oxidative stress, when stefin B is missing.

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FIGURE LEGENDS:

Figure 1 Oligomeric state of the wild-type E31 stefin B samples before (A) and after (B) SPR measurements. A Superdex 75 column (Pharmacia) was used to perform the size-exclusion chromatography. The column was equilibrated in phosphate buffer pH 7.0 containing 0.15 M NaCl. The flow rate was 0.5 ml/min at room temperature. 50 μ l of the E31 stefin B monomers, dimers, tetramers and higher oligomers were injected.

Figure 2 Inhibition of A β fibril formation by stefin B measured by ThT fluorescence. A β peptide concentration was 17 μ M throughout, pH 7.3, 40°C **A)** A β alone, 1:1 molar ratio of A β to Y31 stefin B (complete inhibition) and 1:1 molar ratio of A β to E31 stefin B. **B)** A β alone, 1:1 molar ratio to E31 stefin B monomers, dimers, tetramers and higher oligomers. Protein concentration of stefin B samples was 17 μ M.

Figure 3 Morphology of the aggregates and fibrils as observed by TEM. After A β fibril formation reached steady state (cca. 100 hours at pH 7.3, 40°C) a sample of 15 μ l was spread on the carbon coated grid, 1 % of uranyl acetate was added for a contrast and TEM images were recorded. At the same time point samples were taken of mixtures of A β with the following proteins **A)** Y31 stefin B **B)** E31 stefin B **C)** lysozyme and **D,E)** A β alone.

Figure 4 Binding experiments by SPR. The interaction of A β with the two stefin B iso-forms (Y31 and E31 stefin B) and with isolated oligomers of the wild-type E31 stefin B was assessed at 5 μ l/ml in PBS buffer, pH 7.3. **A)** Binding of Y31 stefin B (analyte) to chip-immobilized A β . The concentration of Y31 stefin B was 0.5, 1, 2, 4 and 20 μ M concentrations (from bottom to top). **B)** binding of 10 μ M A β to chip-immobilized Y31 stefin B. **C)** Binding of monomers (curve a), dimers (b), tetramers (c) and higher oligomers of the wild-type E31 stefin B (d). to chip-immobilized A β . All forms were assessed at 100 μ M concentration. **D)** Binding of the isolated tetramers of the wild-type E31 stefin B at 10, 25, 50 and 100 μ M concentration (from bottom to top).

Figure 5 Complex detected by ESI MS. ESI MS spectra of A β (1–40), stefin B dimer (Y31-variant) and their mixture were recorded: **A)** - 2 μ M A β (1–40), **B)** - the mixture of 2 μ M A β and 2 μ M stefin B and **C)** - 2 μ M stefin B. Peaks corresponding to the A β – stefin B complex are denoted with an asterisk and numbers above the peaks denote charge state of the ions.

Figure 6 Western blots and ELISA of APP and its metabolites upon stefin B co-expression. Western blots showing APP, the C-terminal fragments aCTF, bCF and stefin B in CHO-K1 cells expressing APP wild type **A)** and APP Swedish mutant **B)** and stefin B, respectively, in comparison to mock vector expression. Antibodies to stefin B, aCTF, bCF and APP were used (see, under Materials). In **C)** are shown the levels of A β (1-40) measured by ELISA in cells expressing APP wild type, and the same in cells co-expressing stefin B. Also shown are the levels of A β (1-40) in cells expressing APP-Sw or APP-Sw together with stefin B, which in both cases is 6-fold higher – as expected (26).

Figure 7 Stefins B : A β /APP co-immunoprecipitation. CHO-K1 cells, expressing either wild-type or Swedish mutant APP, were transfected with the vector encoding stefin B and lysed after 24 hours. The lysates

were immuno-precipitated with polyclonal anti-rabbit stefin B antibodies, after which 15% SDS gels and Western blotting with anti-A β antibodies [DE2B4] were performed (see Materials for more details). Lanes 1 or 2 show the results obtained on the whole cell lysates of the APP-wt and APP-Sw, respectively, and lanes 3 and 4 the corresponding samples after co-immunoprecipitation on the anti-stefin B antibodies column. The migration of molecular weight standards is indicated on the left. The anti-A β antibodies [DE2B4] clearly detect a fragment of Mr 15 kDa in the APP-wt cells.

Figure 8 A β peptide co-localization with over-expressed stefin B in A β inclusions. Upper line represents confocal images of CHO-K1 cells immunostained with an antibody against A β (green) and stefin B (red). Overlay represents the overlay of the two corresponding images. Scale bar is indicated on the pictures. Inclusion bodies were marked with different coloured squares (blue, white and yellow). A zoomed view of the selected regions of interest (ROIs) on the side of the colour panels are marked as A, B and C. Only the selected ROIs were used for quantitative co-localization analysis, which was performed by JACoP an ImageJ plug in. The three lines in boxes bellow the colour images marked as **A**, **B**, **C**, represent the results of the JACoPs analysis (see, Table 1).

Table 1

ROI	R_r	$R_r(\text{random})$	P-value	$R = k_{stB} \times k_{a\beta}$			M(threshold)		CCF	ICQ
A	0,37	0,0±0,166	100%	$k_{a\beta}$	0,923	0,739	$M_{a\beta}$	0,46	0,402	0,171
				k_{stB}	0,591		M_{stB}	0,563		
B	0,222	0,001±0,185	100%	$k_{a\beta}$	0,857	0,651	$M_{a\beta}$	0,318	0,301	0,096
				k_{stB}	0,495		M_{stB}	0,463		
C	0,164	0,001±0,155	100%	$k_{a\beta}$	0,762	0,656	$M_{a\beta}$	0,416	0,261	0,072
				k_{stB}	0,565		M_{stB}	0,43		

Table 1: Quantitative co-localization analysis performed with different intensity correlation coefficient based tools, which are grouped in public domain tool JACoP. A, B and C represent selected regions of interest used in quantitative co-localization analysis. R_r - Pearson's correlation coefficient, $R_r(\text{random})$ - Pearson's correlation coefficient determined with Costes randomization based co-localization between random images of the green channel and original image of the red channel with 200 randomization rounds, P-value expressed as a percentage, R - overlap coefficient, $k_{a\beta}$ - overlap coefficient determined for A β (green channel) k_{stB} - overlap coefficient determined for stefin B (red channel), M - Manders coefficient, $M_{a\beta}$ - Manders coefficient determined for A β (green channel), M_{stB} - Manders coefficient determined for stefin B (red channel) CCF - Van Steensel's cross-correlation function, ICQ - Li's intensity correction quotient.

Figure 1

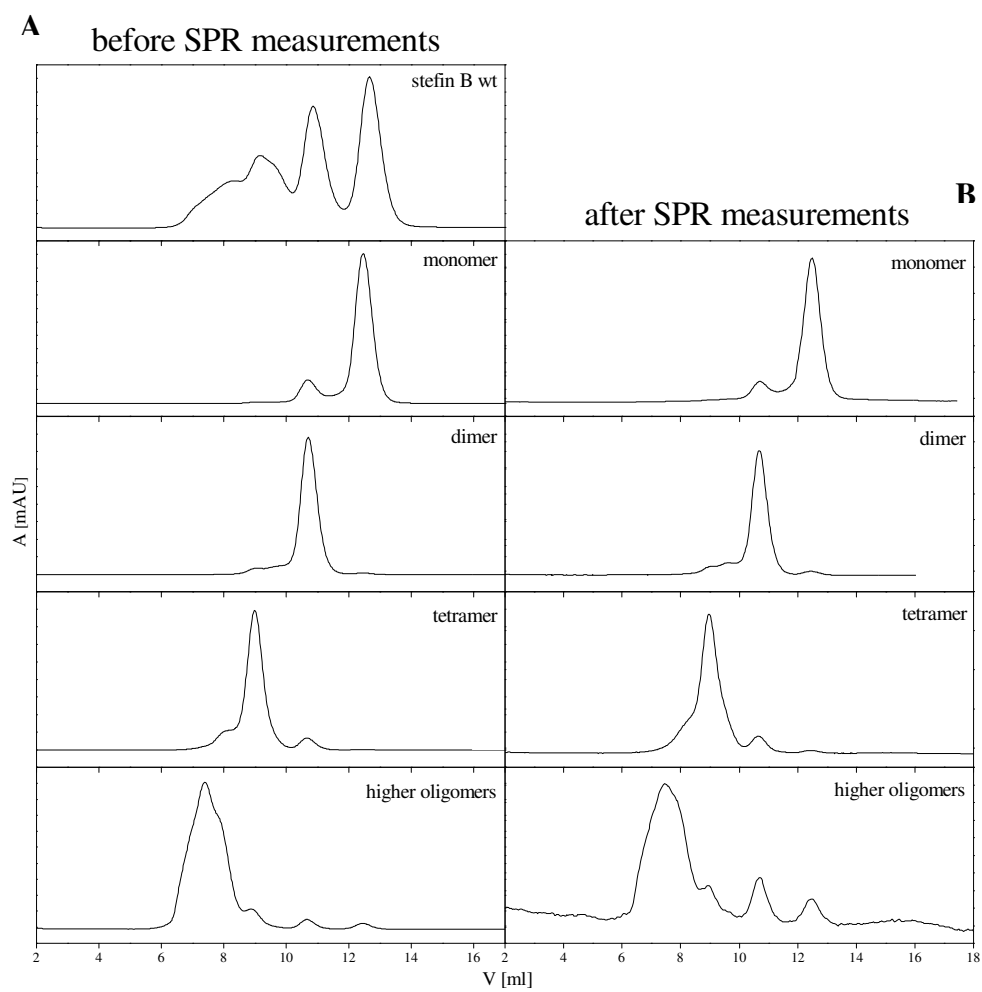


Figure 2

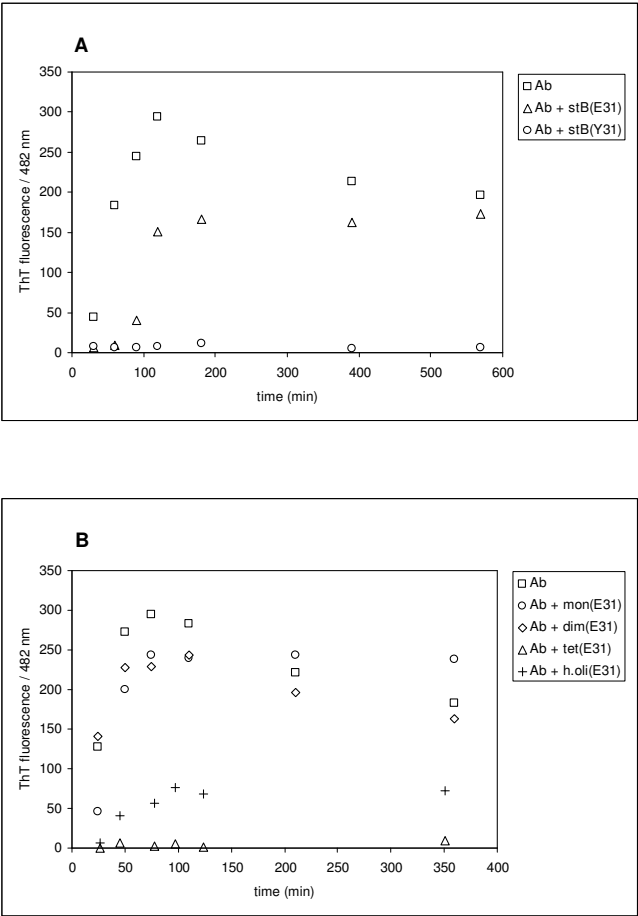


Figure 3

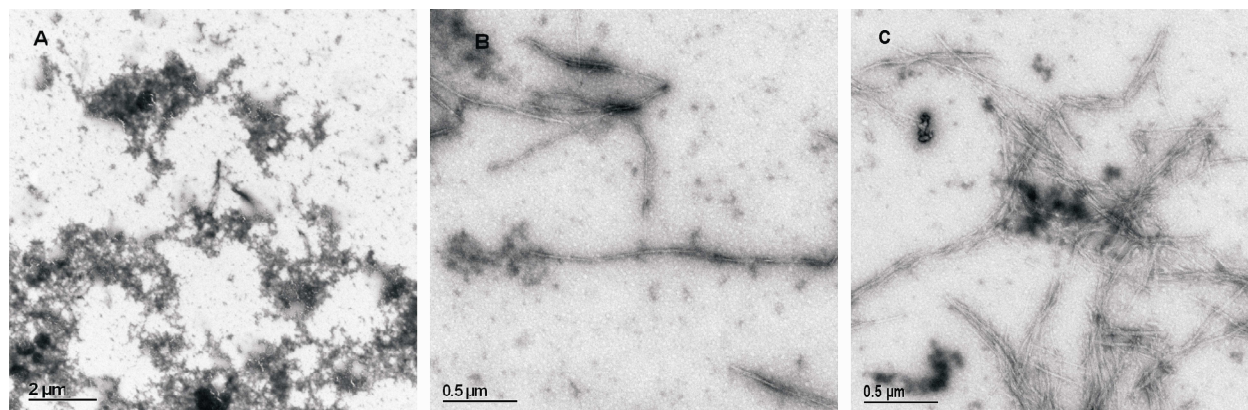


Figure 4

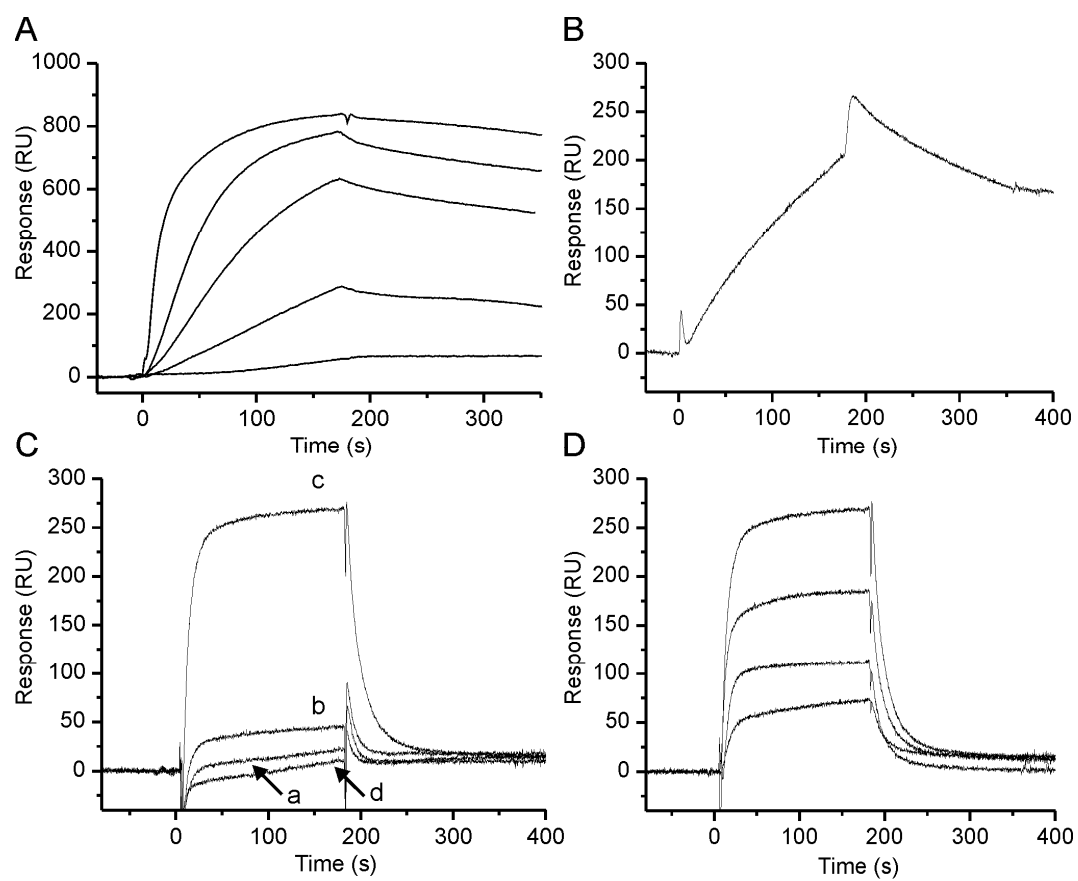


Figure 5

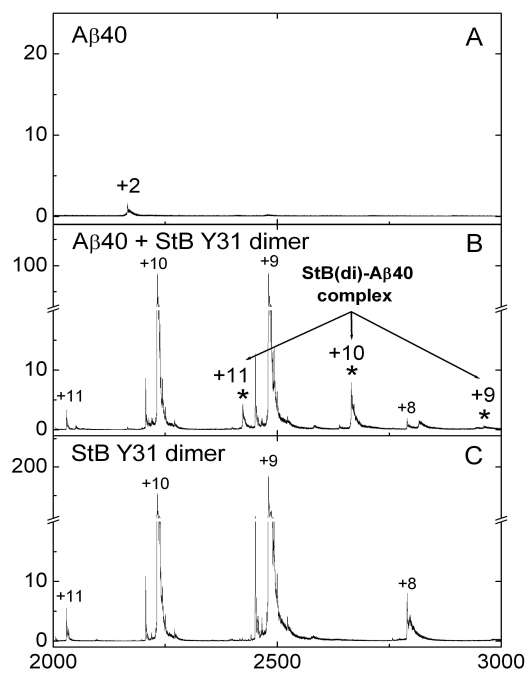


Figure 6

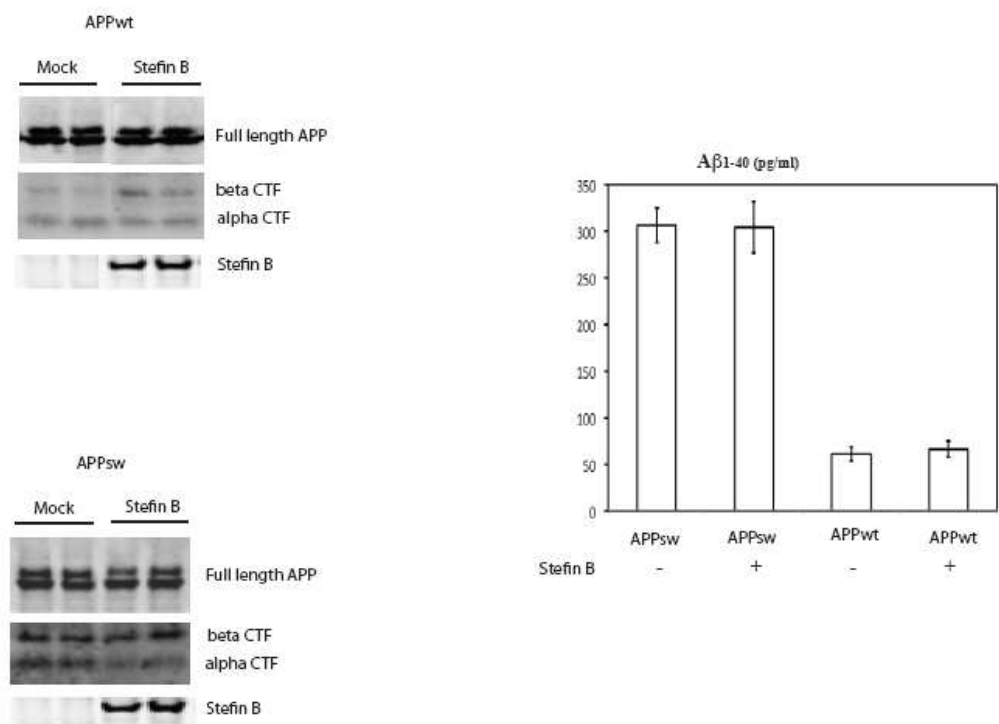


Figure 7:

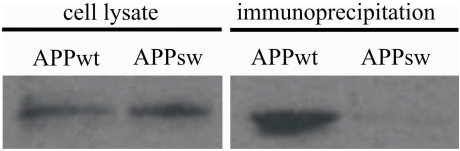


Figure 8

