Amyloid β-peptides interfere with mitochondrial preprotein import competence by a co-aggregation process

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List of Abbreviations:

TMRE, tetramethylrhodamine ethyl ester; TCA, trichloroacetic acid; MPP, mitochondrial processing peptidase; BN-PAGE, blue-native polyacrylamide gel electrophoresis; SDS-PAGE, sodiumdodecylsulfate polyacrylamide gel electrophoresis; TOM, translocase of the outer membrane; TIM, translocase of the inner membrane; OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; IMS, intermembrane space; DHFR, dihydrofolate reductase; AA, amino acid; $\Delta\psi_{mt}$, electric potential across the inner mitochondrial membrane.

Abstract

Aβ peptides play a central role in the etiology of Alzheimer disease (AD) by exerting cellular toxicity correlated with aggregate formation. Experimental evidences showed an intraneuronal accumulation of Aβ peptides and an interference with mitochondrial functions. Nevertheless, the relevance of intracellular Aβ peptides in the pathophysiology of AD remained controversial. Here, we found that the two major species of Aβ peptides, in particular Aβ42, exhibited a strong negative effect on the preprotein import reactions essential for mitochondrial protein biogenesis. However, Aβ peptides only weakly interact with mitochondria and did not affect the inner membrane potential or the structure of the preprotein translocase complexes. Aβ peptides significantly decreased the import competence of mitochondrial precursor proteins through a specific co-aggregation mechanism. Co-aggregation and import inhibition were significantly stronger in case of the longer peptide Aβ42, correlating with its importance in AD pathology. Our results demonstrate that a direct interference of aggregation-prone Aβ peptides with mitochondrial protein biogenesis represents a crucial aspect of the pathobiochemical mechanisms contributing to cellular damage in AD.

Introduction

Beta-amyloid (Aβ) peptides have been associated with severe human pathological conditions like Alzheimer disease (AD) (Murphy & LeVine, 2010), Down syndrome (Head & Lott, 2004) and

cerebral amyloid angiopathy (Weller et al, 2000), all characterized by accumulation and deposition of Aβ peptides in the central nervous system. Due to the diversity of pathological aspects connected with a severe neuro-degenerative disease like AD, the bio-chemical mechanisms resulting in neuronal cell death and the correlation with the accumulation of Aβ peptides are not completely clear (Musiek & Holtzman, 2015).

Aβ peptides derive from a proteolytic process mediated by β- and γ-secretases on the type 1 trans-membrane precursor called amyloid precursor protein (APP). The most common forms in AD are constituted of 40 (Aβ40) and 42 (Aβ42) amino acids (Zhang et al, 2011). Mutations, environmental factors as well as aging could induce changes in the equilibrium between Aβ peptide production and removal (Mawuenyega et al, 2010) as well as an imbalance between amyloidogenic and non-amyloidogenic pathways (Agostinho et al, 2015). This causes an increase of Aβ peptide concentrations promoting aggregation and deposition as senile plaques in brain parenchyma. Kinetic and structural studies about Aβ aggregation *in vitro* have reported that unstructured Aβ monomers have an intrinsic tendency to self-assemble spontaneously by a nucleation-polymerization mechanism into higher-order oligomeric, protofibrillar and fibrillar states (Thal et al, 2015). The aggregation process is enhanced by high peptide concentrations, presence of nucleation seeds, altered pH, ionic strength, or temperature (Stine et al, 2003). Furthermore, a large variety of post-translation modifications of the Aβ sequence influence the aggregation propensity (Kummer & Heneka, 2014; Thal et al, 2015). As Aβ42 oligomers represent the most toxic amyloidogenic peptide species, the main component of AD senile plaques, and the first to deposit during the senile plaques formation, they play a key pathophysiological role in the development of AD (Haass & Selkoe, 2007). Interestingly, although Aβ42 has only small structural differences compared to the other Aβ peptides, it displays distinct clinical, biological and biophysical behaviors (Bitan et al, 2003; Jarrett et al, 1993).

The “amyloid cascade hypothesis” re-presents the major theory to explain the etiology and pathology of AD (Hardy & Selkoe, 2002; Musiek & Holtzman, 2015). This hypothesis, strongly supported by genetic studies on familial AD cases (Hardy & Higgins, 1992), proposed that an aggregation of Aβ peptides is responsible for the initiation of a multistep pathological cascade eventually resulting in neuronal death. A growing body of evidence also suggested the prominent contribution of an intracellular accumulation of Aβ peptides as a trigger of neurodegeneration and AD pathology on the cellular level (Gouras et al, 2010; Wirths & Bayer, 2012; Wirths et al, 2004). Based on their specific biochemical properties, it is likely that intracellular Aβ peptides interact with membranes or other cellular components and induce structural changes of sub-cellular compartments (LaFerla et al, 2007).

Mitochondrial dysfunction is now consensually accepted as a general pathological feature in AD patients (Mattson et al, 2008; Piaceri et al, 2012; Selfridge et al, 2013). In line with this, a modification of the amyloid cascade hypothesis was postulated that supports the correlation between mitochondrial dysfunction with AD. Named “mito-chondrial cascade hypothesis”, it considers how individual mitochondrial dysfunctions, accumulating in aging cells, could influence Aβ peptide homeostasis, aggregation and consequently the chronology of AD (Swerdlow et al, 2014). However, it is still disputed if mitochondrial dysfunctions are early casual events or a consequence of other pathological events in AD patients. Evidences exist that indicate an accumulation of Aβ peptides in mitochondria, interactions with protein components of the mitochondrial matrix, and perturbations of mitochondrial functions (Hansson Petersen et al, 2008; Kaminsky et al, 2015; Lustbader et al, 2004; Mossmann et al, 2014). Nevertheless, the molecular mechanisms behind the accumulation and the effects of Aβ peptides on mitochondria need a critical analysis and clarification. For this reason, we elucidated the biochemistry of

the interaction between the two Aβ peptides species relevant to AD (Aβ40 and Aβ42) with human mitochondria. One of the major cellular processes responsible for maintaining mitochondrial functions is the import of nuclear-encoded mitochondrial precursor proteins from the cytosol (Chacinska et al, 2009). In order to check if and how Aβ peptides directly interfere with the mitochondrial protein import reaction, we utilized an established import assay with isolated intact mitochondria (Ryan et al, 2001). Taken together, our results show a strong and direct inhibitory effect of Aβ peptides on mitochondrial protein biogenesis. This inhibition is not caused by a damaging influence of Aβ peptides on mitochondrial functions, but is correlated to an extra-mitochondrial aggregation phenomenon between Aβ peptides and precursor proteins that severely restricts their import competence.

Results

Aβ peptides interfere with the import of mitochondrial precursor proteins

The import of precursor proteins, synthesized at cytosolic ribosomes, represents a crucial process in maintaining mitochondrial function and activity. In order to test a direct effects of Aβ peptides on mitochondrial protein import, we utilized an established *in organello* assay system that measures the uptake of radiolabeled mitochondrial precursor proteins into intact mitochondria isolated from human cell cultures. This assay enables to directly follow the association, the uptake and the processing of precursor proteins into mitochondria (Chacinska et al, 2009; Ryan et al, 2001).

As precursor proteins, we used the following radio-labeled [^{35}S] polypeptides: a) mitochondrial malate dehydrogenase (MDH2), a key enzyme for the citric acid cycle; b) ornithine carbamoyltransferase (OTC) involved in the urea cycle; and c) Su9(86)-DHFR and d) Su9(70)-DHFR, both artificial, mitochondrially targeted fusion proteins, comprising the pre-sequence of the subunit 9 (Su9) of the F_1F_0-ATP synthase (86 and 70 AA respectively) from *Neurospora crassa* fused to the complete mouse dihydrofolate reductase (DHFR). All these precursor proteins contain an N-terminal pre-sequence that is cleaved by the mitochondrial processing peptidase (MPP) after the polypeptide reaches the matrix compartment. Their mitochondrial import depends on the membrane translocase complexes TOM (Translocase of the Outer Mitochondrial membrane) and TIM23 (Translocase of the Inner Mitochondrial membrane with the core component Tim23) and a functional inner membrane potential ($\Delta\psi_{mt}$) (Chacinska et al, 2009). In addition, we tested a precursor protein of the metabolite carrier family, the adenine nucleotide translocator 3 (ANT3). This protein is constituted by highly hydrophobic transmembrane subunits and lacks an N-terminal presequence. ANT3 is inserted into the inner mitochondrial membrane (IMM) and its import uses a distinct pathway that depends on the TOM and TIM22 complexes (Truscott et al, 2002).

In our import assay, we used the most relevant Aβ peptides found in AD cases, constituted by 40 (Aβ40) and 42 (Aβ42) amino acids. We prepared the Aβ peptides according to a protocol optimized by Stine et al (Stine et al, 2003). The Aβ peptides and the radiolabeled precursor protein were incubated together with energized human mitochondria isolated from cultured HeLa cells lines. After the import incubation, samples were treated with proteases to digest residual non-imported polypeptides represented by the precursor form (*p*), and leaving the completely imported and processed mature form (*m*). Then, import reactions were analyzed by tricine SDS-PAGE and Western blot followed by autoradiography to detect the ^{35}S-labeled imported polypeptides, while the presence of Aβ peptides was detected by immunodecoration with a specific antibody against Aβ. As ANT3 does not contain a N-cleavable presequence and is not processed in the matrix, complete import was analyzed by blue-native gel electrophoresis (BN-PAGE) indicating the formation of a dimeric complex after insertion into the inner membrane.

We found that Aβ peptides strongly interfered with the mitochondrial import of all precursor proteins analyzed (Figure *1*). The two Aβ peptides showed a different

degree of inhibitory effect. Using the same concentration, Aβ40 partially inhibited the import reaction (Figure *1A*), while Aβ42 completely inhibited it (Figure *1B*) as indicated by the absence of the mature (*m*) form of a fully imported and processed precursor protein. ANT3 import was analyzed by BN-PAGE to visualize the $\Delta\psi_{mt}$-dependent formation of the inner membrane dimeric complex around 148 kDa (Figure *1C*, lane *1*). Also in this case, Aβ peptides were able to inhibit to different extent the complex formation and therefore ANT3 import. Again Aβ42 was more effective in inhibiting the import reaction compared to Aβ40. The inhibitory effect of the Aβ42 resulted in a full elimination of the generation of mature forms (m) as well as a complete protease sensitivity of the precursor protein (p) in the import reaction. Taken together, these two criteria indicate a full block of the mitochondrial translocation process and a general phenomenon affecting different import pathways.

In order to investigate the concentration-dependence of the inhibitory effect of Aβ peptides on mitochondrial import, we performed a titration of Aβ peptides amount during the [^{35}S]Su9(86)-DHFR import assay (Figure *1D* and Supplemental Figure *EV1*). After import, samples were digested by trypsin and analyzed by tricine SDS-PAGE, autoradiography and Western blot. We quantified the protease-resistant mature form (*m*) of the imported [^{35}S]Su9(86)-DHFR. We found that the inhibitory effect of Aβ42 was about ten fold stronger than Aβ40 (Figure *1D*). Inhibition of import by Aβ42 started at a concentration of about 0.1 μM, while for Aβ40 a concentration of more than 1 μM was required. It should be noted that only at the highest concentration, the Aβ40 band was detectable also in the mitochondrial fraction (Supplemental Figure *EV1*).

Aβ peptides do not interfere with general mitochondrial functions

Since it was previously reported that *in vitro* Aβ peptides exert direct damage on mitochondria (Hansson Petersen et al, 2008; Lustbader et al, 2004; Mossmann et al, 2014), we assayed the state of specific import-related mitochondrial functions in our experimental setup. An electric potential across the mitochondrial inner membrane ($\Delta\psi_{mt}$) is indispensable for the mitochondrial import of precursor proteins into the matrix as well as the insertion into the inner membrane (Ryan et al, 2001). We measured the $\Delta\psi_{mt}$ in our model by the potential-dependent accumulation of the fluorescent dye tetramethylrhodamine-ethyl ester (TMRE) after incubation of isolated and energized mitochondria with increasing amounts of Aβ peptides (Figure *2A*). Both Aβ40 and Aβ42 did not exhibit any effect on $\Delta\psi_{mt}$, even at high concentrations. As negative control, we incubated the mitochondria with 0.5 μM of valinomycin that causes a complete dissipation of the membrane potential and a concomitant strong reduction of the fluorescence signal. Using native conditions (BN-PAGE), we inspected the structure and the composition of translocase complexes responsible for the import reaction. In the BN-PAGE, the translocase complexes of both the outer membrane (TOM) and the inner membrane (TIM23) migrate as distinct high-molecular weight bands. Incubations with both Aβ peptides did not have any visible effect on the running behavior of the translocase complexes, indicating no significant change in structure and composition (Figure *2B*). Furthermore, the absence of effects in the native PAGE indicated that there is no significant stable interaction between the mitochondrial import complexes and Aβ peptides themselves. It should be noted that in the Western blots of the BN gels (Figure *2B*), a signal localized in the upper part of the stacking gel appeared for Aβ42, but not for Aβ40 consistent with a formation of high molecular weight aggregates. Additionally, we also checked the running behaviors of the five respiratory chain complexes of the inner membrane in native PAGE and again found no significant differences caused be the presence of Aβ peptides (Supplemental Figure *EV2*). These results demonstrated that Aβ peptides did not negatively affect mitochondrial activities that are directly relevant for the import reaction. In line with this, resistance of mitochondrial control proteins against Proteinase K (PK) treatment after import also suggests that mitochondrial

membranes remained largely intact after Aβ treatment.

Aβ peptides affect the initial steps of the mitochondrial import reaction

Based on the observation of a significant inhibition of the overall import process, we set out to identify the particular step of the import reaction that was affected by Aβ peptides. Most cases of the precursor protein import can be generally distinguished into three steps: a) binding to the receptors of the import machinery of the outer mitochondrial membrane (OMM); b) $\Delta\psi_{mit}$-dependent transport through the membranes via the translocase complexes; c) processing of the precursor to the mature form. To investigate the effect of Aβ peptides on the initial step of the import reaction, we dissipated the $\Delta\psi_{mit}$ as an import driving force, allowing only binding of precursor proteins to OMM import receptors and/or insertion into the TOM translocase channel. As the OMM binding reaction is very quick, we incubated the isolated mitochondria with the radioactive precursor protein for short times (range of seconds) in presence of Aβ peptides and tested for a co-fractionation of the precursor polypeptides with the mitochondria. Both Aβ peptides did not negatively affect the binding between the precursor protein [^{35}S]Su9(86)-DHFR (Figure *3A*) and the mitochondria, indicating that the interaction with the mitochondrial surface receptors was not affected. On the other hand, in particular with Aβ42, we consistently observed elevated amounts of precursor protein associated with mitochondria that are proportional to the amount of peptide used (Figure *3B*). Since also non-specific radio–active protein bands generated during *in vitro* translation in addition to the genuine precursor band were found in association with the mitochondrial pellet after centrifugation, the increase in signal intensity of the precursor protein is probably due to an aggregation phenomenon (see below).

Transport and processing reactions were tested utilizing a two-step protocol that separated the binding of the precursor from the actual translocation process. The precursor protein [^{35}S]Su9(70)-DHFR was first incubated with mitochondria where the $\Delta\psi_{mit}$ was dissipated by the addition of CCCP (1 μM). In this way, the precursor protein was able to bind to the TOM machinery without being imported. After removing excess unbound precursor proteins, $\Delta\psi_{mit}$ was restored by taking away the CCCP by binding it to excess amounts of albumin (BSA) and re-energizing the mitochondria, allowing the translocation and processing reaction to proceed. Interestingly, an inhibition of protein import was only observed when Aβ peptides were present already in the first step of the experiment, (Figure *3C*, lanes *11* and *12*). While adding the peptides directly in the second step, after the binding step has been completed, did not show any effect on the import reaction (Figure *3C*, lanes *17* and *18).* This directly demonstrated that Aβ peptides did not negatively affect the later phases of the import reaction, but rather interfered with the first steps of the import reaction that happen at the outer face of the OMM.

Interaction of Aβ peptides with human mitochondria

An association with mitochondria or even an import of Aβ peptides has been claimed already in previous publications (Hansson Petersen et al, 2008; Lustbader et al, 2004; Pagani & Eckert, 2011), although the underlying mechanism of interaction and functional consequences remained ambiguous. In our own experiments, we observed an apparent interaction between Aβ peptides with mitochondria in particular Aβ42 co-purifying with mitochondria more than Aβ40 suggesting a potential association. Since also the degree of import inhibition correlated with the amount of Aβ peptides co-purified with mitochondria, we checked if Aβ peptides maintain the same behavior even in absence of precursor protein. More in details, we pre-treated the isolated mitochondria with Aβ peptides for 30 minutes followed by different washing steps to remove excess unbound material. Then, we performed a normal import reaction using the precursor protein [^{35}S]Su9(86)-DHFR (Figure *4)*. Interestingly, a pre-treatment of mitochondria with Aβ40 did not significantly show any co-purification Aβ-mitochondria and did not affect a later import reaction. On the contrary, the pretreatment with Aβ42

showed a co-purification and a strong, although not complete, inhibitory effect on the import reaction. Furthermore, we were able to detect Aβ42 co-purifying with the mitochondria even after extensive washing, confirming an association with mitochondria.

We investigated in detail the biochemical properties of this association of Aβ peptides with isolated mitochondria. First, we performed a standard mitochondrial import experiment using Aβ peptides to clarify if they were taken up via the canonical import pathway. The import reaction was analyzed by tricine SDS-PAGE followed by Western blot using antiserum against Aβ peptides. As shown in Figure *5A,* the smaller peptide Aβ40 again did not show a significant co-purification with mitochondria even at longer incubation times. In contrast like seen before, with Aβ42, a band of 4 kDa was visible in the samples containing mitochondria already at very short time points (Figure *5B*). The band intensity did only slightly increase with longer incubation times. Due to the small size and the specific properties of the Aβ peptides, any processing event during the import reaction was not expected. However, for Aβ42 an additional band with a slightly higher molecular weight appeared in the presence of mitochondria, which is likely due to a different running behavior of the small peptide in presence of high amounts of mitochondrial proteins or lipids. However, two observations argue strongly against a specific uptake of Aβ peptides via the mitochondrial import machinery: a) the intensity of the co-purifying Aβ signal was not influenced by $\Delta\psi_{mit}$ (Figure *5B*, lane *11*) and b) both Aβ peptides showed a comparable signal also in the mock sample containing no mitochondria at all (Figure *5A* and *5B*, lanes *6* and *12*). Interestingly, both the co-purifying materials as well as the peptides in the mock samples were largely resistant to protease digestion (Figure *5A* and *5B*, lanes *1-6*).

As protection against proteases is a major hallmark of a successful mitochondrial import reaction (Ryan et al, 2001), we characterized the protease digestion behavior of Aβ peptides in more detail (Figure *6A*). We incubated the Aβ peptides with isolated and energized mitochondria followed by solubilization with 0.5% Triton X-100 (Figure *6A*, lanes *5-8*) or ultra-sonication (Figure *6A*, lanes *9-12*). Under these conditions, the mitochondrial membranes are disrupted and would not be able to offer protection against external proteases. A titration with rising amounts of trypsin was performed and then all the samples underwent trichloroacetic acid (TCA) precipitation, tricine SDS-PAGE and detection of present Aβ peptides by western blotting. As showed in control panels, both detergent- and sonication-lysis of mitochondria were successful as endogenous control proteins were efficiently degraded even at the lowest concentration of trypsin (5 µg/ml). In the mock samples, without mitochondria and used as control, we again found a significant protease resistance of both Aβ peptides (Figure *6A*, lanes *1-4*). The protease resistance of both Aβ peptides was decreased in presence of detergent or after ultrasound treatment (Figure *6A*, lanes *6-8* and *10-12*). Aβ42 was found slightly more resistant than Aβ40 after detergent lysis, but remained resistant to trypsin after ultrasound treatment. In presence of mitochondria, the behavior of the two peptides was different. As Aβ40 did not co-purify or pellet with mitochondria, the analysis of Aβ40 susceptibility to protease digestion was not possible. In contrast, Aβ42 showed some co-purification with the mitochondria and also a complete protease resistance that was neither affected by the presence of detergent nor by sonication. This specific intrinsic protease resistance and the band of Aβ peptides still visible in samples without mitochondria (mock) or even after destruction of mitochondrial membranes and proteins suggest that in our experimental setup Aβ peptides are more prone to form sedimentable aggregated material than to associate with the OMM.

The import of nuclear-encoded precursor proteins initially requires a specific interaction with receptor proteins at the surface of the OMM (Endo & Kohda, 2002). To analyze if the interaction of Aβ peptides with mitochondria depends on the involvement of the OMM receptors, we pre-treated isolated intact mitochondria

with trypsin to digest any protein domains exposed on the cytosolic face of the outer membrane. Then, we incubated the mitochondria with Aβ peptides (Figure *6B*). Samples were analyzed by tricine SDS-PAGE followed by Western blot. As control, Tom20 was degraded at the lowest trypsin concentration (5 µg/ml), while the inner membrane protein Tim23 was stable during both protease treatments indicating the intactness of mitochondria. The co-purified amount of Aβ42 with mitochondria did not show any difference between trypsin pre-treated mitochondria versus untreated control samples, indicating that any potential interaction of Aβ42 with mitochondria is not based on a specific binding to the import-related receptor proteins of the TOM complex.

The previous experiments suggest that the association of Aβ peptides with mitochondria rather represents a non-specific interaction with the OMM. We performed an alkaline extraction to assess the membrane interaction properties after incubating Aβ peptides with mitochondria, (Figure *6C*). During alkaline extraction, polypeptides that stably associate with membranes remain in the pellet fraction (*P*), while peripheral membrane proteins are found in the supernatant (*S*). As shown before, Aβ40 did not show a significant signal in presence of mitochondria. However, the mock samples showed that minor amounts of Aβ40 accumulated in the pellet fraction consistent with a generation of small amounts of protein aggregates. The Aβ42 peptides showed a similar behavior in the mock samples. However, in the presence of mitochondria, a significant amount of co-purified material was found in the supernatant fraction excluding integration into the OMM, suggesting at most a peripheral association. The mitochondrial control proteins MPP (soluble) and Tom40 (membrane-integrated) behaved as expected. A non-specific interaction with the OMM, in particular for Aβ42, was also supported by a saturation titration experiment (Figure *5C* and *5D*). Here, we incubated increasing amounts of Aβ peptides with a constant amount of mitochondria and separated soluble and insoluble material by intermediate-speed centrifugation. Increasing the peptide concentration, most of the Aβ40 peptide remained in the supernatant and only a minor amount appeared in the pellet fraction (Figure *5C*) without being influenced by the presence of mitochondria. On the other hand, significant amounts of Aβ42 peptides accumulated in the pellet fraction, both in presence or absence of mitochondria (Figure *5D*). In both cases, the amount of Aβ42 peptides recovered in the pellet fractions did not seem to be saturable, indicating again a non-specific mitochondrial association as well as a pronounced tendency to form sedimentable aggregate material.

From the results above, it was not possible to clearly distinguish between Aβ peptides associated to the OMM and Aβ peptides prone to aggregation that are able to sediment with mitochondria by conventional differential centrifugation methods used in a standard import assay. Thus, we decided to analyze the behavior of Aβ peptides during the mitochondrial import using a specific rate-zonal centrifugation method. Using sucrose gradients (20-50%) the particles are separated by their size and density. After performing an import reaction of precursor protein [^{35}S]Su9(70)-DHFR in presence or absence of Aβ peptides, samples were separated by centrifugation through the sucrose gradient. Fractions from top to bottom were collected and analyzed by Western blot or autoradiography for the presence of the imported precursor protein or Aβ peptides. As controls, we carried out the same experiment in the absence of mitochondria (mock) or in the absence of Aβ peptides (Figure *7B*). From the sedimentation behavior of mitochondrial marker MPP and Tim23, isolated mitochondria were concentrated mostly around the middle of the gradient (Figure *7*, fractions *12-14*). Most of Aβ40 accumulated as monomer or as small, low density and SDS-soluble aggregates at the top of the gradient and no co-sedimentation with the mitochondria was observed (Figure *7A*, upper panels). This observation is consistent with the behavior in the differential centrifugation experiments previously reported. However, Aβ42 behaved significantly different (Figure *7A*, middle panels). In presence of

isolated mitochondria, a small percentage of Aβ42 was found in the gradient fractions together with the mitochondrial markers, suggesting a direct interaction with mitochondria. In the mock samples, most of Aβ42 accumulated on the top of the gradient like Aβ40. In the control import containing only the precursor protein, [^{35}S]Su9(70)-DHFR showed a localization of the mature form (*m*) in the same fractions as the bulk mitochondria (Figure *7B*). As expected, in presence of Aβ40 the amount of mature form was partially reduced (Figure *7C*), while Aβ42 treatment resulted in a complete disappearance of the mature form, demonstrating again a complete inhibition of mitochondrial import (Figure *7D*). Interestingly, in presence of the precursor protein, the amount of Aβ42 bound to the mitochondria was strongly reduced and a band in the bottom of the gradient is appearing for both Aβ42 and [^{35}S]Su9(70)-DHFR (Figure *7D*, lane *23*).

Taken together all these data suggest that there is a differential behavior of the two Aβ peptides concerning their interaction with mitochondria. Under the experimental conditions used, Aβ40 did not show a significant interaction with mitochondria and also only a small aggregation propensity was detected. In contrast, Aβ42 exhibited a small but significant non-specific association with the mitochondrial surface and also a significant tendency to form aggregate assemblies. Interestingly, in presence of mitochondrial precursor proteins, the association of Aβ42 with the mitochondria was reduced together with an increased formation of potential sedimentable preprotein-Aβ42 co-aggregates.

Preprotein import competence is reduced by the formation of Aβ-preprotein co-aggregates

As aggregate formation is a pathological intrinsic property of Aβ peptides (Thal et al, 2015), we reasoned that the induction of preprotein aggregation and the reduction of their solubility in presence of Aβ peptides might significantly contribute to the inhibitory effect on the import reaction. We therefore analyzed the co-aggregation by three types of assays: a) high-speed centrifugation followed by tricine SDS-PAGE, b) filter retardation assay, and c) BN-PAGE. These techniques provide direct information about the aggregation behavior of precursor polypeptides in the presence of the Aβ peptides and partially characterize the nature of the aggregates. After incubation of radiolabeled precursor proteins with Aβ peptides, samples were centrifuged at high speed (45000 rpm; 124500 xg) to separate the insoluble high-molecular weight aggregates from the soluble proteins. The resulting pellets and supernatants were analyzed by Western blot and immunodecoration against Aβ peptides, as well as autoradiography to detect the precursor polypeptides (Figure *8A*). The precursor protein alone partially fractionated to the pellet suggesting an intrinsic aggregation propensity (Figure *8A*, lanes *7* and *17)*. However, in presence of rising concentrations of Aβ42, the amounts of [^{35}S]Su9(86)-DHFR found in the pellet was significantly increased (Figure *8A*, lanes *18-20)*. In contrast, Aβ40 had less severe effects on the distribution of precursor polypeptides in the centrifugation assay (Figure *8A*, lanes *8-10*) where most precursor protein remained soluble in the supernatant (Figure *8A*, lanes *3-5*). Aβ42 itself was mostly found in the pellet fraction suggesting a strong propensity to form insoluble aggregates (Figure *8A*, lanes *16, 18-20*). In the pellet fraction, but not in the supernatant, an additional band was detected for Aβ42 at the top part of the PDVF membrane corresponding to the loading pockets of the tricine gel. This suggested that Aβ42 formed high-molecular weight aggregates that were insensitive to SDS solubilization. For Aβ40, part of the peptides sedimented as insoluble aggregates (Figure *8A*, lanes *6,8-10*) and part remained soluble in the supernatant (Figure *8A*, lanes *1,3-5*). In the supernatant fraction, Aβ40 showed two bands around 20 kDa and 35 kDa in addition to to the predominant band at 4 kDa (Figure *8A*, lanes *3* and *4)*. These bands were present only when Aβ40 was incubated with the precursor proteins, but not with the peptides alone. Similar bands were also detected with Aβ42, but in much lower amounts (Figure *8A*, lanes *12* and *13*).

In the filter retardation assay, different amounts of Aβ peptides were incubated

with the [^{35}S]Su9(86)-DHFR (Figure *8B)* or [^{35}S]OTC (Supplemental figure *EV3*) and subsequently filtered through nitrocellulose or cellulose acetate membranes. With the cellulose acetate membrane, which does not have an intrinsic protein binding affinity, inclusions or aggregates bigger than 0.2 µm are trapped under these conditions, while the smaller complexes pass through and are washed away (Heiser et al, 2000). As most of the added protein should be retained on a nitrocellulose membrane, this type of membrane was used as loading control. Precursor proteins were detected by autoradiography and the presence of Aβ peptides by immunodecoration. The total amount of retained polypeptides was also evaluated by Ponceau red staining of the membranes. As expected from their intrinsic aggregation propensities, Aβ42, but not Aβ40, showed a signal on cellulose acetate membranes, when similar concentrations were loaded (Figure *8B*). While the precursor protein [^{35}S]Su9(86)-DHFR alone showed a light signal on cellulose acetate membrane, a strong signal was detected when it was incubated together with Aβ42 (Figure *8B*). The formation of the precursor protein aggregates increased with the amount of Aβ42 peptides added. [^{35}S]OTC showed a similar behavior (Supplemental Figure *EV3*).

We also applied the samples on BN-PAGE to characterize the complexe formation between Aβ peptides and precursor proteins under native condition. After incubation of the [^{35}S]Su9(86)-DHFR with different concentrations of Aβ peptides, the complete samples were separated by BN-PAGE gradient gel (5-16.5%) and then analyzed by Western blot and autoradiography. The precursor protein [^{35}S]Su9(86)-DHFR alone distributed over a large size range without forming a defined band, a typical behavior for a soluble protein in native PAGE (Figure *8C*, lanes *2* and *9*). In presence of Aβ40, some of the precursor proteins shifted to a higher molecular weight zone of the gel in a concentration-dependent manner (Figure *8C*, lanes *3-7*). In presence of Aβ42, the signals of the precursor protein almost exclusively shifted to an area around 720 kDa (Figure *8C*, lanes *10-13*). Interestingly, the immunodecoration with anti-Aβ serum showed that some Aβ42 material accumulated at the same molecular weight range (Figure *8C*, lanes *10* and *11*). In addition, Aβ42 also exhibited a signal at the highest part of the membrane related to the loading pockets in the gel, representing large insoluble aggregate material (Figure *8C* lanes *8, 10-12*). The fact that in native conditions the precursor protein band together with Aβ42 band shifted to the same area strongly suggests a direct interaction between the precursor protein and Aβ42. The large size of the complex, comprising multiple copies of both molecules was consistent with the formation of Aβ42-preprotein co-aggregates.

Taken together, the data obtained from three different technical approaches clearly confirmed a co-aggregation phenomenon between the precursor proteins and Aβ peptides that reduced the precursor proteins solubility. As solubility of the precursor proteins is a requirement for an efficient mitochondrial import, a formation of co-aggregates between the precursor proteins and Aβ peptides interferes with the insertion of the precursor protein inside the TOM channel. This represents the initial step of an import reaction that was found defective in our experiments in presence of Aβ peptides. Notably, the two Aβ peptides analyzed showed different effects on co-aggregate formation, correlating well with the observed preprotein inhibition efficiency, their aggregation propensity and also the pathological impact in AD patients.

Discussion

An intracellular localization together with their intrinsic physicochemical properties encourages Aβ peptides to interact with organelles such as mitochondria. Indeed, it was previously observed that Aβ peptides a) localize to mitochondria from postmortem AD brains and from several experimental models of the disease (Pagani & Eckert, 2011), b) physically interact with some mitochondrial components (Lustbader et al, 2004), and c) exert harmful effects on mitochondrial function (Kaminsky et al, 2015). Interestingly, unlike plasma membrane,

endoplasmic reticulum (ER), trans-Golgi network and endosome-lysosome system, mitochondria are completely deprived of the amyloid precursor protein (APP) and the metabolic enzymes responsible to release Aβ peptides from the precursor (Sannerud & Annaert, 2009). As an *in situ* production of Aβ peptides in mitochondria themselves seems biochemically unlikely, our study addressed the possible mechanisms of Aβ peptide interaction with mitochondria as well as the correlation between a mitochondrial localization of Aβ peptides and the mitochondrial dysfunctions observed in AD.

Under *in organello* conditions, we observed a clear-cut and strong inhibitory effect of Aβ peptides on mitochondrial import. The inhibitory effect of the Aβ42 was significantly stronger than the related Aβ40, correlating well with the stronger pathogenic effect of Aβ42 in human AD patients (Eckman & Eckman, 2007). Notably, the lowest Aβ42 concentration that resulted in a significant inhibition of mitochondrial import was comparable to the concentration of the peptide that have previously found in AD brains (2 μM for Aβ42 and 200 nM for Aβ40 (Roher et al, 2009). Our experiments also shed a light on the biochemical details of the inhibitory mechanism, in particular which stage of the import process was affected. The inhibitory effect occurred immediately and did not require a prolonged preincubation period. Although previous publications reported that a treatment of mitochondria with Aβ peptides resulted in a reduction of the $\Delta\psi_{mit}$ (Kaminsky et al, 2015), in our model system we did not observe any changes in $\Delta\psi_{mit}$ in the time-frame of the import experiments, excluding an Aβ-related reduction of the membrane potential as a cause for the import inhibition. Neither did we observe changes in the size and composition of the precursor protein translocase complexes in the outer or the inner membrane (TOM and TIM) that are responsible for the precursor protein translocation reaction. Similarly, also the metabolic complexes of the respiratory chain were not affected. The possibility of a direct physical damage on mitochondrial membranes, the oxidative phosphorylation system or the preprotein import machinery by Aβ peptides is very unlikely.

Up to date only scarce information is available about direct effects of Aβ peptides on the mitochondrial protein biogenesis process. Using flow cytometry, it was demonstrated that after long-term exposure to Aβ peptides, differentiated PC12 cells exhibited a reduction of newly synthesized mitochondrially-targeted GFP (Sirk et al, 2007). These results are generally in line with our observations, however, due to the long exposure to potentially toxic molecules, these experiments could not distinguish if the import inhibition was a direct or indirect consequence of the presence of Aβ peptides. The immediate inhibitory effect of Aβ peptides on the import reaction in healthy mitochondria, as observed in our experiments, essentially rules out that the inhibition was caused indirectly by a long-term accumulation of functional defects in the affected mitochondria. A single previous study also used isolated mitochondria pretreated for short time with Aβ peptides, but did not detect a deficiency of the mitochondrial import (Hansson Petersen et al, 2008). Considering the concentration-dependency of Aβ peptides effect on mitochondrial import, the discrepancy between our results and the previous results could be explained only by the amount of Aβ peptides used. Indeed, in these experiments a concentration of Aβ peptides around 0.1 μM was used that were not sufficient to observe a significant import inhibition according to our observations. Interestingly, a defect in mitochondrial protein biogenesis as a potential cause for neurodegenerative disorders was also observed in other pathological situations. It was observed that a mutant form of the protein huntingtin (HTT), involved in Huntington's disease (HD), partially inhibited mitochondrial import through a physical association with TIM23 translocase complexes and lead to neuronal death in a HD mouse model. The import inhibition was concentration dependent and the concentration of the huntingtin used was comparable to the Aβ peptides concentration used in our model (Yano et al, 2014).

It should be noted that an alternative mechanism of AD-related inhibition of mitochondrial precursor protein import had been suggested previously (Anandatheerthavarada et al, 2003; Devi et al, 2006). Here, the precursor protein of Aβ peptides, APP, was shown to interact with the TOM complex and to undergo an incomplete translocation reaction (*in organello* model). The authors suggested that APP would thereby block the translocation of other authentic precursor proteins resulting in the development of mitochondrial dysfunction. However, the significance of this possibility is unclear as APP is typically localized in the cell as an integral membrane protein in the plasma membrane, endosome and ER. Although some mistargeting of ER proteins to mitochondria cannot be excluded, the overall probability would be very low and therefore unlikely to results in a major functional defect.

A recent study proposed that Aβ peptides indirectly interfered with the processing of imported precursor proteins to the mature and active forms (Mossmann et al, 2014), which is an important late step of the mitochondrial import reaction. The proposed model was based on a report that Aβ peptides are degraded by PreP, a peptide-degrading enzyme in the mitochondrial matrix (Falkevall et al, 2006). The authors claimed that an inhibition of PreP (or its yeast homolog Cym1) by Aβ peptides (Alikhani et al, 2011) would result in the accumulation of prepeptides in the mitochondrial matrix that in turn would interfere with the activity of the processing peptidase MPP, required for the maturation of mitochondrial precursor proteins. Eventually this would lead to an accumulation of non-functional mitochondria as observed in AD. This is in strong contrast to our study that showed that Aβ peptides acted on an early step of the import reaction. Two observations from our study directly argue against a mitochondrial processing defect caused by Aβ peptides. A) The precursor form visible in import experiments after Aβ peptide inhibition was always sensitive to digestion by external proteases, indicating that the preproteins never crossed the mitochondrial membranes, consistent with a complete translocation defect. B) Using two-step import experiments, which separated the binding from the translocation and processing reaction, we observed an inhibitory effect of Aβ peptides only in the first step that is independent of the membrane potential, but not in the second translocation step into the matrix that would also comprise the processing reaction. Although Mossmann et al. found an impaired precursor protein processing activity in presence of Aβ peptides using soluble mitochondrial extracts from yeast as well as in total brain extracts from PS2APP mice, a murine model of AD, the relevance of the claimed processing inhibition for the *in vivo* situation is questionable. In addition to the use of soluble extracts instead of intact organelles, very high concentrations of Aβ peptides (10 μM) were utilized in these experiments to result in any significant processing inhibition. Mossmann et al. also observed a very minor accumulation of precursor polypeptides after cellular expression of Aβ in intact yeast cells and also in brain extracts from AD patients. However, as a cytosolic accumulation of unprocessed precursor forms is the typical hallmark of a defective overall import process instead of just a faulty processing reaction, these observations are even consistent with our results of a direct inhibitory Aβ peptides effect.

Despite any obvious deleterious effects on mitochondrial functions, we observed that a pretreatment of intact mitochondria with Aβ42 (but not Aβ40) resulted in a later reduction of preprotein import efficiency even when the Aβ peptide was removed, albeit not a complete inhibition when present during the import incubation per se. This indicated that at least some of the Aβ42 peptide would be able to interact and bind to mitochondria. Although previous experiments indicated a specific and complete import of Aβ peptides into mitochondria (Hansson Petersen et al, 2008), we revisited this question by analyzing the biochemical properties of the interaction of Aβ peptides with isolated and energized mitochondria. Considering that Aβ peptides lack the typical properties of mitochondrial targeting sequences, it is questionable if a specific interaction or

even an uptake by mitochondria might take place. Nevertheless, also in our experiments, Aβ42 exhibited some co-sedimentation with mitochondria during differential centrifugation typically used to re-isolate mitochondria after an import experiment. In contrast, the shorter Aβ40 peptide did not show a significant association with mitochondria in all used assays. In addition, the co-sedimenting Aβ42 showed some degree of resistance against added proteases. Superficially, both observations might argue for a successful import reaction.

However, our experimental results clearly show that both Aβ peptides are not imported into mitochondria because they do not completely satisfy the required criteria of mitochondrial import reaction. Most importantly, the sedimentation of Aβ42 was largely maintained in the absence of mitochondria (mock samples), correlating with its intrinsic tendency to form aggregates. As the removal of outer membrane protein components by a protease pre-treatment did not change the co-sedimenting amount of Aβ42, we exclude any specific interaction between Aβ peptides and proteinaceous components of the OMM, in particular the cytosol-exposed receptors of import machinery. In addition, the amount of co-purifying Aβ42 with isolated mitochondria was dependent on the peptide concentration and did not seem to be saturable, again arguing against a protein-mediated interaction. Aβ peptides behavior in an import reaction did not show any dependence on $\Delta\psi_{mit}$ like for precursor proteins destined to matrix and IMM. Alkaline extraction experiments also indicated a peripheral membrane association. All together these results exclude a complete import of Aβ peptides, but not a peripheral association between Aβ peptides with the OMM.

Our observations of an apparent protease-resistance are independent from the presence of mitochondria and are linked to intrinsic properties of Aβ peptides rather than representing imported protein material. Indeed, in mock samples as well when mitochondria were destroyed by detergent solubilization or by mechanical disruption, the Aβ42 band was still visible even at the highest concentration of proteases. Also in mock samples, Aβ40 showed a similar pattern. In line with our results are data from the literature showing that both Aβ peptides extracted from AD brains as well as synthetic Aβ peptides spiked into brain homogenates acquired detergent-insolubility and resistance to protease digestion (Xiao et al, 2014). Furthermore, it was found that Aβ conformers with the highest amyloidogenic capability and with high content of beta-sheet structure were more resistant to proteolytic digestion (Soto & Castano, 1996). Our experiments indicated that the presence of mitochondria promoted both aggregation propensity and protease-resistance of Aβ42. These results are supported by the literature (Murphy, 2007) (Henry et al, 2015), but further investigation is needed to explore the consequences of this observation.

In order to overcome the technical problems of differential centrifugation as an analysis of Aβ peptide interaction with mitochondria, we utilized density gradient centrifugation as a method to separate protein aggregates from cell organelles like mitochondria (Sehlin et al, 2012). In these gradients, we observed ca. 20% of the total Aβ42 added to the experiment in the intermediate fractions, indicating a direct association with mitochondria (Figure *7E*). In contrast, Aβ40 remained in the top fractions probably as monomers or small SDS-soluble aggregates. Interestingly, the presence of precursor proteins changed the behavior of Aβ42 as the amount of mitochondria-associated material decreased while the amount in the bottom fractions, representing aggregates increased. Additionally, in the presence of Aβ42 a considerable amount of the precursor protein itself was found in the aggregate fraction at the bottom of the gradient, indicating the formation of co-aggregates between Aβ peptides and mitochondrial precursor proteins. We propose that a co-aggregation of precursor proteins and Aβ peptides is the main reason for the strong inhibitory effect of mitochondrial protein import. A formation of high molecular weight aggregates and the concomitant reduction of the precursor solubility would significantly reduce their import competence. Several further observations support this co-aggregation

model: a) correlating with the much stronger import inhibitory effect of Aβ42 compared to Aβ40, also the co-aggregation phenomenon was particularly pronounced in presence of Aβ42; b) The solubility of the precursor proteins was reduced in presence of Aβ42 as assayed by a centrifugation assay; c) together with Aβ42, precursor proteins formed large aggregates that are retarded in a filtration assay; d) in native PAGE experiments, precursor protein signals were shifted to a high molecular weight complex in the range of 700 kDa that co-purified with Aβ42. The aggregation behavior of precursor proteins was dependent on Aβ peptide-concentration, supporting the concept of co-aggregation. Interestingly, recent results showing negative consequences of co-aggregation between cytosolic enzymes and Aβ peptides support this AD-specific pathological mechanism. A co-aggregation between glycolytic glyceraldehyde-3-phosphate (GAPDH) and Aβ peptides accelerated amyloidogenesis and promoted mitochondrial dysfunction as well as cell death *in vitro* and *in vivo* (Itakura et al, 2015). Our work therefore adds an important aspect concerning the deleterious consequences of Aβ co-aggregation reactions during the etiology of neurodegenerative diseases. Many of amyloid diseases involve co-aggregation of different protein species (Penke et al, 2012; Sarell et al, 2013), although the pathological mechanisms are not always entirely clear. It is conceivable that amyloidogenic β-sheet peptides interact with many different endogenous proteins leading to sequestration and functional impairment (Olzscha et al, 2011).

Generally, Aβ peptides have an intrinsic tendency to self-assemble into a range of different aggregates also under the conditions that we applied in our mitochondrial import assay (Snyder et al, 1994; Stine et al, 2003; Thal et al, 2015). Considering the intracellular space as a crowded environment, Aβ peptides likely undergo multiple, largely non-specific interactions with any protein and lipid components of the cytosol. The import-competent state of mitochondrial pre-proteins is represented by an incompletely folded conformation that is prone to irregular interactions with Aβ peptides and subsequent aggregation. Already during the onset of the disease at the point at which the concentration of Aβ peptides is increasing, the formation of co-aggregates with newly synthesized mitochondrial precursor polypeptides might progressively interfere with the import process. This would eventually result in a reduction or even loss of mitochondrial enzyme activities, in turn leading to the multitude of mitochondrial defects observed in AD patients and respective disease models (Wang et al, 2007). Hence, the observed strong inhibitory effect on mitochondrial protein import, in particular in case of the pathogenic Aβ42, strongly supports the hypothesis of a direct mitochondrial toxicity of Aβ peptides on mitochondria in AD.

Material and Methods

Preparation of Aβ peptides and mitochondrial treatment

The *Escherichia Coli* expressed human recombinant Aβ peptides 1-40 (Ultra Pure HFIP; cat. A-1153-2) and 1-42 (Ultra Pure HFIP; cat. A-1163-2) used in this study were purchased from AJ Roboscreen GmbH (Leipzig, DE). Working solutions of both peptides were prepared as described (Stine et al, 2003). Briefly, the lyophilized peptides were dissolved in 100% 1,1,1,3,3,3-Hexafluoro-2-Propanol (Sigma-Aldrich) and distributed in low-binding micro-centrifuge tubes (VWR, DE). The solvent was allowed to evaporate over night at room temperature and the Aβ peptide aliquots were stored at -80°C. Immediately prior to use, each aliquot was warmed to room temperature followed by a resuspension of the peptide film to a stock of 5 mM in dimethyl sulfoxide (AppliChem GmbH, DE) to remove any preexisting aggregated structures and to provide a homogeneous non-aggregated peptide preparation. After mixing well, the Aβ peptide DMSO stock was freshly diluted with ice-cold distilled water to a final concentration of 100 µM. This dilution was mixed and used immediately. All experiments with Aβ peptides were performed in super-clear tubes (VWR, DE).

Cell culture and isolation of mitochondria

HeLa Cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a saturated humidity atmosphere containing 5% CO_2. All the chemicals were bought from Gibco, Life Technologies, DE. The mitochondria were isolated from HeLa cells as described (Becker et al, 2012). Briefly, after harvesting and washing in PBS, cells were incubated for 40 min on ice with HMS-A buffer (0.22 M mannitol, 0.07 M sucrose, 0.02 M HEPES pH 7.4, 1 mM EDTA, 0.2% BSA, 1 mM PMFS). Then, cells were homogenized with a glass/Teflon homogenizer (B. Braun Melsungen AG, DE) followed by differential centrifugation steps to isolated mitochondria. The mitochondria were washed and resuspended in HMS-B buffer (0.22 M mannitol, 0.07 M sucrose, 0.02 M HEPES pH 7.4, 1 mM EDTA, 1 mM PMFS).

Import of radiolabeled preproteins into isolated mitochondria

The import of radiolabeled precursor proteins was performed essentially as described (Becker et al, 2012). Radiolabeled preproteins were synthesized by *in vitro* transcription/translation using the mMESSAGE mMACHINE transcription kit (Life Technologies, DE) and rabbit reticulocyte lysate (Promega, DE) in presence of [^{35}S]-methionine-cysteine (PerkinElmer, DE). For the import reaction, mitochondria were diluted in import buffer (20 mM HEPES-KOH, pH 7.4, 250 mM sucrose, 5 mM magnesium acetate, 80 mM potassium acetate, 5 mM KPi, pH 7.4, 7.5 mM glutamate, 5 mM malate, 1 mM DTT, 2 mM ATP) to a final concentration of 50 µg/100µl. In mock samples, Aβ peptides were incubated under the same buffer conditions but without added mitochondria. Where indicated, mitochondrial membrane potential ($\Delta\psi_{mit}$) was dissipated by adding a mixture of 8 µM antimycin A (Sigma-Aldrich, DE), 0.5 µM valinomycin, and 2 µM oligomycin (Sigma-Aldrich, DE). All the import reactions were performed at 30 °C, stopped by addition of 50 µM valinomycin and placing the samples on ice. Non-imported, protease-accessible mitochondrial proteins were digested by incubation with 100 µg/ml trypsin (Seromed, Biochrom KG, DE) for 30 min on ice and terminated by adding 800 µg/ml of trypsin inhibitor (Sigma-Aldrich, DE) and 1 mM PMFS (Carl Roth, DE). Then, mitochondria were washed in import buffer without substrates. Where indicated, samples were treated with 25 µg/ml proteinase K (PK; Carl Roth, DE) on ice for 30 min before the addition of 1 mM PMSF. After centrifugation for 10 min at 12.000xg and 4 °C, mitochondrial pellets were analyzed by tricine SDS-PAGE, Western blot, digital autoradiography and immunodecoration.

For two-step import reactions, mitochondrial inner membrane potential $\Delta\psi_{mit}$ was first depleted with 1 µM carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Mitochondria were incubated with radiolabeled preprotein for 30 minutes at 30 °C. After washing, the mitochondria were re-incubated for 30 minutes at 30 °C in energized import buffer supplemented with 2 mg/ml BSA to restore the membrane potential in presence or absence of 3.5 µM Aβ peptides. After re-isolation of mitochondria, imported proteins were separated by tricine SDS-PAGE and detected by immunodecoration and digital autoradiography.

BN-PAGE

To analyze mitochondrial protein complexes and Aβ peptide aggregation states under native conditions, samples were analyzed by blue native (BN)-PAGE (Wittig et al, 2006). Isolated mitochondria, as well as co-aggregates containing Aβ peptides and radiolabeled preproteins were solubilized in BN-lysis buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1% digitonin, 1 mM PMFS). BN gel loading buffer (100 mM Bis-Tris, pH 7.0, 500 mM ε-amino-n-caproic acid, 5% w/v Coomassie Brilliant Blue G250) was added and the samples were loaded on 5-16.5% BN gels. Native unstained protein standard (Novex, Life Technologies, DE) was used to estimate molecular weights of protein complexes. After running over-night, gels were equilibrated in SDS buffer (1% (w/v) SDS, 0.19 M glycine, 25 mM Tris) and blotted on PDVF membrane (Carl Roth GmbH, DE)

followed by immunodecoration and digital autoradiography.

Sodium carbonate extraction

After incubation of isolated and intact mitochondria with 3.5 µM Aβ peptides, a further incubation in 0.1 M Na_2CO_3 solution (pH 11) was performed on ice for 30 min. Then, after withdrawal of a total sample, an ultra-centrifugation step was done in a Beckman TLA-55 at 45000 RPM (123,000 xg) for 40 min at 4 °C. The pellets were resuspended in tricine sample buffer while the supernatants were precipitated with 72% trichloroacetic acid (TCA) followed by tricine SDS-PAGE, western blot and immunodecoration.

Sucrose density gradient centrifugation

After incubation with Aβ peptides (35 µM) and/or [^{35}S]Su9(70)-DHFR, isolated mitochondria and mock samples were loaded on a continuous sucrose gradient (25-50%) and centrifuged in a Beckman SW41 rotor at 33,000 rpm (135,000 xg) for 1h at 4° C. Then, fractions of 500 µl were collected from the top of each gradient followed by 72% TCA precipitation. Protein pellets were re-suspended in tricine loading buffer, separated by tricine SDS-PAGE and analyzed by Western blot and immuno-decoration.

Membrane potential measurement in isolated mitochondria

Mitochondrial membrane potential ($\Delta\psi_{mit}$) was analyzed by potential-sensitive fluorescent dye tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Invitrogen, DE). After incubation with Aβ peptides, isolated mitochondria were resuspended in potential buffer (0.6 M sorbitol, 0.1% BSA, 10 mM $MgCl_2$, 20 mM KPi, pH 7.2, 5 mM malate, 10 mM glutamate) and incubated with 1 µM of TMRE for 30 min at 30 °C on ice. After washing away the excess of TMRE, the TMRE fluorescence was measured in a microplate reader (excitation 540 nm, emission 585 nm; Infinite M200 PRO, TECAN, DE).

Filter retardation assay

To visualize the formation of aggregates and co-aggregates, a modified filter retardation assay (Scherzinger et al, 1997) was used. After incubation of radiolabeled precursor proteins with different amounts of Aβ peptides for 30 min at 30 °C in energized import buffer, samples were filtered directly through cellulose acetate membrane (0.2 µm pore size; GE Healthcare, DE) or nitrocellulose membrane (GE Healthcare, DE) using a dot blot filtration unit (SCIE-PLAS, DE). Proteins retarded on the membranes were analyzed by immunodecoration and digital autoradiography.

Miscellaneous methods

All the chemicals using in this study were from Carl Roth GmbH or Sigma-Aldrich. Standard techniques were used for tricine SDS-PAGE, Western blot, and immunodecoration. After performing a tricine SDS-PAGE, samples were transferred on PVDF membrane (Carl Roth GmbH) followed by blocking in TBS/Tween (0.9% NaCl, 10 mM Tris/HCl pH 7.4, 0.25% Tween 20) with 5% milk and immunodecoration with andibodies appropriately diluted in TBS/Tween. Signal detection was performed by enhanced chemiluminence (SERVA Light Eos Ultra, Serva, DE). Used antibodies were: Aβ 6E10 (Covance SIG-39320); Tim23 (BD Bioscience 611222), Tom 20 (Santa Cruz SC-11415), Tom 40 (Santa Cruz SC-11414), SMAC (Santa Cruz SC-22766), MPP (Sigma-Aldrich HPA021648), Complex-I (Invitrogen 459100), Complex-II (Invitrogen 459200), Complex III (Santa Cruz SC-23986), Complex-IV (Cell Signaling 3E11), $F_1\beta$ (Invitrogen A21351), Rabbit IgG-Peroxidase (Sigma Aldrich A6154) and Mouse IgG-Peroxidase (Sigma Aldrich A4416). Digital autoradiography was performed using a FLA5100 phosphorimaging system (Fujifilm, DE). Quantitative analysis was done by ImageJ 64 (NIH, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc, USA).

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Author contributions

GC performed most of the experiments of this manuscript. CR performed the ANT3 import. GC and MB performed the sucrose density gradient experiments. GC and WV designed the study, supervised the experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. Effect of Aβ peptides on mitochondrial import of nuclear-encoded precursor proteins. [^{35}S]-labeled radioactive precursor proteins were incubated with energized and isolated mitochondria from HeLa cell cultures in presence of same amounts (3.5 µM) of Aβ40 and Aβ42 peptides. (**A**, **B**) Import of the precursor proteins mitochondrial malate dehydrogenase (MDH2), the artificial reporter construct Su9(86)-DHFR, and ornithine carbamoyltransferase (OTC) for the indicated incubation times. After the import reaction, half of the samples (*lanes 4-6* and *9,10*) were treated with trypsin (100 µg/ml) to remove non-imported preproteins. Imported proteins were analyzed by tricine SDS-PAGE followed by Western blot, digital autoradiography and immunodecoration against Aβ peptides. (**C**) Import of the adenine nucleotide translocator 3 (ANT3) in comparison with Su9(70)-DHFR. After import, all samples were treated with proteinase K (PK; 50 µg/ml) and analyzed either by BN-(ANT3) or SDS-PAGE (Su9(70)-DHFR), Western blot and digital autoradiography. As control, immunodecoration against Tim23 was carried out. (**D**) Quantification of import inhibitory effect of Aβ peptides. Import experiments with the precursor protein [^{35}S]-Su9(86)DHFR and different amounts of Aβ peptides (0.007 up to 7.0 µM) were performed as described above. The signals of processed and protease-resistant preprotein bands (*m-form*) were quantified using Image J. The amount of imported protein in the absence of Aβ peptide was set to 100%. Mean values and standard deviation (*S.D.*) were determined for n = 3 independent experiments. *p*, precursor protein; *m*, mature processed form; *L*, loading control; *WB*. Western blot.

Figure 2. Effect of Aβ peptides on import-related mitochondrial functions. (**A**) Mitochondrial membrane potential ($\Delta\psi_{mit}$) was evaluated after treatment of energized mitochondria with increasing amount of Aβ peptides as indicated, followed by incubation with the potential-dependent fluorescent dye TMRE. After removal of excess TMRE, fluorescence was determined by a spectrofluorometer (Infinite M200 Pro, TECAN). Mean values and standard deviation were determined from three independent experiments. (**B**) After treatment of isolated and energized mitochondria with Aβ peptides (3.5 µM), structure and composition of import translocase complexes were analyzed by BN-PAGE, SDS-PAGE, and western blotting techniques. Before loading, mitochondria were solubilized in a buffer containing 1% digitonin. Immunodecorations against components of the translocase complexes TOM and TIM23, responsible for the import of presequence-containing preproteins through the mitochondrial membranes, Tom20, Tom40, Tim23 (*lanes 1-6 and 9-14*) and Aβ peptides (*lanes 7,8 and 15,16*) were performed.

Figure 3. **Mitochondrial import steps affected by Aβ peptides.** (**A**) Binding of the precursor protein to the OMM import machinery receptors. After removing the $\Delta\psi_{mit}$, mitochondria were incubated for short time points (range of seconds) with Aβ peptides (3.5 µM) and precursor protein [^{35}S]-Su9(70)DHFR. Half of the samples were incubated with proteinase K (PK; 50 µg/ml) to digest not imported precursor protein. (**B**) Isolated mitochondria without $\Delta\psi_{mit}$ were incubated with increasing amounts of Aβ40 and Aβ42 (as indicated) and precursor protein [^{35}S]-Su9(70)DHFR. (**C**) Separation of preprotein binding (*Binding*) to OMM from inner membrane translocation and processing steps (*Chase*). For precursor binding and insertion into the OMM, $\Delta\psi_{mit}$ was dissipated by CCCP (1 µM) during incubation with [^{35}S]-Su9(70)DHFR in presence (lanes *11,12*) and absence of Aβ peptides (lanes *10* and *13-18*). To assay inner membrane translocation and processing (*Chase*), the $\Delta\psi_{mit}$ was restored by addition of albumin (BSA; 2 mg/ml; lanes *10-12* and *16-18*) in presence (lanes *17,18*) and absence of Aβ peptides. For comparison, a complete one-step import reaction of precursor protein [^{35}S]-Su9(70)DHFR was performed (lanes *1-9*). All samples were analyzed by Tricine SDS-PAGE followed by Western blot, digital autoradiography and immunodecoration against Aβ peptides and Tim23. *p*, mitochondrial precursor protein; *m*, mitochondrial mature form; *Mock*, control experiment in the absence of mitochondria; *WB*, Western blot.

Figure 4. **Pretreatment of isolated mitochondria with Aβ peptides.** Isolated mitochondria were pre-treated with Aβ peptides (3.5 μM) for 30 minutes. After several washing steps, mitochondria were re-isolated and incubated in an energizing buffer with precursor protein [^{35}S]-Su9(86)DHFR for an import reaction in the absence of Aβ peptides (*lanes 8-16*). For comparison, the precursor protein [^{35}S]-Su9(86)DHFR was directly incubated with isolated and energized mitochondria and in mock samples (*mo*) in presence or absence of Aβ peptides (*lanes 1-7*). Half of the samples were treated with proteinase K (PK; 50 μg/ml) to digest not imported precursor protein. Samples were analyzed by Tricine SDS-PAGE followed by western blotting, digital autoradiography and immunodecoration against Aβ peptides and control mitochondrial Tim23. *p*, precursor protein; *m*, mature form; *WB*, Western blot.

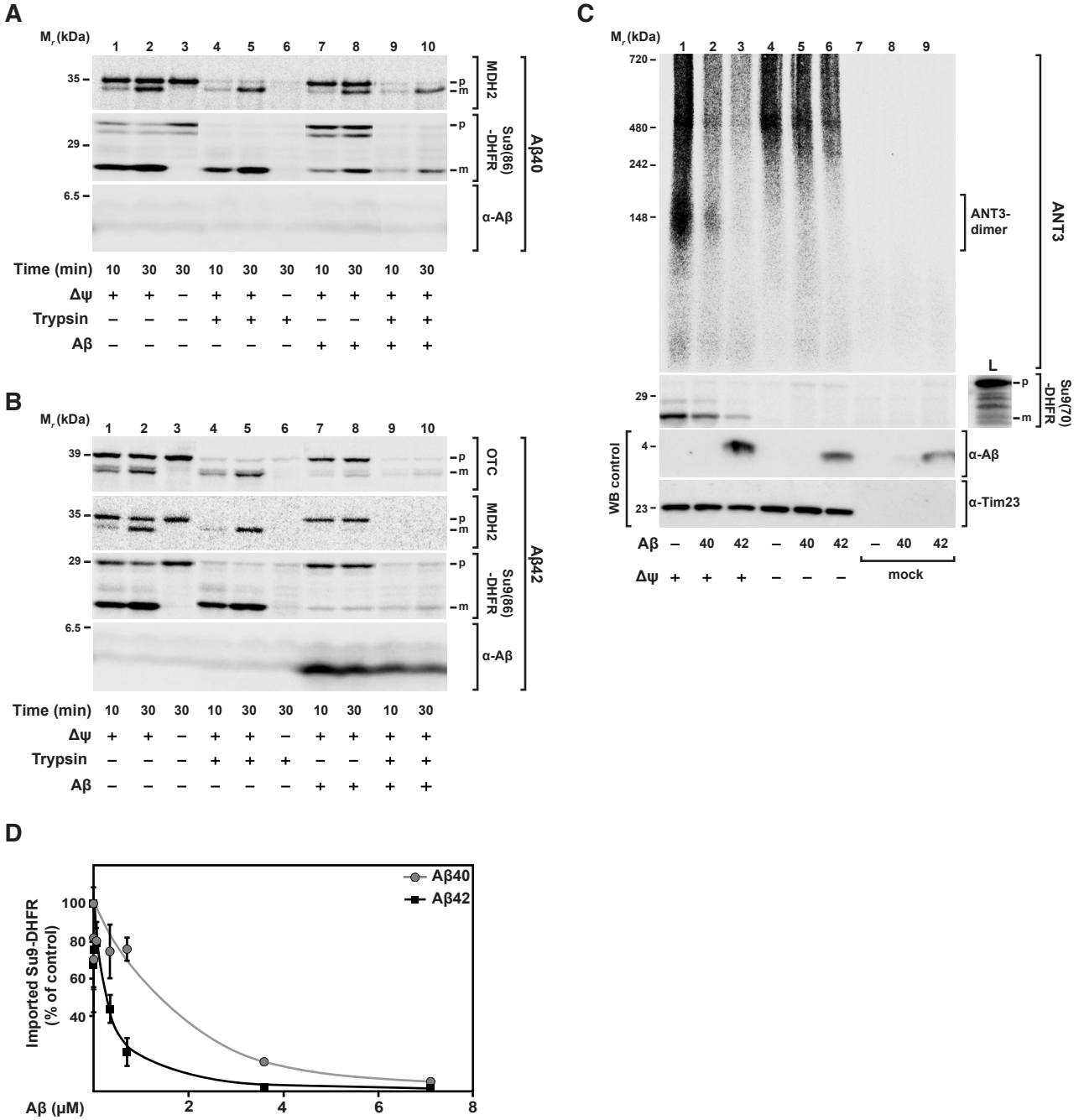
Figure 5. **Analysis of Aβ peptides interaction with human mitochondria.** Isolated and energized mitochondria and mock (*mo*) samples (*lanes 6*, *12*) were incubated with the same amount of Aβ40 (**A**) and Aβ42 (**B**) peptides (3.5 μM) for different time points. $\Delta\psi_{mit}$ was dissipated where indicated (*lanes 5 and 11*). Half of the samples were then treated with trypsin (100 μg/ml; *lanes 1-6*). Increasing amounts of Aβ40 (**C**) and Aβ42 (**D**) peptides were incubated for 30 min in presence or absence (*Mock*) of energized mitochondria and separated in insoluble (*Pellet*) and soluble (*Supernatant*) fractions. All samples were processed by Tricine SDS-PAGE followed by Western blot. As control, immunodecoration against mitochondrial Tom20, Tim23 and Tom40 proteins was performed. *WB*, Western blot.

Figure 6. **Membrane interaction behavior of Aβ peptides.** (**A**) Aβ peptides (3.5 μM) were incubated with or without (*Mock*) intact and energized mitochondria followed by digestion with increasing amounts of trypsin (*lanes 1-4*). As controls, mitochondria were lysed by solubilization with 0.5% Triton X-100 (*lanes 5-8*) or by sonication (*lanes 9-12*) before the addition of the trypsin. All the samples underwent TCA precipitation. (**B**) Dependence of the interaction between Aβ peptides and isolated mitochondria on peripheral OMM receptors. Isolated mitochondria were pre-treated with the indicated trypsin concentrations to digest exposed OMM proteins. After trypsin inactivation, isolated mitochondria were re-isolated and incubated in an energized buffer with Aβ peptides (3.5 μM). (**C**) Alkaline extraction of Aβ peptides from mitochondria and mock samples. Aβ peptides (3.5 μM) were incubated in presence or absence (*Mock*) of isolated and energized mitochondria. After reisolation, mitochondria and mock samples were subjected to alkaline extraction as described under "Material and Methods" section. All samples were analyzed by Tricine SDS-PAGE and Western blot. As control, immunodecoration against the endogenous mitochondrial proteins such as SMAC (IMS), MPP (matrix) and Tom40 (OMM) was carried out. *T*, total; *P*, pellet; *S*, supernatant; *L*, loading control; *WB*, Western blot.

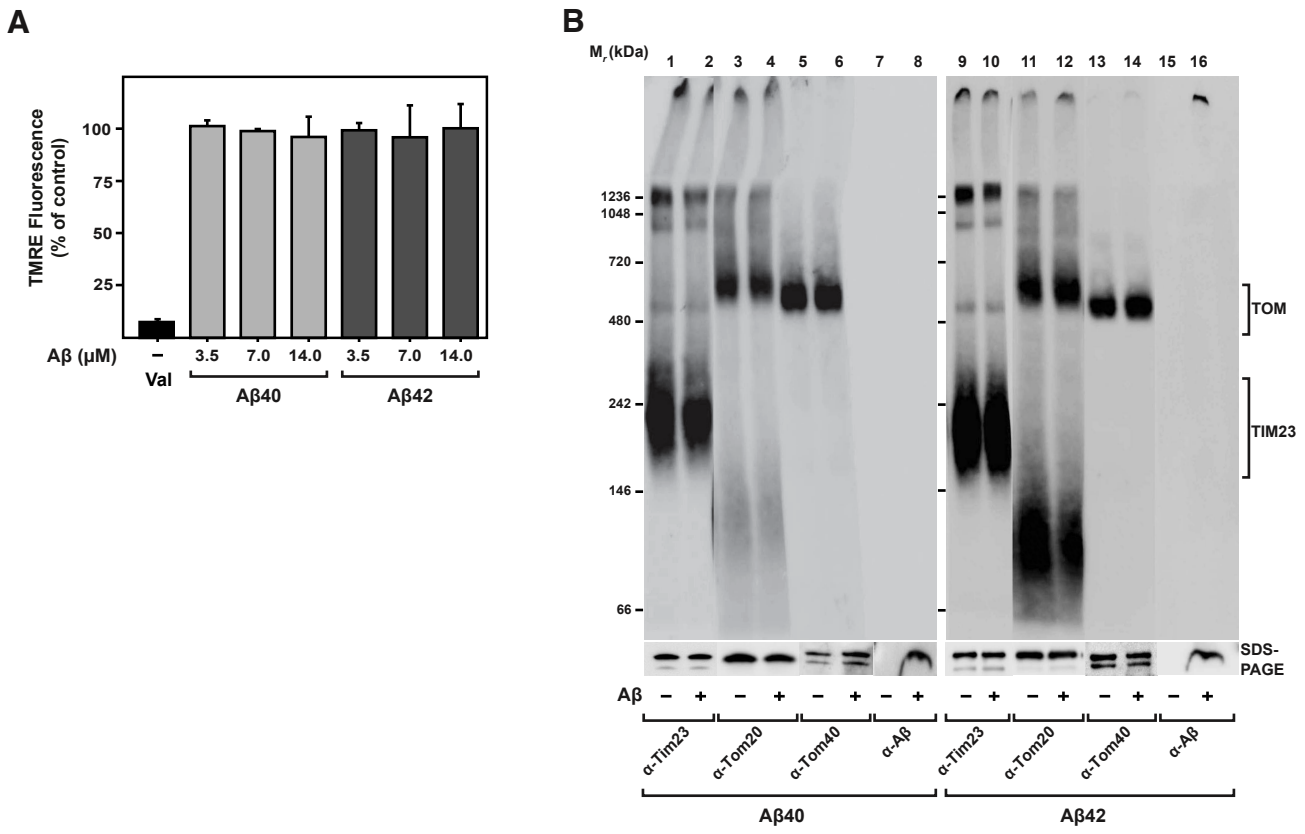
Figure 7. **Analysis of the interaction between Aβ peptides and mitochondrial precursor proteins with mitochondria through density gradient centrifugation.** (**A**) Sucrose gradient centrifugation of 3.5 μM Aβ40 (upper panels) and Aβ42 (lower panels) incubated with and without (Mock) isolated and energized mitochondria. (**B**) As control, a sucrose gradient of precursor protein [^{35}S]-Su9(70)DHFR incubated with or without (*Mock*) isolated and energized mitochondria in the absence of Aβ peptides was performed. (**C, D**) Sucrose gradients with or without (*Mock*) mitochondria incubated with precursor protein [^{35}S]-Su9(70)DHFR in the presence of Aβ40 (**C**) or Aβ42 (**D**). Density gradient fractionations were performed as reported in "Materials and Methods" section. Samples were analyzed by tricine SDS-PAGE and Western blot. As control, immunodecorations against MPP and Tim23 were used. (**E**) Quantification of the Aβ42 band intensities incubated with mitochondria in absence (**A**) or presence (**D**) of precursor protein [^{35}S]-Su9(70)DHFR. Each value is the ratio between the intensity of the Aβ42 band in each fraction and the total sample (*T*). *WB*, Western blot; *p*, precursor form; *m*, mature form of the preprotein.

Figure 8**. Co-aggregation between Aβ peptides and mitochondrial precursor protein.** Precursor protein [^{35}S]-Su9(86)-DHFR was incubated for 30 min at 30 °C in import buffer in

presence or absence of the indicated amounts of Aβ peptides. After incubation, samples were analyzed by the following techniques: (**A**) Tricine SDS-PAGE. Soluble fractions (*Supernatant*) were separated from the insoluble (*Pellet*) by centrifugation for 40 min at 123000 xg and 4 °C. Samples were analyzed by tricine SDS-PAGE. (**B**) Filter retardation assay. Samples were filtered directly through cellulose acetate and nitrocellulose membranes using a dot blot filtration unit as described in “Material and Methods” section. Proteins bound to both membranes were stained with Ponceau S. Bound Aβ peptides were detected by immunodecoration and the precursor protein by digital autoradiography. (**C**) BN-PAGE. Samples were loaded on native PAGE as described in “Materials and Methods” and analyzed by Western blot. The precursor protein signal was detected by digital autoradiography and the Aβ peptides by immunodecoration.



Cenini *et al.*, Fig. 1



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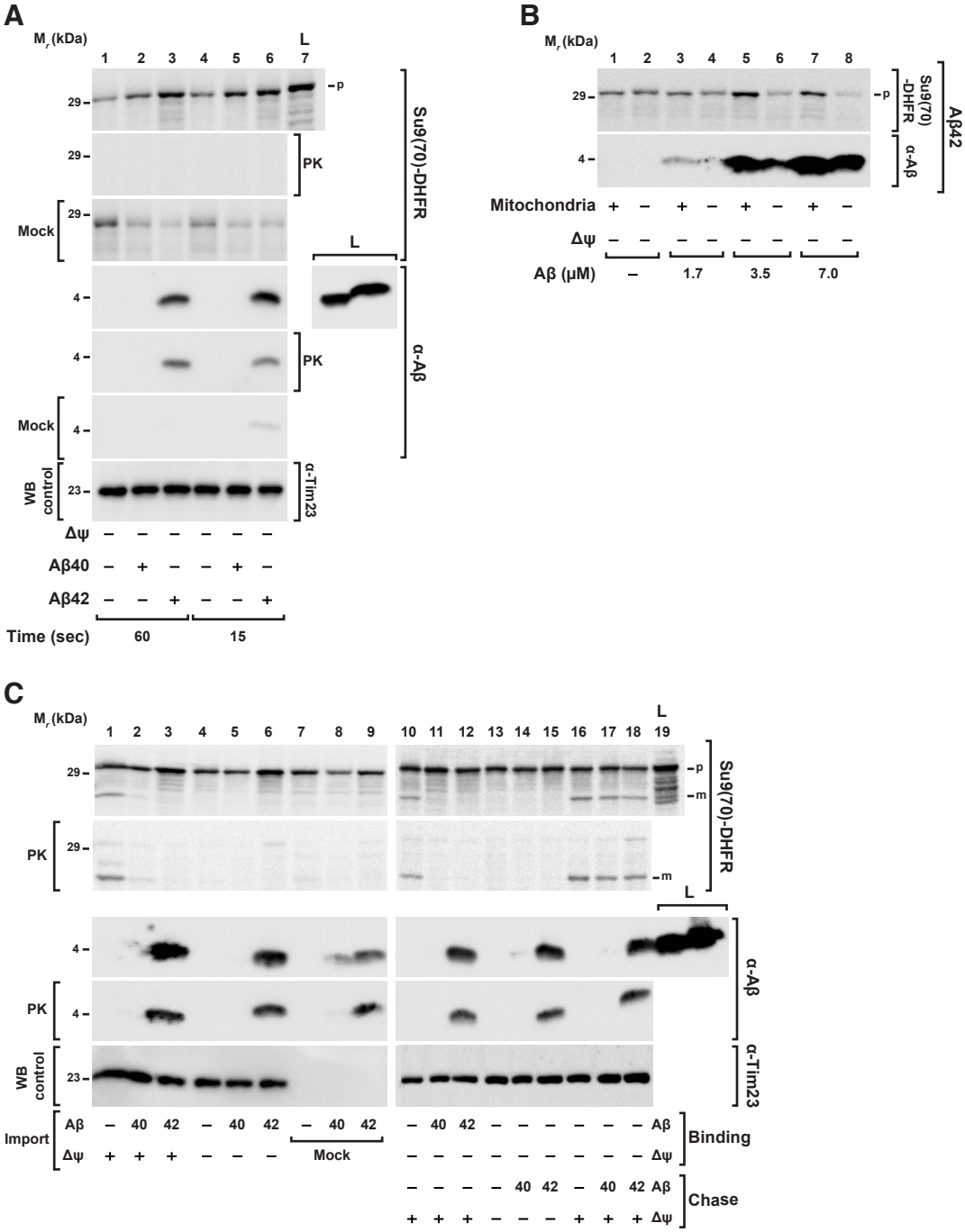

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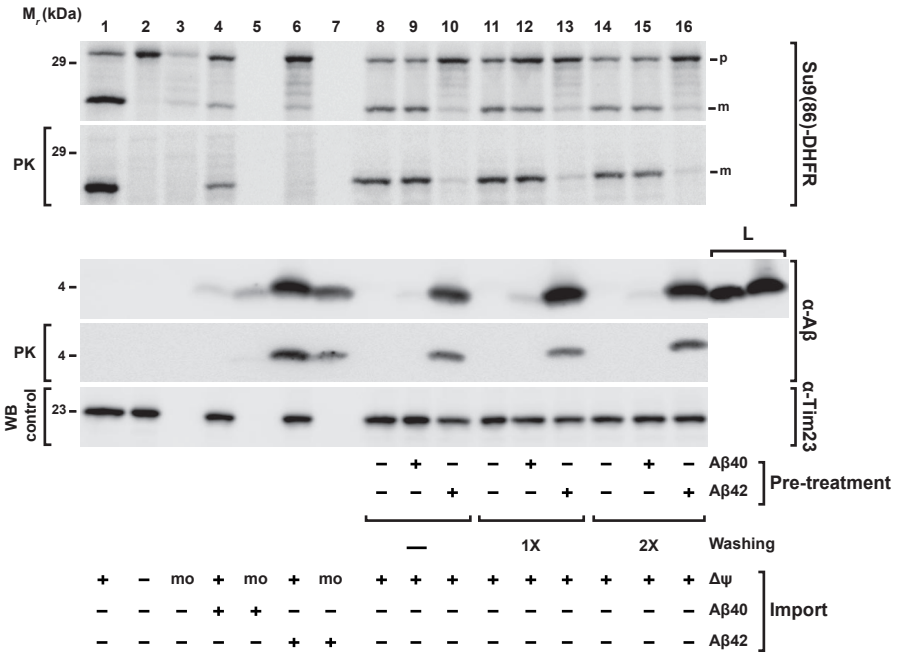

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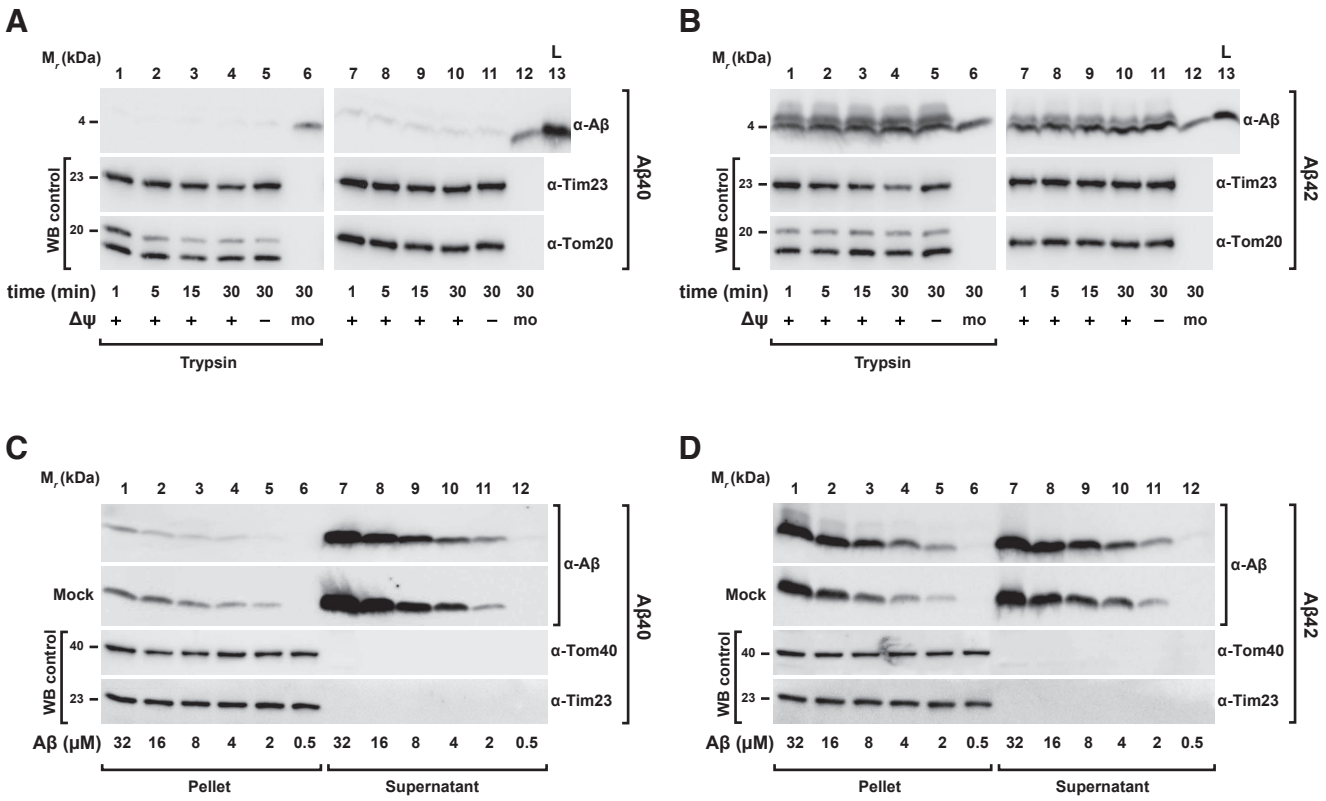

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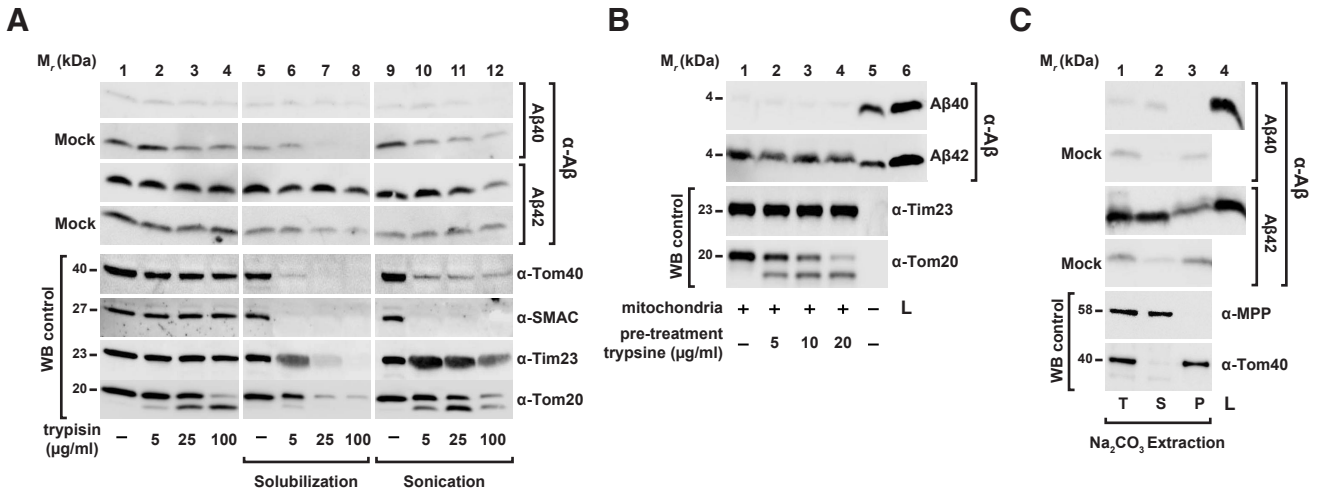

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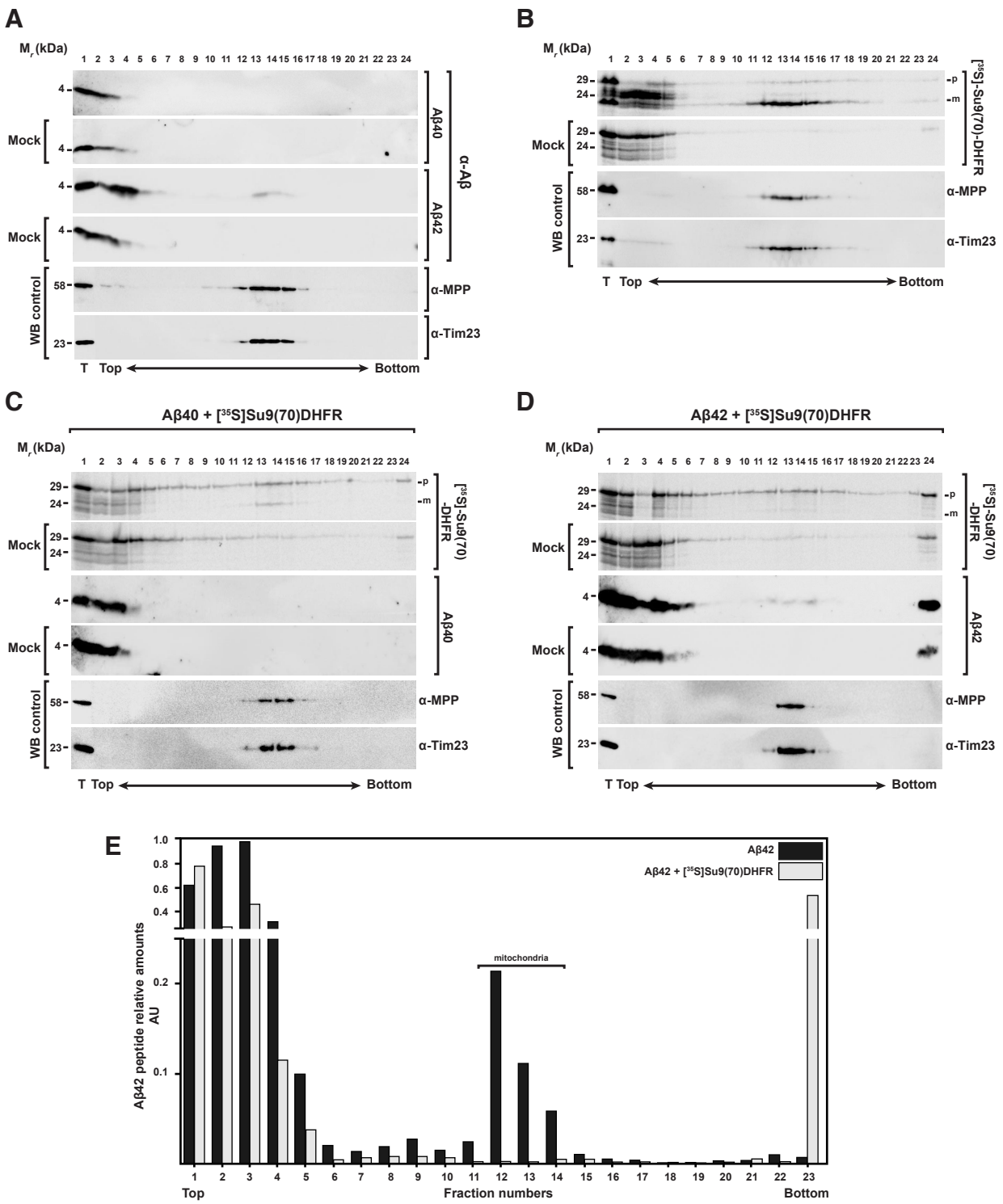

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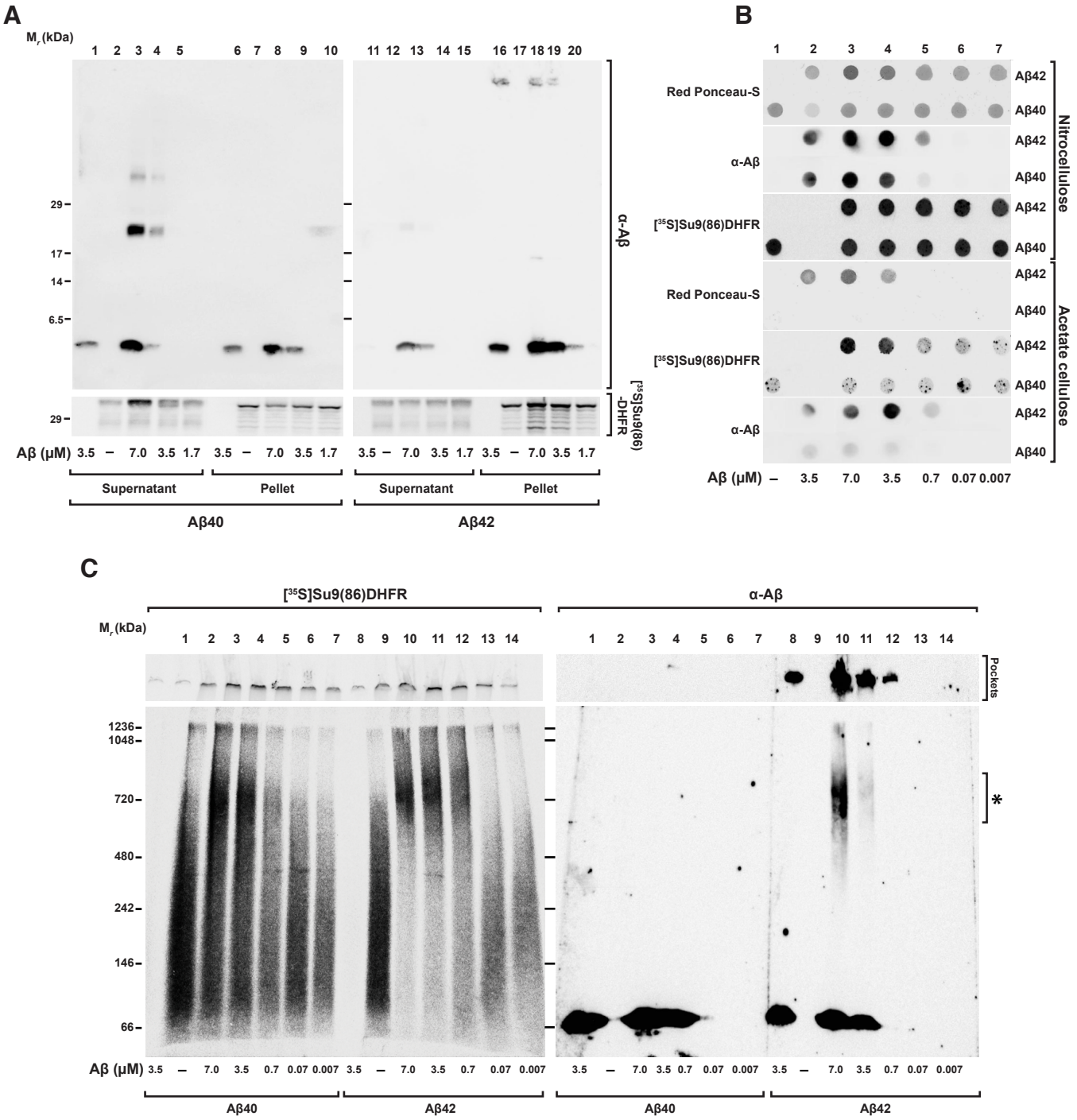

Cenini *et al.*, Fig. 8