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Interaction of human stefin B in the prefibrillar oligomeric form with membranes

Correlation with cellular toxicity

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Protein aggregation is central to most neurodegenerative diseases, as shown by familial case studies and by animal models. A modified 'amyloid cascade' hypothesis for Alzheimer's disease states that prefibrillar oligomers, also called amyloid-β-derived diffusible ligands or globular oligomers, are the responsible toxic agent. It has been proposed that these oligomeric species, as shown for amyloid-β, β_2-microglobulin or prion fragments, exert toxicity by forming pores in membranes, initiating a cascade of detrimental events for the cell. Interaction of granular aggregates and globular oligomers of an amyloidogenic protein, human stefin B, with model lipid membranes and monolayers was studied. Prefibrillar oligomers/aggregates of stefin B are shown to cause concentration-dependent membrane leaking, in contrast to the homologous stefin A. Prefibrillar oligomers/aggregates of stefin B also increase the surface pressure at an air–water interface, i.e. they have amphipathic character and are surface seeking. In addition, they show stronger interaction with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] monolayers than native stefin A or nonaggregated stefin B. Prefibrillar aggregates interact predominantly with acidic phospholipids, such as dioleoylphosphatidylglycerol or dipalmitoylphosphatidylserine, as shown by calcein release experiments and surface plasmon resonance. The same preparations are toxic to neuroblastoma cells, as determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay, again in contrast to the homologue stefin A, which does not aggregate under any of the conditions studied. This study is aimed to contribute to the general model of cellular toxicity induced by prefibrillar oligomers of amyloidogenic proteins, not necessarily involved in pathology.

Common cellular and molecular mechanisms underlie a variety of neurodegenerative diseases, from Alzheimer's disease (AD), Parkinson's disease and amyotrophic lateral sclerosis, to sporadic prion diseases. The molecular mechanisms include aberrant protein folding and aggregation in the form of extracellular

Abbreviations
A- β, amyloid-β peptide; AD, Alzheimer's diesase; BRBC, bovine red blood cells; CCAA, cystatin C amyloid angiography; DMEM, Dulbecco's modified Eagle's medium; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)]; DPPS, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine]; IAPP, islet amyloid polypeptide; LTP, long-term potentiation; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PtdCho, phosphatidylcholine; PtdG, phosphatidylglycerol; PtdSer, phosphatidylserine; SUV, small unilamellar vesicle; TEM, transmission electron microscopy.

plaques or intracellular inclusions [1]. A deeper understanding of the detailed mechanism of protein aggregation and the resulting cellular toxicity should lead to rational drug design for this type of disease.

Protein aggregation can result from external insults or aging, however, inherited forms of neurodegenerative diseases, such as familial Parkinson's disease, Huntington's disease or familial AD, are directly linked to the aggregation of mutant proteins. Protein aggregates, in the form of amyloid plaques, neurofibrillary tangles, intracytoplasmic or intranuclear inclusions [1] lead to increased production of reactive oxygen species and dysfunction of the ubiquitin/proteasome system. Finally, mitochondrial dysfunction and cell death are observed (http://www.nature.com/focus/neurodegen/).

The mechanism of amyloid fibrillation has been studied for several individual proteins and a number of models have been proposed [2,3]. Dobson and co-workers proposed that a 'generic' mechanism, common to all proteins, may exist [4,5], which justifies using proteins not involved in any pathology as models. A generic mechanism has similarly been proposed for amyloid-induced toxicity [6–8], with prefibrillar oligomers as the most likely toxic agent. Recently, an antibody was raised against amyloid-β peptide (A-β) that recognizes the structure of the prefibrillar oligomers of a number of amyloidogenic proteins [9], further supporting a generic mechanism.

A mechanism for toxicity was proposed based on the observation that some amyloidogenic proteins have been seen to form so called 'amyloid pores' or 'amyloid channels', which might be cation selective [10]. That the interaction with membranes is involved in amyloid-induced toxicity is supported by the finding that cholesterol can modify this interaction and cytotoxicity [11].

We have looked for a correlation among amyloid fibril formation, interaction with phospholipids, and cellular toxicity, using a model amyloidogenic protein, human stefin B. Stefin B is a member of the I25 family of cystatins (MEROPS classification), the cysteine proteinase inhibitors [12]. Its main pathology is a rare monogenic epilepsy EPM1, so-called Unverricht-Lundborg disease [13]. The most prevalent mutation is a dodecamer repeat expansion in the promoter region of the gene, leading to reduced protein expression. No amyloid pathology of stefin B has been demonstrated *in vivo*, although the analogous human cystatin C is a well-known amyloidogenic protein, causing cystatin C amyloid angiopathy (CCAA) [14].

It has been shown previously that human stefin B readily forms amyloid fibrils *in vitro* [15,16], in contrast to its homolog, stefin A [17,18]. By following the kinetics of fibril formation, conditions were defined in which the protein exists in the form of prefibrillar oligomers/aggregates, which persist during the lag phase. These have been confirmed by both transmission electron microscopy (TEM) and atomic force microscopy [15].

In this study, we measured the interaction of stefin B with various combinations of phospholipid monolayers and bilayers. Interaction of stefin B in the prefibrillar aggregated state with model lipid membranes was probed using the calcein permeation assay, surface pressure measurements and surface plasmon resonance. Stefin A, a protein of 54% identity and 80% similarity to stefin B, which does not form aggregates under any of the conditions studied here, was always used for comparison. In parallel, the toxicity of the prefibrillar preparations of stefin B was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, with stefin A as a negative control. Stefin B exhibits a weak, yet significant, surface-seeking activity, especially when in the prefibrillar form. This property correlates with its weak toxicity to the cells. Stefin A (which remained native) showed neither surface activity nor toxicity.

Results

Preparation of prefibrillar oligomers/aggregates

Stefin B can be induced to form amyloid-like fibrils at pH 4.8 or 3.3 [15–17], which parallels the two acid-induced intermediates of the protein [19]. The lag phases of the fibrillation reaction, where prefibrillar aggregates accumulate, were determined for up to 2 weeks at pH 4.8 and room temperature, and for 1–2 days in pH 3.3 buffer at room temperature. TEM pictures taken during the lag phase at pH 4.8 and 3.3 are shown in Fig. 1. At pH 4.8 (Fig. 1A), a granular aggregate composed of loosely bound oligomeric blocks can be seen and, at pH 3.3 (Fig. 1B), necklace-like structures built from basic ellipsoid blocks (similar to protofibrils) are observed. At pH 7.3, oligomers of stefin B might be present as well, particularly dimers, which have been shown by gel-filtration to be the predominant species [20].

Toxicity of the aggregates

Decrease in cell viability after exposure to prefibrillar oligomers/aggregates of stefin B, prepared at various pH values as described above, was determined using the MTS assay (Fig. 2). Cells were incubated with the

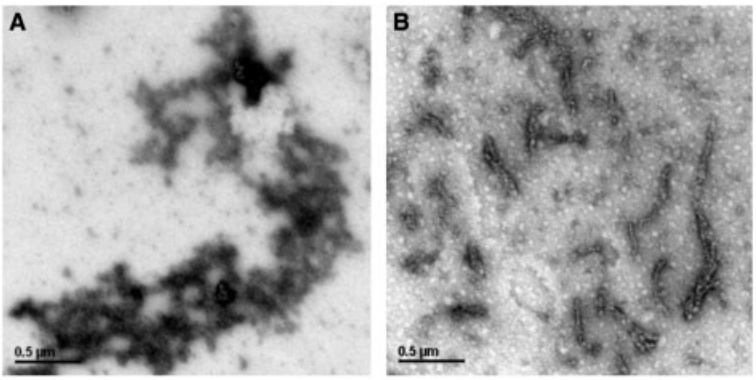

Fig. 1. TEM pictures of prefibrillar oligomeric aggregates of human stefin B. (A) pH 4.8 and (B) pH 3.3. Samples were prepared as described previously [15]. TEM measurements were made with a Philips CM 100 transmission electron microscope at 80 kV and magnifications from ×10 000 to ×130 000. Images were recorded by Bioscan CCD camera Gatan, using DIGITAL MICROGRAPH software.

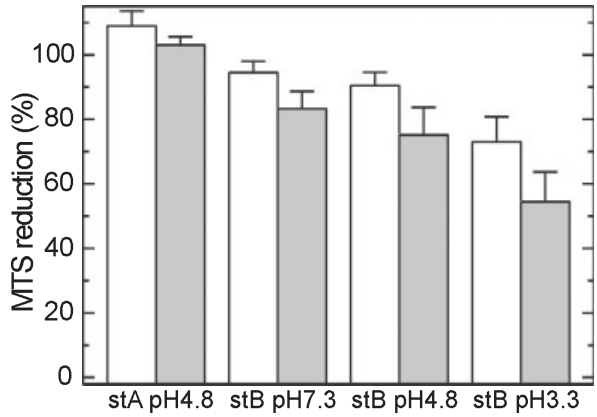

Fig. 2. Viability of SH-SY5Y neuroblastoma cells exposed to human stefin preparations. Cell viability was measured by the MTS test. Cells were exposed overnight to native stefin A (pH 4.8), native stefin B (pH 7.3) and to prefibrillar aggregates of stefin B, both, at pH 4.8 and 3.3. Protein concentration in each case was 22 μM (light bar) and 41 μM (dark bar). Values shown are averages of five independent experiments, whereas in each experiment each value was determined in triplicate.

toxic agent (in our case prefibrillar aggregates and protofibrils) for 16 h before the MTS reagent was added. Cell-mediated reduction of MTS was then measured at 490 nm within a few hours, resulting in lower readings if cells were not viable. Overnight incubation took place in the medium at pH 7.3, therefore, no fibrils other than those present initially could form. From previous experiments we have shown that fibrils do not form within the lag phase and this is confirmed by the images shown in Fig. 1.

It has been shown that stefin A does not form prefibrillar aggregates at pH 4.8 or 7.3, so stefin A was used as a control in determining the effect of native proteins on cell viability. Buffers at pH 3.3, 4.8 and 7.3 without the protein had no effect on cell viability (data not shown). Stefin A does not diminish cell viability (but rather slightly increases it). In contrast, stefin B prefibrillar aggregates prepared at pH 4.8 and 3.3 (for morphology see Fig. 1), caused a significant, protein-concentration-dependent reduction in cell viability (Fig. 2). Toxicity was maximal with the prefibrillar aggregates obtained at pH 3.3 (up to 40% loss of viable cells). Therefore, the MTS test appears suitable for discriminating the cytotoxic effect of the stefin prefibrillar forms. In order to determine whether the prefibrillar aggregates of stefin B exert their toxic effect via lipid membrane interactions, a lipid vesicle permeabilization assay, insertion into lipid monolayers, and binding observed by surface plasmon resonance were employed.

Permeabilization of small unilamellar vesicles

The permeabilizing activity of prefibrillar stefin B aggregates on small unilamellar vesicles (SUV) of various lipid compositions was monitored using the calcein release method. Phosphatidylcholine (PtdCho) vesicles were largely resistant to leakage for all tested variants of stefin B. In contrast, native stefin B and its aggregates were active against liposomes containing negatively charged lipids, such as phosphatidylglycerol (PtdG) or phosphatidylserine (PtdSer) (Fig. 3). When measuring the kinetics of release from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine/1,2-dipalmitoyl-*sn*-glycero-3-[phospho-L-serine] (DOPC/DPPS) 2 : 1 (mol/mol) SUV, up to 25% of permeabilization was measured for stefin B aggregates at pH 4.8 at a lipid/protein molar ratio of ≈ 1 (30 μM concentration of both protein and lipid). After overnight incubation, aggregates at both pH 3.3 and 4.8 showed maximal release on 1,2-dioleoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] (DOPG) vesicles. Interestingly, native stefin B at pH 7 also showed considerable permeabilization ($\approx 60\%$) of these vesicles. Stefin A and pure buffers were used as negative controls and did not show any permeabilizing activity for any

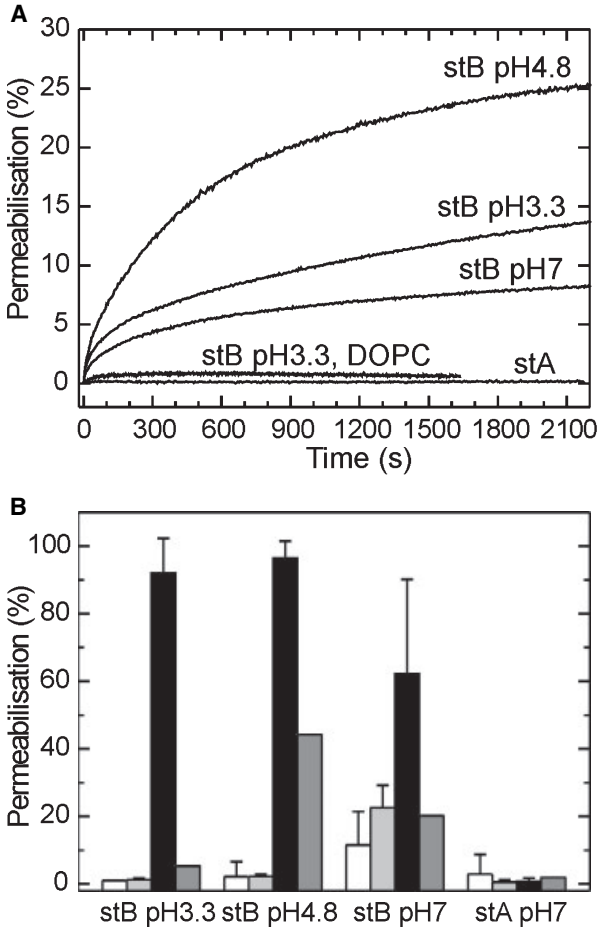

Fig. 3. Permeabilization of SUV by prefibrillar stefin B. (A) Kinetics of SUV permeabilization. SUV were composed of DOPC/DPPS (2 : 1, mol/mol). Protein (30 μM) and lipids (30 μM) were in 140 mM NaCl, 20 mM Tris/HCl, pH 8.5, 1 mM EDTA. (B) Permeabilization of liposomes of different compositions after overnight incubation with stefin A (stA) and B (stB). White, DOPC; light gray, DOPC/DOPG (1 : 1, mol/mol); black, DOPG; dark gray, DOPC/DPPS (2 : 1; mol/mol). The results are mean ± SD, $n = 1$–4. The degree of permeabilization is expressed as the percentage of the maximal value obtained at the end of the assay by the addition of 2 mM Triton X-100. The excitation and emission wavelengths were set to 485 and 520 nm. Both slits were set to 5 nm.

lipid mixture or concentration tested. Release from the vesicles was dose dependent, but none of the aggregates was active at lipid/protein ratios > 10, i.e. the percentage of release for stefin B aggregates at pH 4.8 was 96.4, 19.8, 5.6 and 3.7 at lipid/protein ratios 1, 2, 4 and 8, respectively.

None of the samples used was hemolytically active towards bovine red blood cells at concentrations up to 40 μM, which is consistent with the low content of negatively charged phospholipids in the outer membrane lipid leaflet.

Insertion in monolayers

The ability of stefins and their aggregates to insert at the air–water interface, i.e. in the absence of lipids, was determined first, as this may give an indication about the amphipathicity of the protein. Stefin B aggregates obtained at pH 4.8 or 3.3 insert much more readily into an air–water interface than do the native states of stefins A and B obtained at pH 7 (Fig. 4). The lowest degree of insertion was observed with stefin A, reaching only half the value for aggregated stefin B. This indicates that the prefibrillar oligomers may be organized in such a way that they are more amphipatic than the native protein and therefore acquire a higher surface-seeking potential.

Insertion into lipid monolayers was next measured using monolayers composed of DOPC or DOPG. The insertion of proteins into the monolayer generated an increase in surface pressure, $\Delta\pi$, from the chosen initial pressure, π_0 (Fig. 5A). At $\pi_0 = 5$ mN·m^{-1}, insertion of the proteins differed markedly. Whereas stefin A inserted poorly, stefin B, at pH 7 and in the forms aggregated at pH 4.8 and pH 3.3, inserted readily and to a higher final pressure. Stefin B at pH 7 and aggregates at pH 3.3 showed slower kinetics of insertion than the aggregates at pH 4.8. The kinetics observed for these two cases were quite complex and it is possible that interaction with the monolayer induces cooperative conformational rearrangements or further oligomerization on the surface of the monolayer.

The increase in pressure was measured as a function of π_0 (Fig. 5B,C). Extrapolation to $\Delta\pi = 0$ gives the

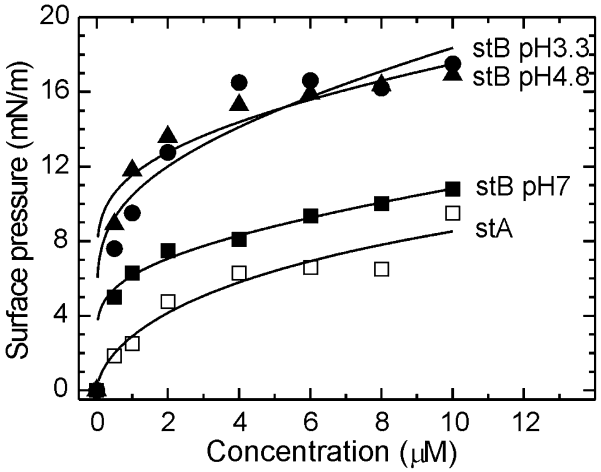

Fig. 4. Insertion of stefin B in prefibrillar form into an air–water interfaceInsertion into the air–water interface was measured in 10 mM Hepes, 200 mM NaCl, pH 7.5 with constant stirring at room temperature. Open squares, stefin A, pH 7; solid squares, stefin B, pH 7; triangles, stefin B pH 4.8; circles, stefin B pH 3.3.

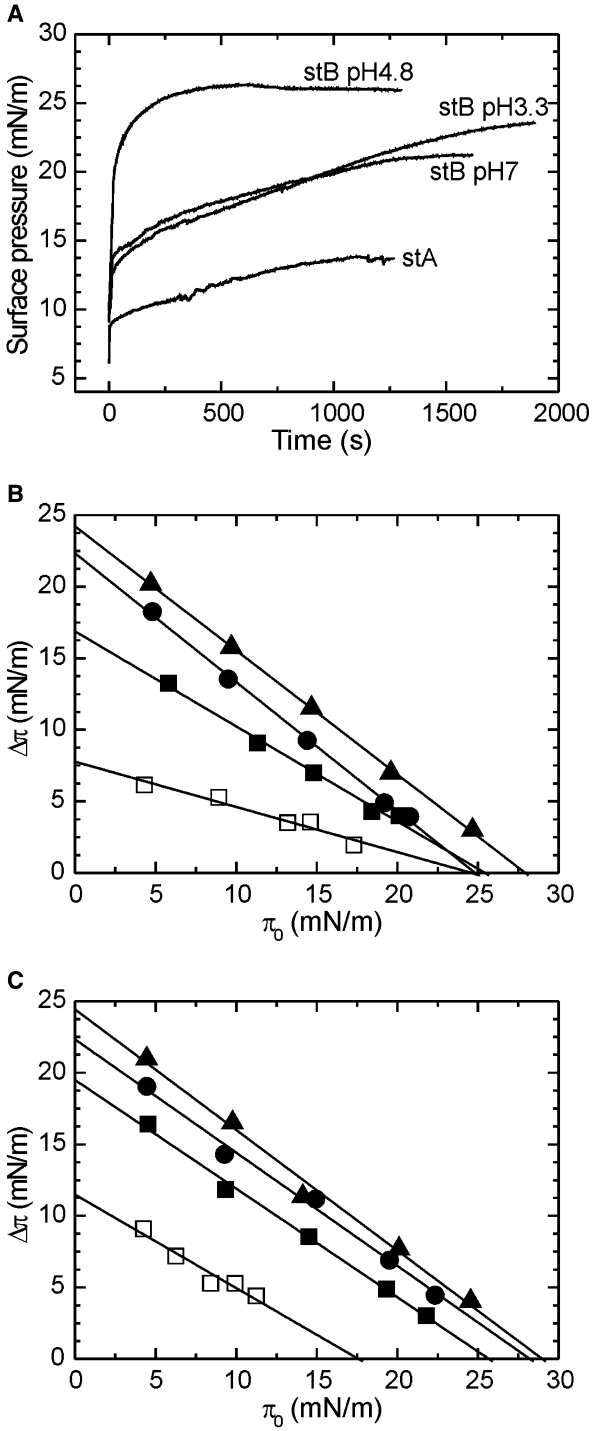

Fig. 5. Insertion of stefins into DOPC and DOPG monolayers. (A) Kinetic traces of the insertion into DOPG lipid monolayers at initial pressure of 5 $mN{\cdot}m^{-1}$. The proteins were injected into the subphase composed of 10 mM Hepes, 200 mM NaCl, pH 7.5 with constant stirring at room temperature. (B) Critical pressure plots for DOPC monolayers. (C) Critical pressure plots for DOPG monolayers. Open squares, stefin A, pH 7; solid squares, stefin B, pH 7; triangles, stefin B pH 4.8; circles, stefin B pH 3.3.

Table 1. Critical pressures for the insertion of stefins into lipid monolayers. Stefin B at pH 3.5 or 5 is prefibrillar (see Results). Stefin B at pH 7 is native and dimeric and stefin A at pH 5 or 7 is native monomeric. These are actual pH readings of protein solutions and not values of the buffers.

Protein	DOPC ($mN{\cdot}m^{-1}$)	DOPG ($mN{\cdot}m^{-1}$)
Stefin B pH 3.5	24.8	28.2
Stefin B pH 5.0	27.9	29.0
Stefin B pH 7.0	25.4	25.7
Stefin A pH 7 or 5	24.6	17.6

critical pressure, π_C, i.e. the pressure at which protein cannot insert into the monolayers (Table 1). Once more, the critical pressure of the proteins differs markedly. The lowest critical pressure was observed for stefin A at pH 7 on both membranes, whereas the highest was observed for stefin B aggregate at pH 4.8. In DOPG membranes, critical pressure increased by $\approx$2–5 mN, reaching almost 30 $mN{\cdot}m^{-1}$, which is similar to the surface pressure encountered in biological membranes [21].

Binding to supported lipid membranes

Binding to liposomes was measured by surface plasmon resonance using Biacore X and L1 chip. Liposomes were retained on the surface of the chip by lipophilic groups on the chip dextran matrix and served as a ligand for the proteins to be bound. Proteins were injected across a prepared surface at 5 μM for 1 min and the dissociation was followed for 5 min. This technique allows direct estimation of rate and dissociation constants [22]. In our case, the quality of the data does not allow quantitative analysis, but nevertheless, some conclusions can be drawn. Neither stefin A nor stefin B native states at pH 7 bound to any membrane used as the signal hardly changes during the injection and was the same as before the injection during the dissociation phases. Weak binding at the micromolar range was observed for stefin B at pH 3.3 and 4.8 (Fig. 6) for negatively charged liposomes (DOPC/DOPG, 1 : 1), but the best for both were DOPG liposomes. Stefin B aggregates at pH 3.3 bound the most of all, as the signal increase during the injection phase was the largest and there was low dissociation after the end of injection.

Discussion

The main hypothesis for pathology in AD and other neurodegenerative diseases is the modified ‘amyloid

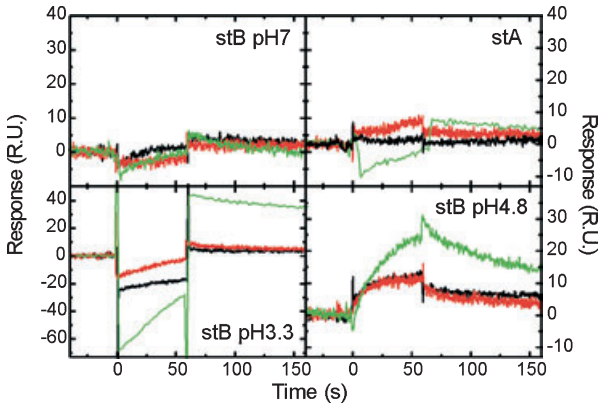

Fig. 6. Binding of stefins to liposomes measured by surface plasmon resonance. Binding of stefin A (stA) and B (stB) was measured using captured liposomes composed of DOPC (black), DOPC/DOPG (1 : 1; mol/mol) (red) and DOPG (green) in 140 mM NaCl, 20 mM Tris/HCl, pH 8.5, 1 mM EDTA at 25 °C. The concentration of protein injected was 5 μM. The association was followed for 1 min.

cascade' hypothesis, which states that the primary reason for the initiation of events detrimental to the cell are prefibrillar species [23,24]. It is now believed that globular oligomers, also called A-β-derived diffusible ligands [25,26] are the responsible toxic agents. These are thought to interact with inner cellular membranes or even the plasma membrane, making pores or channels.

The channel hypothesis of AD has a decade-long history [10]. It was first shown by Arispe *et al.* [27] that A-β [1–40] can form channels *in vitro* in lipid bilayers. The pores of A-β formed *in vitro* were cation selective for Ca^{2+}, whereas Zn^{2+} blocked them [28]. Therefore, it was proposed that Ca^{2+} influx could lead to neuronal death in AD and other neurodegenerative diseases [29,30]. These results were extended by Kourie *et al.* [31] who described several distinct channel subtypes. The channel hypothesis of AD and neurodegeneration in general, is not incompatible with other key elements of toxicity, as, for example, the deregulation of Ca^{2+} homeostasis and generation of reactive oxygen species [10]. In contrast, mechanisms of toxicity as derived from channel hypothesis seem quite likely. Even small changes in plasma membrane potential may alter the electrical properties of neurons, which are very sensitive to ion gradients. Ca^{2+} influx would trigger apoptosis and alter signaling. If amyloid toxin could disrupt mitochondrial membranes, this again may lead to apoptosis. The channels were predicted to occur easily in low pH compartments, such as lysosomes.

At least six proteins or peptides other than A-β were shown to form channels, including islet amyloid polypeptide (IAPP) [32], β_2-microglobulin [33] and the fragment PrP 106–126 of the prion protein [34,35]. It also was shown that A-β, IAPP and the prion protein fragment evoke free calcium elevation in neuronal cell lines [36] and that α-synuclein interacts with lipids [37]. Our aim in this study was to contribute to the general model of cellular toxicity induced by prefibrillar oligomers of amyloidogenic proteins not necessarily involved in pathology. Prefibrillar preparations of stefin B were shown to be toxic to cells, in contrast to the homologous stefin A, which is not amyloidogenic.

Prefibrillar oligomers/aggregates of stefin B obtained in the lag phase at pH 4.8 or 3.3 differ in morphology, producing more protofibrils at pH 3.3 (Fig. 1B) and having more loosely bound oligomers (the so called granular aggregate) at pH 4.8 (Fig. 1A). This probably results in a different effect on cell viability (Fig. 2), with the protofibrils producing a maximal effect (up to 40% less viable cells). However, even stefin B at pH 7.3, where it is native and predominantly dimeric [20], exhibits some toxicity. This might be due to the inherent toxicity of lower oligomers or it could be due to the influence of the low pH at the membrane surface, which would trigger partial unfolding with subsequent aggregation. It should be noted here that even small oligomers of A-β up to tetramers were shown to change neural plasticity and block long-term potentiation (LTP) [38], without extensive cell death. Toxicity to cells is not limited to amyloidogenic proteins with known pathology. It has been shown for at least some other nonpathological amyloidogenic proteins, such as apomyoglobin [7], SH3 domain from bovine phosphatidylinositol-3′-kinase, and HypF N-terminal domain [6,8].

Prefibrillar oligomers of human stefin B obtained at pH 4.8 or 3.3, in addition to toxicity, cause membrane leaking in a protein-concentration-dependent manner. Surface pressure measurements have shown that the aggregated stefin B increases the surface pressure of the lipid monolayer, reaching almost 30 $mN \cdot m^{-1}$ for DOPG membranes, a value encountered in natural membranes [21]. Surface plasmon resonance experiments confirm the binding of the aggregated forms, albeit to a much smaller extent than that observed for some proteins that bind specifically to membranes, such as the small membrane-binding domains involved in cell signaling [39,40] or domains used by pore-forming toxins for attachment to the membranes [41,42]. In all our experiments, stefin B prefibrillar oligomers interacted predominantly with acidic phospholipids, such as DOPG and DPPS. As in the toxicity experiments, stefin B at pH 7.3, a pH at which it is native and predominantly dimeric [20], exerted some membrane binding.

All the effects observed were specific to stefin B, relative to its homolog, stefin A, which is not transformed into prefibrillar oligomers/aggregates under any of the conditions studied and is not toxic. Electrostatic interaction with negatively charged lipids due to global or local charge could explain the greater binding of stefin B which is more basic, with an isoelectric point of ~ 8, than stefin A, with an isoelectric pont of ~ 5. An additional factor may be the much higher stability of stefin A which also may count for stefin A not forming aggregates under mild conditions. This difference would mean that stefin B, but not stefin A, could (partially) unfold under the conditions at the membrane surface to which it could subsequently bind. A third factor may be the oligomeric state. Only stefin B forms dimers easily, whereas stefin A remains monomeric under all the conditions studied. If the dimers (most likely domain swapped) arrange into higher oligomeric complexes these may form anular structures observed with some other aymloidogenic peptides/proteins.

With our experiments we cannot unambiguously prove the channel hypothesis for stefin B aggregates, i.e. that prefibrillar oligomers of stefin B induce membrane leakage by forming channels. The preference for acidic lipids suggests that the membrane might be destabilized simply by surface interactions. However, the permeabilization by stefin B prefibrillar oligomers of vesicles made of acidic phospholipids resembles pore formation by A-β [27] and liposome permeabilization of α-synuclein [43]. The toxic activity exerted by prefibrillar forms of stefin B and other amyloidogenic proteins is much lower than that of some specialized proteins, such as pore-forming toxins. For example, leakage from liposomes is routinely observed at submicromolar concentrations with pore-forming toxins, such as actinoporins from sea anemones [44], and cholesterol-dependent cytolysins [45], which is at least one order of magnitude larger. However, pore-forming toxins have evolved to act acutely, whereas exposure to amyloidogenic proteins, and therefore their deleterious effects, may be chronic.

Recently a study by Zhao *et al*. [46] has shown that endostatin binds predominantly to PtdSer PtdG liposomes. The authors show that at acidic phospholipids surface (but not at PtdCho), the protein transforms into fibrous material, which binds Congo Red and exhibits characteristic green birefringence. It is worth mentioning that PtdSer is exposed on the surface of cancer cells, whereas PtdG is present in microbial membranes. Zhao *et al*. [46], propose that microbial peptides and cytotoxic proteins (such as endostatin and stefin B) might share similar molecular mechanisms of permeabilization with the well-known pore-forming toxins.

Conclusions

We have shown that human stefin B, an amyloidogenic protein not involved in any known amyloid pathology, is toxic to cells. We have also shown that the toxic effects of stefin B are correlated to its interaction with acidic phospholipids, found predominantly in the cytosolic site of the plasmalema (PtdSer) and inner mitochondrial membrane (cardiolipin and PtdG). Lessons from comparison of homologous proteins, in our case human stefins B and A, may help to clarify factors involved in membrane permeabilization and cytotoxicity.

Experimental procedures

Materials

DOPC, DOPG and DPPS were from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals were from Sigma (St Louis, MO, USA) unless stated otherwise. The CellTiter $96^{(R)}$ AQ_{ueous} One Solution Reagent from Promega (Madison, WI, USA) contains a tetrazolium compound (inner salt; MTS) and electron coupling reagent (phenazine ethosulfate). The concentration of PtdCho was determined with Free Phospholipids B kit according to the manufacturer's instructions (Wako Chemicals, Dusseldorf, Germany).

Recombinant proteins

Recombinant human stefins A and B were produced in *Escherichia coli* and isolated as described previously [47,48]. For this study the usual recombinant variant S3Y31 of stefin B was used.

Preparation of prefibrillar aggregates

Buffers used were 0.015 M acetate, 0.15 M NaCl, pH 4.8 and 0.015 M glycine, 0.26 M Na_2SO_4, pH 3.3 [15,16]. The protein concentration for growing oligomers was always 100 μM. Dilution of the bulk protein solution to the buffers gave pH values higher by 0.2 pH units.

Neuronal cell culture

SH-SY5Y neuroblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) and 10% (v/v) fetal bovine serum unless otherwise stated, in a 5% (v/v) CO_2 humidified environment at 37 °C.

Measurement of toxicity to neuroblastoma SH-SY5Y cells

The CellTiter 96$^{(R)}$ AQ$_{ueous}$ One Solution Cell Proliferation Assay, a colorimetric method based on MTS reagent, was used to determine of the number of viable cells after exposure to 'amyloid' toxins (prefibrillar aggregates of stefin B) or native proteins (stefin A). Cell-mediated reduction of MTS was measured at 490 nm, resulting in lower readings if cells were not viable.

The SH-SY5Y cells were plated on to 96-well plates at a density of 10 000 cells per well in 100 μL fresh medium. After 24 h incubation, the culture medium was exchanged with 100 μL serum free medium DMEM (OPTIMEM) to prevent cell duplication. 10 and 20 μL of concentrated prefibrillar protein in buffers of different pH was added to the wells (containing 100 μL of culture medium each), giving 22 and 41 μM final protein concentration. As a negative control, cells without the prefibrillar protein, and as a positive control cells with added staurosporine, were taken. Further controls were buffers without protein. The 96-well plates were incubated overnight. Twenty microliters of MTS reagent was then added to each well. The plate was incubated for 2–3 h at 37 °C in a 5% (v/v) CO_2 humidified environment. The absorbance of formazan was measured at 490 nm using an automatic plate reader. Control experiments were performed by exposing cells to solutions of the nonprefibrillar protein (stefin A) for the same length of time and the same concentrations.

Liposome permeabilization assay

Lipid mixtures, dissolved in chloroform, were spread on a round-bottom glass flask of a rotary evaporator and dried under vacuum for at least 3 h. The lipid film was resuspended in 1 mL of 60 mM calcein in vesicle buffer (140 mM NaCl, 20 mM Tris/HCl, pH 8.5, 1 mM EDTA) and freeze–thawed six times. The resulting multilamellar vesicles were converted to SUV by sonication (MSE 150 W ultrasonic disintegrator, MSE, Butte, UT) of the suspension at room temperature. The SUV suspension was centrifuged at 12 000 ***g*** for 15 min to remove titanium particles released from the probe. The excess of calcein was removed from the calcein-loaded liposomes by gel filtration on a small G-50 column. Vesicles were stored at 4 °C immediately after preparation and used within 2 days. For calcein release experiments, liposomes at 30 μM final concentration were mixed with protein in 0.5 mL and incubated overnight at room temperature. Vesicle buffer (0.5 mL) was then added to the samples, which were centrifuged for 10 min at top speed in a benchtop centrifuge. The supernatant was transferred to another tube and the released calcein measured using a Jasco FP-750 spectrofluorimeter (Jasco, Easton, MD), with excitation and emission at 485 and 520 nm. Excitation and emission slits were set to 5 nm. For time course measurements protein was incubated at desired concentrations in a 1 mL cuvette and stirred at 25 °C. Vesicles were added at the required concentration and the time course was followed for 30 min. The permeabilization induced by the proteins was expressed as a percentage of the maximal permeabilization obtained at the end of the assay by the addition of Triton X-100 to a final concentration of 2 mM.

Hemolytic activity

Hemolytic activity was measured turbidimetrically using a microplate reader (MRX; Dynex Technologies, Deckendorf, Germany). A suspension of bovine red blood cells (BRBC) with $A_{630} = 0.5$ in hemolysis buffer (0.13 M NaCl, 0.02 M Tris/HCl, pH 7.4) was prepared from well washed BRBC. One hundred microliters of BRBC suspension were added to 100 μL of twofold serially diluted proteins. Hemolysis was monitored by measuring the attenuance at 630 nm for 20 min at room temperature.

Surface pressure measurements

Surface pressure measurements were carried out with a MicroTrough-S system (Kibron, Helsinki, Finland) at room temperature. The aqueous sub-phase consisted of 500 μL of 10 mM Hepes, 200 mM NaCl, pH 7.5. Lipids dissolved in chloroform/methanol (2 : 1, v/v) were gently spread over the sub-phase. The desired initial surface pressure was attained by changing the amount of lipid applied to the air–water interface. After 10 min, to allow for solvent evaporation, the desired stefin variant was injected through a hole connected to the sub-phase. The final stefin concentration in the Langmuir trough was 10 μM. The increment in surface pressure vs. time was recorded until a stable signal was obtained.

Surface plasmon resonance

The binding to the supported lipid membrane was measured using a Biacore X (Biacore). L1 chip was equilibrated in vesicle buffer. Large unilamellar vesicles were prepared by extrusion as described previously [49]. They were passed at 0.5 mM lipid concentration across the chip for 15 min at 1 μL·min^{-1}. Loosely bound vesicles were eluted from the chip by three injections of 100 mM NaOH. Unspecific binding sites were blocked by one injection of 0.1 mg·mL^{-1} bovine serum albumin. For the binding experiment proteins were injected at 5 μM concentration for 60 s at 30 μL·min^{-1}. Blanks were injections of buffer without protein.

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