A yeast model for Aβ aggregation exemplifies the role of membrane trafficking and PICALM in cytotoxicity

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ABSTRACT

Alzheimer’s disease is the most common neurodegenerative disease, associated with aggregation of Aβ peptides. The exact mechanism of neuronal cell dysfunction in Alzheimer’s disease is poorly understood and numerous models have been used to decipher the mechanisms leading to cellular death. Yeast cells might be a good model to understand the intracellular toxicity triggered by Aβ peptides. Indeed, yeast has been used as a model to examine protein functions or cellular pathways that mediate the secretion, aggregation, and subsequent toxicity of proteins associated with human neurodegenerative disorders. In the present study, we use the yeast *Saccharomyces cerevisiae* as a model system to study the effects of intracellular Aβ in fusion with the green fluorescent protein. We sent this fusion protein into the secretory pathway, and showed that intracellular traffic pathways are necessary to generate toxic species. Yeast PICALM orthologs are involved in cellular toxicity, indicating conservation of the mechanisms of toxicity from mammals to yeast. Finally, our model demonstrates the capacity for intracellular Aβ to cross intracellular membranes and target mitochondrial organelles.
INTRODUCTION

Alzheimer’s disease (AD) was first described 100 years ago and is a progressive neurodegenerative pathology leading to gradual cognitive and behavioral changes and loss of memory (Selkoe and Podlisny, 2002). It is associated with the presence of region-specific amyloid-β (Aβ) deposits in the brain. These amyloid plaques form one of the neuropathological hallmarks of AD. The APP gene encodes the amyloid precursor protein (APP) which encompasses the Aβ peptides. Differential cleavage of APP produces amyloid peptides of 40 (Aβ40) or 42 (Aβ42) amino acids in length; the Aβ40 species are considered to be less toxic. Although Aβ aggregation is correlated with the extracellular deposition of terminal amyloid plaques in AD patients and AD mouse models, Aβ species also accumulate within the cell, including inside multivesicular bodies (Almeida et al., 2006; Langui et al., 2004; Takahashi et al., 2002), lysosomes, or other vesicular compartments (Nixon, 2007; Shie et al., 2003). Recent publications have confirmed the important role played by intracellular Aβ, whether it is produced through the secretion pathway, by transfection, or by uptake from the media (Echeverria et al., 2004; Hansson Petersen et al., 2008; Hu et al., 2009; Kandimalla et al., 2009; Rebeck et al., 2010).

Different biological model systems including Aβ-transgenic Caenorhabditis elegans worm (Link, 1995), Drosophila melanogaster flies (Crowther et al., 2005; Iijima et al., 2004), and mammalian cell cultures (Magrané et al., 2004) have been used to study the role of intracellular Aβ. These biological systems have identified general effects such as mitochondrial organization (Zhao et al., 2010; Iijima-Ando et al., 2009) or folding machinery (Fonte et al., 2002; Magrané et al., 2004) as targets or regulators of toxic Aβ species. These
findings have been confirmed in vivo (Hoshino et al., 2011) and they are similar to the changes found in pathogenic situations. This supports the notion that part of the complex process leading to AD can be reliably studied at the cellular level. However, the different cell models used so far have not revealed any molecular mechanisms that could account for the toxicity of Aβ. In addition, pharmacological approaches based on molecules that interfere with Aβ amyloid formation have not been productive. This has raised several questions concerning the paradigm and the models used for these strategies. So far, no simple organism that can be manipulated for a high throughput screening can be used as a “gold standard” for Aβ toxicity.

Yeast cells are suitable for such screening and have been widely used to pinpoint gene networks and chemical compounds that can modulate amyloid toxicity. This was particularly the case for Parkinson’s disease (Willingham et al., 2003; Cooper et al., 2006; Franssens et al., 2010), and ALS disease (Sun et al., 2011; Ju et al., 2011; Fushimi et al., 2011). Although this experimental model has been successfully used to monitor the aggregation pattern of Aβ (Bagriantsev and Liebman, 2006; Caine et al., 2007; von der Haar et al., 2007), these first yeast systems has failed to recapitulate the toxic properties of this peptide. In these previous studies, Aβ was expressed in the yeast-cell cytoplasm. Very recently, a new screen based on secreted form of Aβ in yeast revealed the importance of endocytic pathway in cellular toxicity (Treusch et al., 2011).

In the present study we demonstrate that targeting Aβ in the secretory pathway produces toxic species. The toxicity depends on the allele expressed, arctic mutants being more harmful than wt Aβ. Disturbance of cell-traffic routes (i.e. secretion, endocytosis, recycling, or traffic between Golgi and vacuoles) reduced the toxic effects of Aβ. Altogether, these results
indicate that in our yeast model, the cellular toxicity is not due to endocytosis dysfunction, but rather implicate another cellular target.
RESULTS

Aβ becomes toxic when expressed with a secretory sequence

It has been clearly established that Aβ_{40} or Aβ_{42} expressed in frame with reporter genes such as GFP (Caine et al., 2007) or the functional domain of Sup35 (von der Haar et al., 2007; Bagriantsev and Liebman, 2006) does not lead to yeast death. We fully reproduced this result since, regardless of whether the Aβ_{42} or arctic mutant was expressed, the number of colonies formed on galactose (inducing conditions for expression of Aβ-GFP) or on dextrose (repressing conditions) was the same (Fig 1). In both cases, the pattern of aggregation was similar with the presence of bright dots. It was therefore challenging to test the toxicity to yeast of Aβ species entering the secretion pathway as Aβ toxicity is observed when this peptide is secreted in the fly model of Alzheimer's disease (Finelli et al., 2004; Iijima et al., 2004). When GFP was fused to the mating factor α prepro-leader sequence (MFα) secretion signal derived from the precursor of the *Saccharomyces cerevisiae* mating alpha-factor (Kurjan and Herskowitz, 1982), there was no marked effect on the colony forming unit (CFU) capacity. Previous studies based on four different yeast secretion signal sequences (*i.e.*, INU1, SUC2, PHO5, and MELI) did not report any toxicity for secreted GFP (Li et al., 2002). In contrast, we observed that expression of MFα-Aβ_{42}-GFP reduced viability (decrease in CFU), and extended the time required to obtain visible colonies (Fig 1). Both of these effects were exacerbated when Aβ_{ARC} was expressed under the same conditions. One of the problems with GFP chimeric proteins is that cellular properties such as toxicity could be due to GFP itself. We tested this hypothesis by removing GFP from the plasmids (Fig S1), and we observed the same toxic phenotypes. The toxicity was clearly lower, but the arctic mutant was still more deleterious than wild-type (WT) Aβ. Western-blot analysis revealed that the level of
expression was greatly diminished without GFP (Fig S1). GFP probably helps to stabilize Aβ peptide but it is clear that all of the toxic properties of Aβ-GFP can be attributed to the Aβ part of the chimeric protein.

The MFα-GFP chimer exhibited a low and non-diffuse fluorescence pattern, whereas the MFα-Aβ-GFP proteins could not be detected by fluorescence. This low signal (or even absence of signal) may be due to a low level of expression, improper GFP folding, post-translational modification, or a combination of these factors. To test the possibility of GFP misfolding due to Aβ aggregation (Wurth et al., 2002), we engineered additional constructs with a linker sequence inserted between Aβ and GFP. The two new chimeric proteins were as toxic as the two previous constructs (Fig 1) and could now be detected in yeast cells. This finding rules out the possibility of GFP misfolding due to its expression throughout the secretory pathway. The same Aβ-GFP chimeric proteins behave differently in the cytoplasm (formation of foci and non toxic) and in the secretory pathway (non-punctiform and toxic).

We next checked the processing of MFα-Aβ-GFP proteins.

**Processing of MFα -Aβ-GFP**

Expression of GFP, Aβ42GFP, or AβARC GFP resulted in one species (26 or 31 kDa, respectively) which cross-reacts with anti-GFP antibodies (Fig 2, panel A). In contrast, the secreted forms of Aβ42GFP or AβARC GFP resulted in three bands (Fig 2, panel A). The smallest (about 34 kDa) and largest species (about 50 kDa) were the most abundant. During galactose induction, these two species were detected first (Fig 2, panel B); the incapacity to detect the intermediate species early on during induction was probably due their low concentration (below the sensitivity threshold). The mobility of the smallest species is consistent with the production of the mature proteins. This process requires Kex2p, an endoprotease in the late Golgi compartment (Redding et al., 1991) involved in processing
alpha-factor (Julius et al., 1984). To test this hypothesis, we analyzed the different protein species produced in a Δkex2 strain (Fig 3, panel A). Indeed, the smallest species were no longer detected as expected if it was produced by Kex2p. Among the three bands revealed by anti-GFP antibodies, the highest weight band is bigger than the MFα-Aβ42-GFP species (41 kDa). The prepro-alpha-factor is glycosylated through the yeast secretory pathway (Julius et al., 1984), and the higher mobility could be due to glycosylation of prepro-Aβ-GFP species. To test this hypothesis, we added a deglycosylation mix to the crude extract. After enzymatic treatment, the proteins were analyzed by western blot (Fig 3, panel B). The highest mobility band was no longer detected whereas the intermediate band was more intense, as expected if this band corresponds to the unglycosylated form of prepro-Aβ-GFP. The ratio of these 3 bands depended on the preparation method of the crude extracts (data not shown). This may have been due to a particular protection against proteases and we thus tested the sensitivity of these proteins to proteases. The extracts prepared from yeast spheroplastes revealed four bands instead of three. Proteinase K analysis of the P13 fraction (corresponding to the 13000g pellet fraction of yeast lysate prepared in mild conditions) identified the lowest-mobility band as PK-resistant entities (Fig 3, panel C). This finding is consistent with an intra-membranous localization of glycosylated prepro-Aβ-GFP, but indicates most of the mature form (Aβ-GFP) is sensitive to the protease (i.e. is not imbedded in the membranous compartment). As the proteins enter the secretory pathway, we searched for the presence of GFP species in the medium. It has been previously reported that GFP expressed in frame with different secretory signal peptide did enter the secretory pathway but was not detected in the medium (Li et al., 2002). With our constructs based on the prepro-alpha factor peptide, we also did not detect any GFP species in the medium using either fluorescence or western blot of concentrated medium (data not shown).
Disturbance of cell-traffic pathways decreases the toxicity triggered by Aβ chimeric proteins

Prepro-Aβ-GFP enters the secretory pathway and is clearly toxic to yeast cells. Moreover, the PICALM gene has been recently described as a new susceptibility gene for AD (Harold et al., 2009). In mammalian cells, the neuron-specific AP180 protein and its ubiquitously expressed homolog, clathrin assembly lymphoid myeloid leukemia protein (PICALM) are adaptor proteins that participate in clathrin-mediated endocytosis. Adaptors determine the trafficking itinerary of cargos and their steady state distributions within the cell (Fig 4). The yAP180 proteins are yeast homologues of PICALM (Wendland and Emr, 1998). They share 48% identity, and 25.5% (YAP1801) and 26.5% (YAP1802) identity with PICALM protein. Deletion of YAP1801 and YAP1802 clearly decreased the toxicity induced by MFα-Aβ42-GFP and MFα-Aβarc-GFP by lowering the growth inhibition (table 3 and Fig S2). We investigated whether mammalian PICALM gene could complement the YAP1801 and YAP1802 mutants. PICALM has two splice variants designated long (L) and short (S) according to the number of amino acids. The two mouse cDNA were subcloned into a multi-copy galactose-induced plasmid. The L isoform on its own was toxic in yeast (data not shown) and could thus not be used. Expression of the S isoform of PICALM in YAP1801/YAP1802 double-deleted yeast mutants partially restored the growth inhibition observed when MFα-Aβ42-GFP or MFα-Aβarc-GFP were expressed in WT cells (Fig 5). This suggests that mouse PICALM protein is able to complement YAP loss of function and that mammal and yeast proteins share a critical role in Aβ toxicity.

In mammalian cells, CALM interacts with the clathrin-associated adaptor protein complex-2 AP-2 (Owen et al., 2000), and depletion of PICALM triggers delocalization of the AP-1 adaptor complex (Meyerholz et al., 2005).
MFα-Aβarc-GFP was partially suppressed when different subunits of the AP-1 and AP-2 complex were deleted. Two other classes of adaptors participate with AP-1 in clathrin-mediated transport between the trans-Golgi network (TGN) and endosomes (Fig 4): Gga2p protein and epsin-like protein Ent5p (Costaguta et al., 2006). The absence of these two proteins also restored growth (table 3). The only identified cargo that is internalized by Yap1801 and Yap1802 is the vSNARE Snc1p (Burston et al., 2009). Snc1p recycles to the plasma membrane and this recycling requires Rcy1p (Galan et al., 2001). We observed that the toxicity induced by MFα-Aβ42-GFP and MFα-Aβarc-GFP was strongly suppressed when RCY1 was deleted (table 3 and Fig S2). Our findings indicate that defects in the endocytic or recycling pathway reduce the toxicity triggered by the Aβ-GFP chimeric proteins. It is also quite clear that MFα-Aβ42-GFP and MFα-Aβarc-GFP are still toxic in such background.

Cellular localization of Aβ-GFP

After induction of MFα-Aβ42-GFP and MFα-Aβarc-GFP in WT cells, proteins present a dual pattern of punctuate and filamentous fluorescence structures (Fig 1). These two patterns are not found systematically because filamentous organization is observed in most of the fluorescent cells whereas punctuate foci are only present in some of them. In mutants which are lower in Aβ toxicity and defective for traffic pathways, the ratio of the two fluorescence patterns is inversed. Most of the cells present small foci and we occasionally observed the filamentous pattern (Fig 6). This correlates the presence of filamentous aggregates with Aβ toxicity and suggests that the punctuate structures correspond to membrane vesicles.

In mutant strains defective in the early stages of endocytosis such as the Δsnf7 mutant, part of GFP fluorescence is associated with the plasma membrane (Fig 6, panel Δsnf7). In such mutants, membrane invagination into multi vesicular bodies (MVB) is impaired (Babst et al., 2002), and this decreases the rate of endocytosis. This result shows that the chimeric proteins
are able to reach the plasma membrane. Since none of chimeric proteins were detected in the extracellular medium, we speculate that the proteins could be internalized once they had reached the plasma membrane.

The use of yeast cells expressing red fluorescent protein fused to proteins whose localization has been characterized previously (Huh et al., 2003) identified the ER as the sole compartment clearly labeled by Aβ-GFP species (Fig S3 “RFP”). This finding does not rule out the localization of Aβ-GFP on other membrane compartments since the speed of the vesicles makes it unlikely to detect both fluorescent signals when they are not observed simultaneously.

These findings show that disturbances of the endocytic or recycling pathways modify the aggregation pattern of MFα-Aβ42-GFP and MFα-Aβarc-GFP as well as their cellular toxicity.

**Hsp104p plays a role in Aβ chimeric protein toxicity**

Hsp104p is critical for the toxicity and aggregation of the poly-glutamine Huntingtin protein in yeast (Meriin et al., 2002). As Hsp104p plays a pivotal role for the aggregates formed in yeast, we tested whether this chaperone protein is necessary for the toxicity of Aβ-GFP chimeric proteins. As indicated in table 3 and FigS2, deletion of HSP104 partially restored the viability of cells expressing MFα-Aβ42-GFP and MFα-Aβarc-GFP. This result was unexpected because Hsp104p is a cytosolic protein and MFα-Aβ42-GFP and MFα-Aβarc-GFP were associated with the secretory pathway. We therefore conclude that some of the toxic species formed by Aβ-GFP chimeric proteins become cytosolic. This is consistent with the observation that the mature form (Aβ-GFP; Fig 3C) detected by western-blot analysis is sensitive to proteinase K.
In the Δhsp104 strain, the fluorescent profile of Aβ-GFP chimeric proteins was filamentous and no foci were observed (Fig 6). These results suggest that Hsp104p plays a role in the toxicity of MFα-Aβarc-link-GFP, probably by favoring the conversion of big aggregates into smaller and toxic ones.

**Respiratory rate of cells expressing MFα-Aβarc-link-GFP is affected**

Mitochondrial dysfunction plays a key role in AD. We therefore tested whether the toxicity triggered by the expression of Aβ-GFP chimeric proteins in yeast was associated with a mitochondrial disorder. To this end, we monitored the growth and consumption of oxygen in aerobic conditions at different time points after Aβ induction. Yeast cells transformed with the chimeric constructions were grown with lactate as a carbon source and 0.2% galactose was added at the mid-log phase to induce expression of the chimeric protein. Under these conditions, the growth of cells expressing MFα-GFP or MFα-Aβarc-link-GFP was the same as that for cells carrying an empty vector during the first 4 h of the induction. After this time, the growth rate of cells expressing the two chimeric proteins slowed, and this slowdown was stronger for cells expressing MFα-Aβarc-link-GFP (Fig 7A). We then evaluated the respiratory rate of the cells at different times during Aβ induction (Fig 7B). The consumption of oxygen by cells with an empty vector or expressing MFα-GFP was unchanged 4 h after induction (addition of galactose). In contrast, the respiratory rate decreased in the cells expressing MFα-Aβarc-link-GFP. This reduction in oxygen consumption was higher after 8 h of induction, whereas the respiratory rates of the two controls (empty vector and MFα-GFP) decreased only by a small amount in both cases. This difference was not due to cell lethality since the viability (measured by the number of colonies formed after plating) was the same after 8 h of induction (data not shown). Such decline of oxygen consumption can be due
either to a decrease in mitochondria contents or to a defect in electron transport chain. We measured the mitochondrial content by recording the optical absorption spectra of mitochondrial cytochromes. The concentration of these different cytochromes is not specifically changed during Aβ expression (Fig 7C), as expected if the deleterious effect depends on the inhibition of the respiratory chain.
DISCUSSION

Aβ must enter the secretory pathway to become toxic

In well-established model organisms such as Caenorhabditis elegans (Link, 1995), or D. melanogaster (Crowther et al., 2005; Iijima et al., 2004), Aβ is always expressed in frame with a signal peptide. This expression is thought to represent the mammalian situation and is expected to yield extracellular Aβ. These models have shown that physiological impairments can happen before the occurrence of large extracellular deposits and are instead correlated with the intracellular accumulation of Aβ in worm (Fay et al., 1998) and fly (Crowther et al., 2005). Consistent with these findings, the transgenic mouse AD model (Tg2576) displayed defects in morphology, behavior, and memory months before Aβ12 plaque deposition was apparent (Jacobsen et al., 2006). In addition, plaque prevalence does not strictly correlate with dementia in AD (Schmitt et al., 2000; Davies et al., 1988). All of these results imply a pivotal role for intracellular Aβ, but its production is always puzzling because the protein is directed towards the extra-cellular space. Despite the usefulness of model organisms, there are no published reports that specifically address the role of the secretory pathway in generating toxic amyloid species. In the present study, we confirm that Aβ (expressed alone or stabilized by the GFP tag) produced in the cytoplasm does not significantly impair yeast growth. In our hands and even under harsher conditions (37°C), yeast growth was comparable regardless of whether Aβ1-42, Aβarc, or GFP alone was expressed. In contrast, translocation of the same species into the secretory pathway could dramatically altered their toxicity. Our results are in complete agreement with those published recently in a yeast system (Treusch et al., 2011).
**Chimeric Aβ-GFP are relevant for toxicity study**

Our biological system is based on translational fusion between Aβ and GFP. With this system, the results are at the opposite of a study recently published in which PICALM orthologous genes protect from Aβ toxicity (Treusch et al., 2011). In this model, Aβ is supposed to transit through the secretory pathway to the plasma membrane. Aβ toxicity is observed only when a huge amount of this peptide is produced. This high production results both from multiple tandem copies insertion and codon optimization for expression in yeast. At a lower level of expression (one integrated copy), Aβ toxicity can not be detected anymore (Fig S1 (Treusch et al., 2011)). Interestingly, the results found with this system were validated in *C. elegans* and rat hippocampal neurons. In these three models, cells are also exposed to huge amount of Aβ produced into the ER or added in the medium as pre-formed oligomeric Aβ species. In contrast, when we expressed Aβ without GFP, we get a modest toxic effect. These results indicate that PICALM may protect cells in presence of large amounts of Aβ, but it does not rule out the possibility that the same properties (increasing endocytosis) may lead to an opposite effect when Aβ concentration is below some threshold value. In our system, the MFrα pre sequence warrants us to follow the efficient entry and processing into the secretory pathway since this sequence is glycosylated and cleaved in the *trans* Golgi. It might also change the aggregative properties of Aβ that behaves, during this traffic, as part of the cargo protein and mimics its properties during APP trafficking. We think that this difference with the other yeast system may also explain some discrepancies. The presence of a folded protein, such as the functional domain of the yeast translation termination factor Sup35p, downstream Aβ does not change the ability of the natural peptide to aggregate (Bagriantsev and Liebman, 2006). We can not rule out that the presence of GFP may interfere with the aggregative properties of Aβ, but this presence does not change the relative toxic properties of Aβ (wt vs arctic). As Aβ-GFP enters the secretory pathway, such toxicity could be due to a saturation of
the ER-associated degradation (ERAD) system. By determining the expression of a reporter gene, we found that the unfolded protein response (UPR) is higher when GFP is secreted alone (data not shown), though it is less toxic. In addition, MFα-Aβarc-GFP is more toxic than MFα-Aβ-GFP and both species are expressed at the same level. It is therefore clear that the toxicity is due to a qualitative effect rather than induced by an overwhelming UPR. This conclusion is consistent with our experiment based on mutants acting downstream to the ER translocation; the toxicity in some of these mutants was clearly lower indicating that critical steps for deleterious effects are required in addition to the translocation event.

**The yeast model gives new insight for the role of PICALM in AD**

Recent genome-wide association studies have identified new genetic risk factors for AD. These studies have focused our attention on PICALM (phosphatidylinositol-binding clathrin assembly protein, also known as CALM, clathrin assembly lymphoid-myeloid leukemia gene), which was identified independently by different groups as a locus associated with AD risk (Lambert et al., 2011; Harold et al., 2009). In contrast with other genes such as APOE, CLU, and CR1 which are also risk factors for AD and which have many data available in the context of AD, little is known about PICALM. The Yap180 proteins include Yap1801p and Yap1802p and are the yeast homologues of PICALM (Wendland and Emr, 1998). Like PICALM, these proteins are involved in the assembly of clathrin cages at sites of formation of endocytic vesicles. Deletions of one or both genes encoding these proteins reduce the toxicity of Aβ. This decrease in toxicity was clearly reversed when mouse PICALM was expressed in yeast, demonstrating a direct role of the corresponding proteins in Aβ toxicity. In a different yeast model (Treusch et al., 2011), PICALM has the opposite effect. Their finding led them to conclude that Aβ expression led to a deficiency in clathrin-mediated endocytosis that could be reversed by YAP overexpression. However, this idea must be tempered by the fact that this mechanism is not essential in yeast and that its failure does not lead to an altered growth
phenotype, at least in normal growth conditions. Finally, the functional characterization of PICALM mutations associated with an increase in AD risk will allow to assert the exact role of this protein in Aβ toxicity.

**Translocated and non-translocated Aβ forms distinct cytoplasmic aggregates**

We established that almost all of the synthesized Prepro-Aβ-GFP enters the secretory pathway. The 41kDa species (corresponding to the unglycosylated form of Prepro-Aβ-GFP) appeared only as a faint band on western-blot analysis. This band was also barely detectable in the ΔKEX2 strain whereas it is the predominant species following deglycosylation treatment of the crude extract. These biochemical properties are consistent with the GFP profile exhibiting a clear ER pattern. Prepro-Aβ-GFP is thus not diverted to the cytoplasm (or only an extremely small amount is diverted). This putative traffic, if it exists, may be antagonized by selective degradation of the species, but the net result would be the same *i.e.* almost all of the detectable protein goes into the secretory pathway. Despite being correctly processed into the secretory pathway, most of the Aβ peptide is not secreted into the liquid medium, but is retained within the cell. Some mutants showed a clear accumulation of chimeric GFP proteins at the plasma membrane. The protein is thus transported within membrane vesicles to the periphery of the yeast cells. Secreted Aβ-GFP is processed by the glycosylation machinery and is finally released into the lumen of the secretory vesicle budding from the trans-Golgi network where Kex2p is localized (Redding et al., 1991). Aggregation of the protein may occur when Aβ is still linked to its pre-sequence. However, such aggregation could impair Kex2p processing and it therefore seems reasonable to postulate that aggregation will occur only after cleavage of the pre-sequence. We found that Aβ toxicity was partially controlled by Hsp104. Hsp104 is a cytoplasmic chaperone that
solubilizes large aggregates into smaller species and has been reported to inhibit the fibrillization of monomeric and protofibrillar forms of Aβ in vitro (Arimon et al., 2008). The function of Hsp104 in Aβ toxicity is probably direct, leading to the production of smaller entities that might be toxic to yeast cells. This result argues for a cytoplasmic localization of the toxic species. This intracellular localization is somewhat surprising given that the Aβ_{42} constructs are based on a signal sequence that should have led to the extracellular release of Aβ_{42}. The same Aβ-GFP chimeric proteins behave differently when expressed directly in the cytoplasm (formation of foci and not toxic) or in the secretory pathway (undetectable under fluorescence microscope and toxic). The presence of a linker between Aβ and GFP restores the fluorescence of the secreted protein and we therefore postulate that the lack of fluorescence is due to a particular aggregation of the Aβ moiety when the protein is secreted.

It is already known that partners that aggregate into insoluble material interfere with the ability of GFP to achieve its native fluorescent structure (Waldo et al., 1999). This property has been used to search for Aβ variants with reduced tendencies to aggregate (Wurth et al., 2002). Our results suggest that the cytoplasmic aggregates formed by the secreted Aβ are different from the aggregates formed during cytoplasmic expression of Aβ (without the signal sequence).

With our yeast model, we were able to expand our finding and to demonstrate that the endocytosis and recycling pathways are involved in Aβ toxicity. In the yeast model, this effect would change the rising of toxic species produced during vesicle transport between the plasma membrane and the Golgi apparatus. These species would then escape from the membrane compartment. It has been recently shown in a cellular model that bundles of Aβ fibrils formed from soluble extracellular Aβ penetrate the vesicular membrane and extend into the cytoplasm prior to cell death (Friedrich et al., 2010). The same mechanism in yeast could
yield cytoplasmic aggregates. Such aggregates could be then taken in charge by Hsp104. In the absence of this disaggregase, the equilibrium would be in favor of large and non toxic aggregates. In contrast, the presence of Hsp104 would increase the ratio of small and toxic aggregates versus larger aggregates. These species would then interact with cellular compartments such as mitochondria. In our model, the change of oxygen consumption was observed very early after Aβ expression. As *S. cerevisiae* is a facultative anaerobe, mitochondrial integrity may have been affected without any dramatic change in cell viability. However, the same events could have more severe consequences in mammalian cells.

Our study provides convincing arguments to use yeast cells as a cellular model for scoring Aβ toxicity. Our yeast system is now suitable for screening procedures and will enable many different leads to search for chemical or genetic modifiers of Aβ toxicity. Our study also offers a conceptual framework to highlight the role of PICALM and endocytosis in cellular injury and confirms the capacity for Aβ to cross cellular membranes. This initial analysis paves the way for further research that should be as powerful as that used to define molecular mechanisms underlying other amyloid related diseases.
MATERIALS AND METHODS

Yeast strains and media

Yeast strains (listed in Table 1) were transformed with plasmids carrying the different chimeric constructs under the GAL10 promoter and were grown overnight on dextrose medium (0.67% yeast nitrogen base, 2% dextrose) supplemented with 0.67% casaminoacids or with some of the following aminoacids: 20 mg/L histidine (H), 20 mg/L lysine (K), and 60 mg/L leucine (L). Upon reaching exponential phase (OD600=1), the cells were placed in galactose medium supplemented with either 0.67% casaminoacids or HKL to induce the expression of chimeric proteins. After 6 h, cells were either collected obtain total cell extracts or were observed under epifluorescence.

The Δyap1801/1802 strain was constructed by mating the two simple deleted strains Δyap1801 and Δyap1802. The mating type of the Δyap1801 strain was changed by transforming the strain with a plasmid carrying the HO gene. The two strains were then crossed by patching them mixed on a selective medium. After verifying mating by microscopy, the diploid strain was patched onto a sporulation medium. After 3 days of growth, tetrads were dissected, and the resistance of the spores to G418 was tested. The G418 resistant spores from the double recombined tetrads were kept as Δyap1801/1802 strains. To lose the plasmid carrying the HO gene, Δyap1801/1802 was then plated onto rich medium containing 5FOA.

Oligonucleotides are listed in Table 2. The Aβ1-42 sequence was amplified by PCR from pSG5-APP (a kind gift from A. Hémar) using oligonucleotide 792, which introduces a BamHI restriction site at the 5’-end of the fragment and an ATG codon at the beginning of the Aβ1-
42 sequence, and oligonucleotide 794. The PCR fragment was then inserted into the plasmid pYecHetsYGFP (Couthouis et al., 2009) — which had been previously linearized by BamHI — using gap repair method (Orr-Weaver and Szostak, 1983). The pYeαAβYGFP and pYeαAβARCYGFP plasmids were constructed by cloning a synthetic sequence in a BamHI/BstXI-digested pYeAβYGFP plasmid. These synthetic sequences, made by GeneScript, were composed of a BamHI restriction site followed by αfactor prepro sequence, Aβ WT or Arctic mutant coding sequence, the 5'-end of the GFP sequence, and a BstXI restriction site. The pYeAβARCYGFP plasmid was created by overlapping PCR using pYeαAβARCYGFP as a template (with oligonucleotides 705, 706, 859, and 860). This allowed the amplification of a PGAL-AβARC-GFP fragment, which was introduced by gap repair method into a BamHI/BstXI-digested pYeAβYGFP plasmid. Similarly, PGAL-αfactor prepro-GFP and PGAL-GFP sequences were created by overlapping PCR using pYeαAβYGFP and pYeAβYGFP as templates (with oligonucleotides 705, 706, 856, 857 and 858). The fragments were respectively inserted into pYeαAβYGFP and pYeAβGFP BamHI/BstXI digested plasmids. Each of these plasmids is a multicopy yeast-expression plasmid with the URA3 selectable marker and a GAL10 promoter in a pYeHFN2U backbone (Cullin and Minvielle-Sebastia, 1994). CALM long splice variant (CALM-L; GenBank ID BC011470) and short splice variant (CALM-S; GenBank ID BC021491) from the American Tissue Culture Collection (ATCC) were amplified by PCR using oligonucleotide 951 which introduced a BamHI site at the 5’ end of the cDNA and oligonucleotide 952 which introduced a NotI site at the 3’ end of the cDNA. After digestion by BamHI and NotI, the PCR fragment was inserted into the pYeHFN2L plasmid. This plasmid is a multicopy yeast-expression plasmid with an LEU2 selectable marker and a GAL10 promoter (Cullin and Minvielle-Sebastia, 1994).

**Spotting assay**
All spotting assays were performed under the same conditions. Tenfold serial dilutions starting with an equal number of cells (1 OD; \( l = 600 \text{nm} \)) were performed in sterile water. Spotting assays were derived from a pool of three independent fresh transformants. Ten \( \mu l \) drops were then plated onto the appropriate SD or SG medium.

**Fluorescence microscopy**

Cells were washed in water and resuspended in medium. An Axioskop 2 plus (Zeiss) fluorescence microscope was coupled with an AxioCam (Zeiss) black and white camera. The following filters were used: LP-GFP (GFP) and N3 (RFP).

**Protein extraction, deglycosylation, and western blotting**

The alkaline lysis method was used for protein extraction. Briefly, 5 OD (\( l = 600 \text{ nm} \)) units of yeast cells in exponential growth phase were permeabilized with 500 \( \mu L \) of 0.185 M NaOH, and 0.2% \( \beta \)-mercaptoethanol. After 10 min of incubation on ice, Trichloroacetic acid (TCA) was added to obtain a final concentration of 5%, and the samples were incubated for an additional 10 min on ice. Precipitates were then collected by centrifugation at 13000 g for 5 min. Pellets were dissolved in 35 \( \mu L \) of dissociation buffer (4% sodium dodecyl sulfate, 0.1 M Tris-HCl pH 6.8, 4 mM EDTA, 20% glycerol, 2% 2-mercaptoethanol, and 0.02% bromophenol blue) and 15 \( \mu L \) of 1 M Tris-base. For deglycosylation assays, pellets were suspended in 20\( \mu L \) Glycoprotein Denaturing Buffer (Biolabs), incubated for 10 min at 100°C, and transferred for few minutes at 4°C. We then added 5\( \mu L \) 10X G7 Reaction Buffer, 5\( \mu L \) 10% NP40, and 5\( \mu L \) Deglycosylation Enzyme Cocktail (PNGase F, 500,000 u/ml; endo-\( \alpha \)-N-acetylgalactosaminidase 400,000,000 u/ml; neuraminidase 50,000 u/ml; \( \beta 1-4 \) galactosidase
8,000 u/ml; β-N-acetylglucosaminidase 4,000 u/ml) to obtain a volume of 50µl and the samples were then incubated for 4 h at 37°C before adding 15µl of sample buffer.

Yeast proteins were incubated for 5 min at 100 °C and separated by SDS-PAGE in a 12 % polyacrylamide gel. Proteins were electrically transferred onto nitrocellulose membranes (Optitran BA-S83, Schleicher & Schuell) in the presence of transfer buffer (39 mM glycine, 48 mM Tris-base, 2% EtOH, and 0.037% SDS) and were probed with monoclonal anti-GFP antibodies (Sigma) or anti Aβ (Tebu) antibodies. Peroxidase-conjugated anti-mouse antibodies (Sigma) were used as secondary antibodies. Binding was detected with the SuperSignal reagent (Pierce) and the VersaDoc Imaging system (BioRad).

**Fractionation and proteinase-K treatment**

250 OD yeast culture in exponential growth phase in SG medium supplemented with 0.67% casaminoacids was collected by centrifugation (4000g), washed in 20 mL H₂O, and resuspended in 12 ml of spheroplasting buffer (1.4 M sorbitol, 50 mM Tris-Cl, pH 7.5, 40 mM 2-mercaptoethanol, and 0.4 mg/ml zymolyase 20T) and incubated for 20 min at 30 °C without shaking. Spheroplasts were centrifuged for 3 min at 1500 g at 4°C. The pellet was resuspended in 20 ml of cold lysis buffer (20 mM triethanolamine, 1 mM EDTA, pH 7.2, 0.8 M sorbitol, 2 mM phenylmethylsulfonyl fluoride, and 5 mg/ml each of leupeptin, chymostatin, aprotinin, pepstatin A, and antipain). The spheroplasts were then lysed with a Dounce homogenizer (20 strokes). Lysates were cleared by centrifugation at 500 g for 5 min at 4°C and the supernatant was centrifuged at 13000 g for 10 min. The P13 fraction was then submitted to protease protection assays. P13 fractions were resuspended in lysis buffer and incubated with combinations of 0.3125 mg/ml proteinase K (Roche) and/or 5 % Triton X-100 for 30 min at 30°C with gentle shaking. The reactions were then stopped with 10%
trichloroacetic acid for 10 min at 4°C. Samples were then centrifuged at 13000g for 10 minutes at 4°C and the pellets were suspended in 10µl protein sample buffer and separated by SDS-PAGE polyacrylamide gel electrophoresis.

**Oxygen consumption assays**

Cells were grown aerobically at 28 °C in the following medium: 0.175% yeast nitrogen base, 0.5% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.2% DL-lactate (w/v), pH 5.5. Respiration assays of growing cells were performed in the growth medium. Samples of cells were harvested throughout the growth period, washed twice in distilled water and their dry-weight was determined. Oxygen consumption was measured polarographically at 28 °C using a Clark oxygen electrode in a 1-ml thermostatically controlled chamber. Respiratory rates (JO₂) were determined from the slope of a plot of O₂ concentration versus time and were expressed as natO/min/mg of dry weight.

For determination of cytochrome content, cells were harvested after 8 h, washed twice with distilled water and concentrated to obtain 2 ml of a cell suspension of about 50 optical density units at 600 nm. They were placed in a dual spectrophotometer (Aminco DW2000) and a differential spectrum (from 500 to 650 nm) was performed between 1 ml of cells in the presence of 1 µl of H₂O₂ 70% (w/v) (oxidised state) and 1 ml of cells in the presence of a few grains of dithionite (reduced state). Calculations of cytochrome c+c₁ and cytochrome b contents were performed using an extinction coefficient of 18,000 M⁻¹ cm⁻¹ for the wavelength pairs 550–540 nm and 561–575 nm, respectively. The calculation of cytochrome a+a₃ contents was performed using an extinction coefficient of 12,000 M⁻¹ cm⁻¹ for the 603–630 nm interval.
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Axel Edelman & co have proofread the manuscript.

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AUTHOR CONTRIBUTIONS

FDA and HV performed all of the experiments apart from the EM experiments which were performed by BS. JDM set up the centrifugation assays and PK analysis and contributed to scientific discussions. AD supervised the respiratory analysis. CM and CC have conceived and partially performed the work and wrote the paper.

REFERENCES


FIGURE LEGENDS

Table 1: Yeast strains used in this study

Table 2: Oligonucleotides used in this study

Table 3: Growth capacity of different strains expressing MFα-Αβ_{42}-GFP or MFα-Αβ_{arc}-GFP
The growth capacity was evaluated by spotting assays and was compared to WT strains expressing the same chimeric proteins or carrying an empty vector.

Figure 1: Aβ aggregation and yeast growth

Ten-fold dilutions of exponentially growing cultures of BY4742 cells transformed with plasmids carrying the different chimeric constructions under the GAL10 promoter were spotted onto SD (-) or SG (+) agar supplemented with 20 mg/L histidine, 20 mg/L lysine, and 60 mg/L leucine. The cells were incubated at 30°C for 3 days. The cells were also grown for 6 h in SG liquid medium supplemented with 0.67% casaminoacids to induce the expression of the chimeric proteins and were examined by epifluorescence microscopy. *: position of arc mutation.
Figure 2: Maturation of secreted forms of Aβ.

(A) BY4742 cells expressing the different chimeric proteins (6 h of expression) were collected to perform total-protein extracts. Equal quantities of proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and were then transferred onto a nitrocellulose membrane and exposed to monoclonal anti-GFP antibodies (Sigma). (B) At different time points of the induction, cells were collected to perform total-protein extracts. Equal quantities of proteins were separated by SDS-PAGE on a 12% polyacrylamide gel and were then transferred onto a nitrocellulose membrane and exposed to monoclonal anti-GFP antibodies (Sigma).

Figure 3: The secreted forms are glycosylated and processed by Kex2

(A) BY4742 WT or Δkex2 cells expressing the different chimeric proteins (6 h of expression) were collected for total protein extracts. Equal quantities of proteins were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred onto a nitrocellulose membrane, and then exposed to monoclonal anti-GFP antibodies (Sigma). (B) BY4742 expressing the different chimeric proteins (6 h of expression) were collected for total-protein extracts and submitted or not to a deglycosylation enzyme mix. Equal quantities of proteins were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred onto a nitrocellulose membrane, and exposed to monoclonal anti-GFP antibodies (Sigma). (C) After spheroplast fractionation of BY4742 cells expressing MFα-Aβarc-GFP, P13 fractions were treated with proteinase K (0.3125mg/ml) in the presence or absence of 5% Triton X-100, resolved by SDS-PAGE, and then analyzed by immunoblotting.

Figure 4: Schematic view of endocytosis and recycling in yeast

Cellular compartments are indicated: late endosome (LE), multivesicular body (MVB) shown with intralumenal vesicles, vacuole (Vac), and trans-Golgi network (TGN). The proteins
involved in some of the steps of endocytosis and recycling are indicated next to the arrows (note that not all proteins are included, only those tested in this study).

Figure 5: Mammalian PICALM enhances the toxicity of Aβ in yeast

A Ten-fold dilutions of exponentially growing cultures of BY4742 cells transformed with plasmids carrying the different chimeric constructions under the GAL10 promoter and, as indicated, a plasmid containing or not the short form of the PICALM cDNA under the GAL10 promoter were spotted on the same plate onto SD (-) or SG (+) agar supplemented with 20 mg/L histidine, 20 mg/L lysine.

B Deleted strains expressing the different chimeric proteins (6 h of expression) with or without PICALM protein were collected for total protein extracts. Proteins were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred onto a nitrocellulose membrane, and then exposed to polyclonal anti-PICALM antibodies (CALM H-134, Santa Cruz).

Figure 6: GFP pattern of aggregation in yeast mutants

WT or strains deleted for YAP1802, HSP104, RCY1, or SNF7 were grown for 6 h in SG liquid medium supplemented with 0.67% casaminoacids to induce expression of the chimeric proteins and were then examined by epifluorescence microscopy.

Figure 7: Aβ expression leads to a decrease in O2 consumption without changing cytochrome content

A. BY4742 cells transformed with an empty vector (empty) or plasmids carrying the different chimeric constructions under the GAL10 promoter (MFα-GFP and MFα-Aβarc-link-GFP) were grown aerobically in medium containing 2% DL-lactate and 0.2% galactose. B. Respiratory rates of BY4742 cells transformed with an empty vector (empty) or plasmids
carrying the different chimeric constructions under the GAL10 promoter (MFα-GFP and MFα-Aβarc-link-GFP) Results are mean ±S.D. of at least two measurements performed on two independent cell cultures. C. Cytochrome content of transformed BY4742 cells 8h after induction. Results are mean ±S.D. of at least two measurements performed on two independent cell cultures.

Translational Impact box

The amyloid cascade hypothesis put in light the fundamental role of Amyloid β (Aβ) in Alzheimer disease (AD). Although found mainly as extracellular deposits, the role of Aβ seems to impair intracellular mechanisms following the efficient uptake of the peptide. To date, no simple cellular system have been used to extensively study the toxic mechanisms induced by intracellular Aβ and in particular to address the role of its secretion/endocytosis on cellular toxicity. The yeast model has been previously used to study amyloid infectiosity and toxicity in other neurodegenerative disease. More recently, S. cerevisiae has been used successfully to pinpoints genes involve in Aβ toxicity and the role of most of them was confirmed in other eukaryotic models. Here, we present a different approach based also on yeast expression of Aβ. We have set up a new and versatile yeast model in which Aβ toxicity depends both on the allele expressed (arctic mutant being more toxic than the wild type) and on the presence of genes such as PICALM whose the mammalian orthologues are already known as susceptibility factor for AD. Interestingly, in our yeast model PICALM orthologues increase Aβ toxicity, whereas in the other yeast model, it prevents the deleterious effects. These contradictory results highlight the importance of the model used (level of expression, stabilization by translational fusion…) allowing a rational explanation for such discrepancies. In our yeast model Aβ enters the secretory pathway, goes to the plasmic membrane and becomes toxic in further steps. We also provide some evidences arguing for the existence of
different aggregates (toxic and harmless) that depend on the capacity of the protein to be translocated into the secretory pathway or not. Finally, we also demonstrate that Aβ is able to cross the membranes and target mitochondria.
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PICALMs + Ø
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MFα-AB2 -GFP + PICALMs
MFα-ABarc -GFP + Ø
MFα-ABarc -GFP + PICALMs

- GAL

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+ GAL

Ø + Ø
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Figure S1: Expression and toxicity of amyloid beta without GFP.

(A) Ten-fold dilutions of exponentially growing cultures of BY4742 cells transformed with plasmids carrying the different chimeric constructions under the GAL10 promoter were spotted onto SD (-) or SG (+) agar supplemented with 20 mg/L histidine, 20 mg/L lysine, and 60 mg/L leucine. The cells were incubated at 30°C for 3 days. (B): BY4742 cells expressing the different chimeric proteins (6 h of expression) were collected for total-protein extracts. Equal quantities of proteins were separated by SDS-PAGE on a 12 % polyacrylamide gel, transferred onto a nitrocellulose membrane and exposed to anti-Aβ (Tebu) antibodies.
Figure S2: A Ten-fold dilutions of exponentially growing cultures of deleted strains transformed with plasmids carrying the different chimeric constrictions under the GAL10 promoter were spotted on the same plate onto SD (-) or SG (+) agar supplemented with 20 mg/L histidine, 20 mg/L lysine, and 60 mg/L leucine. B Cells expressing the different chimeric proteins (6 h of expression) were collected for total protein extracts. Equal quantities of proteins were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred onto a nitrocellulose membrane, and then exposed to monoclonal anti-GFP antibodies (Sigma).
Figure S3: targeting of Aß in ER.

WT or RFP-tagged strains were grown for 6 h in SG liquid medium supplemented with 0.67% casaminoacids to induce the expression of the chimeric proteins and were then examined by epifluorescence microscopy.