RESEARCH ARTICLE

Alzheimer's Aβ fused to green fluorescent protein induces growth stress and a heat shock response

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Abstract

The 42 amino acid Alzheimer's Aβ peptide is involved in the progression of Alzheimer's disease. Here we describe the effects of intracellular Aβ, produced through its attachment to either end of a green fluorescent protein, in yeast. Cells producing Aβ exhibited a lower growth yield and a heat shock response, showing that Aβ fusions promote stress in cells and supporting the notion that intracellular Aβ is a toxic molecule. These studies have relevance in understanding the role of Aβ in the death of neuronal cells, and indicate that yeast may be a new tractable model system for the screening for inhibitors of the stress caused by Aβ.

Introduction

The 42 amino acid peptide known as Aβ1–42 (herein referred to as Aβ) is considered to be critical in the development of Alzheimer's disease (AD) (Thal *et al.*, 2006) for three reasons. First, it is a predominant protein in the plaques that typify AD, and there appears to be a correlation between the amounts of Aβ and the onset of AD (Kuo *et al.*, 1996). Second, mutations in Aβ known as familial AD mutations lead to an earlier onset age of AD (Tanzi *et al.*, 1993). Third, the addition of Aβ1–40 and Aβ1–42 is toxic to neuronal cells, and may be linked to the death of brain cells (Huang *et al.*, 1999; Sun *et al.*, 2003). Clearly, the ability to investigate the biological effects of Aβ in a eukaryotic model system would be valuable. Yeast serves as an excellent model for the study of human cell biology, and in particular the study of human neurologic disease (Walberg, 2000).

In this study, we have chosen a system to produce Aβ fused to green fluorescent protein (GFP) in the yeast cytosol to monitor its presence and properties. Ideally, we would have worked with native Aβ, but we have not detected any production of native Aβ in yeast, despite the use of a copper-inducible expression system that has previously proved suitable for the production of toxic proteins such as HIV-1 Vpr (Macreadie *et al.*, 1995). Our current thoughts are that a yeast protease may degrade native Aβ as fast as it is produced; however, we have no evidence to support this contention. It is noteworthy that mammalian cells can degrade cytoplasmic Aβ (Buckig *et al.*, 2002; Lee *et al.*, 2006).

Our site of expression of Aβ is the cytosol, whereas native Aβ derives from the amyloid precursor protein APP, which is secreted into the endoplasmic reticulum, processed, and eventually cleaved by proteases to liberate Aβ. Regardless of this difference, we consider that our cytosolic location is worthy of strong consideration, as extracellular Aβ can re-enter and damage neuronal cells, while presumably being exposed to the cytosol. In addition, there are numerous reports of Aβ having a cytoplasmic localization and of this Aβ initiating AD (Glabe, 2001; Wirths *et al.*, 2004). Cytoplasmic Aβ has been found in the eye lenses and brains of AD patients (Gouras *et al.*, 2000; Goldstein *et al.*, 2003), CHO cells (Buckig *et al.*, 2002), human neuroblastoma cells (Lee *et al.*, 2006) and brains of Aβ transgenic mice (Billings *et al.*, 2005).

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In any investigation of the effects of a pathogenic protein such as Aβ, it is important to consider possible detrimental effects, and finding such effects in yeast can be very useful for the advancement of knowledge. Thus, we examined the effects on growth and looked for a specific stress response. Previous studies have shown that Aβ fused to GFP causes GFP misfolding in *Escherichia coli* (Wurth *et al.*, 2002), whereas Aβ fused to mitochondrial release factor (MRF) and caused its oligomerization in yeast (Bagriantsev & Liebman, 2006). *In vitro*, Aβ is known to be involved in the generation of reactive oxygen species (ROS) (reviewed in Adlard & Bush, 2006). Therefore, we had some expectation that Aβ could induce a heat shock response (HSR), as ROS and protein misfolding are both known to be inducers of HSR (Lee *et al.*, 2000; Trotter *et al.*, 2002). Our results show that Aβ fused to GFP causes a stress on growth and induces HSR. It is likely that the HSR can be exploited in further studies to look for inhibitors of Aβ effects.

Materials and methods

Bacterial and yeast strains

For plasmid amplification, *E. coli* strain MC1061(Δ*araA-leu*) 7697 *araD*139 *galK*16 *galE*15 *mcrA*0 *relA*1 *rpsL*150 *spoT*1 *mcrB*9999 *hsdR*2 l^{-} F^{-} was used. Expression in yeast was performed in W303-1a (*MATa*, *ura3*-52, *leu2*-3112, *ade2*-1, *his3*-11, *trp1*-1) and BY4743 *(MATa/*Δ, *his3*Δ*1/his3*Δ*1*, *leu2*Δ*0/leu2*Δ*0*, *lys2*Δ*0/LYS2*, *MET15/met15*Δ*0*, *ura3*Δ*0/ura3*Δ*0*).

Construction of plasmids

For the construction of pAS1N.AβGFP, sequences encoding Aβ were amplified from pBSAPP (obtained from R. Cappai, University of Melbourne) using primers 5′-CGCGATCATG-GATGCAGAATTCCGACATGACTCAGGA and 5′-CGCG ATCCGCTATGACAACACCGCCCACCAT. Following cleavage with MboII to create GATC overhanging ends, the cleaved PCR product was cloned into the BglII site of pAS1N (Prescott *et al.*, 1997). The N-terminal Aβ fusion, pAS1N.GFPAβ, was constructed by inserting sequences encoding Aβ into pAS1N using EagI. The GFP termination codons and other extra codons were deleted to create an in-frame fusion with GFP, as shown in Fig. 1. Deletions were carried out by site-directed mutagenesis on double-stranded DNA using PCR, and this was followed by sequencing to ensure fidelity of sequences. Plasmids encoding wild-type and mutant heat shock element (HSE)-*lacZ* (Sorger & Pelham, 1987) were a gift of Dr Dennis Winge (University of Utah, Salt Lake City, UT).

Organelle localization

For visualization of staining in live yeast, cells were examined by fluorescence microscopy, using 4′,6-diamidino-2-phenylindole for the staining of the nuclear and mtDNA. The staining of lipid particles in yeast was achieved by Nile Red staining using procedures described by Verstrepen *et al.* (2004).

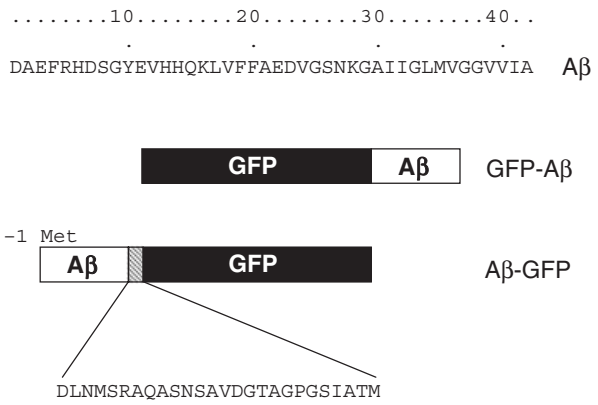

Fig. 1. Schematic diagram of the Aβ–GFP and GFP–Aβ proteins produced in yeast. Expanded sequence refers to linker.

Electron microscopy

Yeast cells were prepared for transmission electron microscopy and immunogold labeling according to the protocol described by Wright (2000). The protocol involves light fixation with aldehydes to preserve antigenicity, followed by ethanol dehydration and embedding in LR White resin. Thin sections were mounted on nickel 200-mesh grids, and were labeled first with either anti-GFP antibody (Chemicon) or anti-Aβ antibody (W02, a gift from R. Cappai, Melbourne University), and then with Aurion Ultrasmall Gold, according to Aurion's supplied protocol. Silver enhancement was performed for 20 min using the Aurion R-Gent SE-EM silver enhancement kit.

Some grids were examined without further processing in a Tecnai 12 transmission electron microscope operating at 120 keV, and images were obtained using a Soft Imaging Systems MegaView III CCD camera. Following initial evaluation of the sections, some were further stained with 2% uranyl acetate and Reynolds lead citrate for increased contrast.

Protein assays

Cells of yeast strain W303-1a transformed with GFP, GFP-Aβ and Aβ-GFP were grown to mid-log phase, and the cells were harvested by centrifugation. The cells were then vortexed with 0.5-mm glass beads in 20 mM Tris (pH 8.0) containing Complete Protease Inhibitor (Roche). The resulting extract was centrifuged at 17 500 ***g*** to give soluble supernatant and pellet fractions. The fractions were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (4–20% Gradipore polyacrylamide gel) and electrophoretically transferred to nitrocellulose for Western

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blot analysis. Blots were probed individually with anti-GFP and anti-Aβ (WO2) monoclonal antibodies for 60 min and then with secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP). Chromogenic detection of bound HRP was accomplished using the color development substrate 3,3′,5,5′-tetramethyl benzidine (McKimm-Breschkin, 1990).

β-Galactosidase assays

β-Galactosidase activity was determined by a modification of the method of Guarente (1983). Yeast cultures (5 mL) were grown overnight in selective media at 30 °C. Cell density ($A_{600\,nm}$) was recorded, and cells were harvested at 4 °C by centrifugation for 1 min at 17 500 ***g***. The cells were resuspended in 1 mL of chilled Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 40 mM β-mercaptoethanol, pH 7.0), and permeabilized at 28 °C for 15–30 min with 0.15 mL of chloroform ($CHCl_3$) and 0.05 mL of 0.1% sodium dodecyl sulfate. The permeabilized cells were then incubated in the presence of 0.2 mL of *o*-nitrophenyl-β-D-galactoside (4 mg mL^{-1}), and the reaction was stopped by addition of 0.5 mL of 1 M Na_2CO_3. Released *o*-nitrophenol (ONP) was measured spectrophotometrically at 420 nm. The ONP-β-D-galactoside-hydrolyzing activity was determined using the formula $A_{420\,nm}/(A_{600\,nm})vt$, where $A_{420\,nm}$ represents the degree of color development during the incubation with ONP-β-D-galactoside, $A_{600\,nm}$ represents the density of culture used, v is the volume of lysate used in each reaction, and t is the time elapsed during the reaction. Specific activities are reported in Miller units ($1000 \times A_{420\,nm}/A_{600\,nm}$ of culture per milliliter of culture per minute of reaction) (Miller, 1972).

Results

Generation of GFP–Aβ in yeast

A yeast expression vector encoding native Aβ did not produce detectable levels of Aβ (data not shown); however, constructs encoding N-terminal and C-terminal fusions of Aβ with GFP were successfully expressed. A yeast episomal vector encoding GFP, pAS1N, was used to produce both fusions, as depicted in Fig. 1. Vector-encoded *LEU2* sequences allowed selection of LEU$^+$ transformed yeast cells in selective media. Both N-terminal and C-terminal fusion proteins were detected by Western blot analysis using anti-GFP antibody (Fig. 2a, lanes GA and AG, pellets). The N-terminal and C-terminal GFP fusions (lanes AG and GA) were *c.* 6 and 4 kDa larger than the GFP control (lane G). Only the fusion proteins were immunoreactive to WO2, an Aβ-specific antibody (Fig. 2b). Further analysis of Fig. 2a shows that, unlike GFP, which is a cytoplasmic protein and in the supernatant, both of the Aβ–GFP fusion proteins were almost entirely localized in the pellet fraction after centrifugation, indicative of Aβ/GFP fusion proteins being localized to an organelle and/or Aβ causing GFP misfolding and/or aggregation.

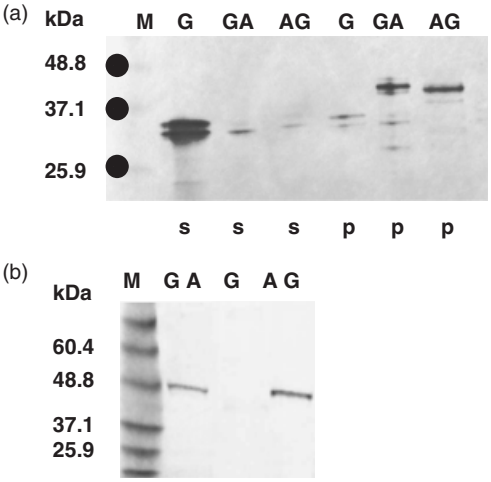

Fig. 2. Western blots of lysates of W303-1a yeast cells that produce GFP, Aβ–GFP and GFP–Aβ. Lanes: M, size markers; G, GFP; AG, Aβ–GFP; GA, GFP–Aβ. (a) Probed with antibody to GFP. (b) Probed with antibody to Aβ. s, supernatant fraction; p, pellet fraction.

Microscopy of yeast with GFP–Aβ

Microscopy on yeast transformants showed that both the N-terminal and C-terminal fusion proteins were fluorescent and present in punctate patches, with possibly small amounts also being cytoplasmic (Fig. 3b). In newly transformed cells, these patches were small and numerous (Fig. 3b), but as cells aged, the fluorescence appeared predominantly in larger patches (Fig. 3c). In contrast, GFP was distributed uniformly throughout the cytoplasm, a localization also observed in older cells (Fig. 3a and e).

In order to further characterize the localization, we examined GFP fluorescence in the presence of various organelle-specific stains. Colocalization of Aβ–GFP with lipid particles was observed using Nile Red stain (Fig. 3d) (Verstrepen *et al.*, 2004). There was minimal staining with Nile Red in cells producing GFP alone (Fig. 3e) and in a proportion of Aβ–GFP-transformed cells that were not fluorescent (Fig. 3c and d). No colocalization of Aβ–GFP with the nucleus or mitochondria was evident when using DNA staining (data not shown).

The contrasting distribution of Aβ–GFP and GFP was confirmed by immunoelectron microscopy with the C-terminal Aβ fusion (N-terminal fusion not done) (Fig. 4). Gold-labeled GFP was dispersed throughout the cytoplasm, whereas gold-labeled Aβ–GFP was observed in amorphous patches: four such patches can be seen in Fig. 4. There did not appear to be an association of Aβ–GFP with any major organelle. Areas within the cell labeling positively for

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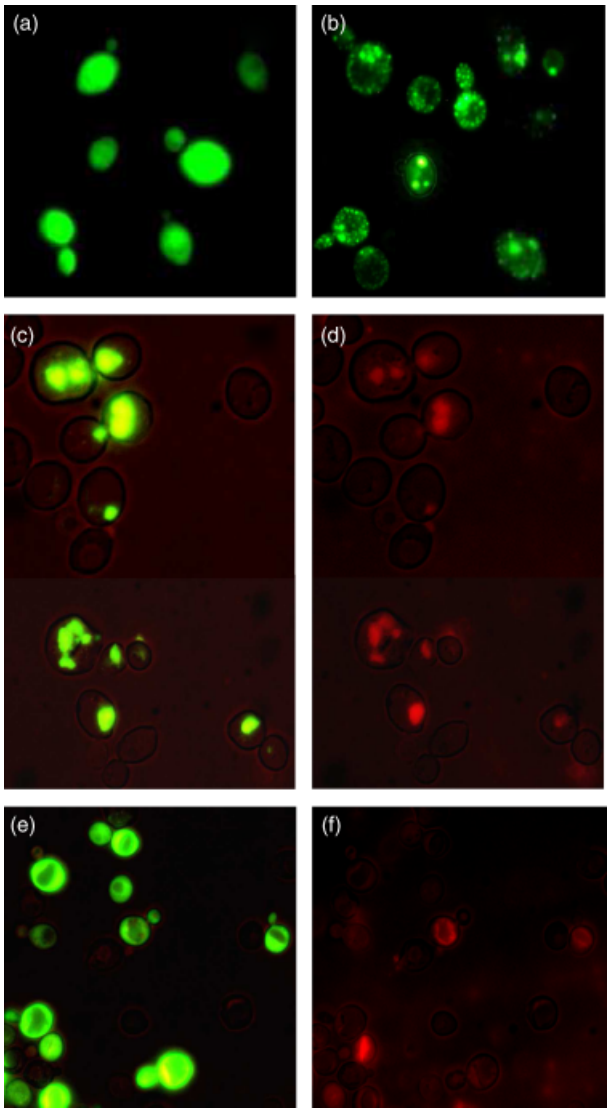

Fig. 3. Fluorescence microscopy of W303-1a yeast cells. New transformants producing GFP (a) and GFP–Aβ (b). Comparison of green fluorescence (c) and Nile Red staining (d) in older transformants producing GFP–Aβ, and the same comparisons in older transformants producing GFP (e, f).

Aβ–GFP appeared to have a smoother texture than the general cytoplasmic area, consistent with Aβ–GFP being localized to lipid particles.

Effects of Aβ on growth and expression of heat shock genes

To gain further insights into the effects of Aβ, it was of interest to look for detrimental effects of the Aβ/GFP fusions. Transformants were examined in growth assays where Aβ fused to GFP was present. As compared to cells producing just GFP, GFP–Aβ caused a 4% decrease in growth (*t*-test, $P = 0.004$) when cells were cultured on synthetic minimal medium (Fig. 5). Similarly, growth of cells producing Aβ–GFP was reduced by 5% (*t*-test, $P = 0.002$) as compared to cells producing GFP alone. It can be concluded that Aβ fused to either end of GFP caused a small but statistically significant reduction in growth.

To investigate whether GFP–Aβ or Aβ–GFP caused a heat shock response (HSR), we cotransformed cells with a reporter construct into the yeast transformants. The reporter comprises a plasmid encoding the yeast heat shock element (HSE2) fused to a downstream β-galactosidase (*lacZ*) gene. When such a plasmid was present in cells where GFP was produced, levels of β-galactosidase were low. In contrast, in cells producing GFP–Aβ or Aβ–GFP, the β-galactosidase levels were increased three-fold (Fig. 6). The mediation of this effect specifically through the HSE was demonstrated via a control construct wherein the HSE was mutated (HSE12). β-Galactosidase activity in all constructs with a mutated HSE was present at levels that were a third of those with wild-type HSE and GFP, and even lower than those with Aβ/GFP fusions.

Discussion

The production of Aβ in yeast has been achieved by fusing of sequences encoding GFP to either end of the Aβ sequence. In yeast producing such fusions, fluorescence was observed, readily allowing the detection of Aβ *in vivo*. This fluorescence contrasts with results from an *E. coli* study where an

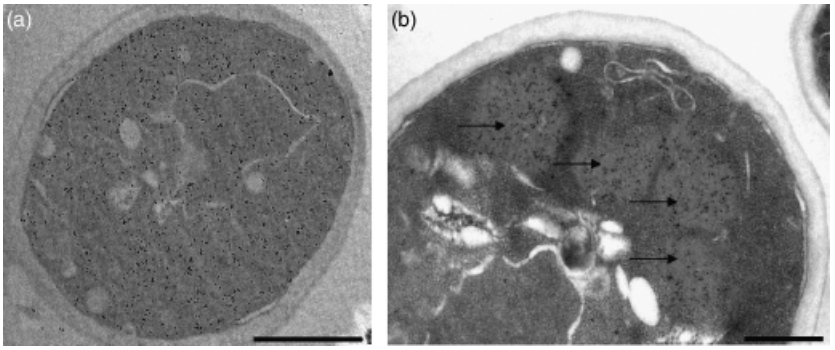

Fig. 4. Immunoelectron microscopy of W303-1a yeast transformants. Antibody to GFP was gold-labeled and probed against (a) cells expressing GFP alone, and (b) cells expressing Aβ–GFP. Note the labeling (arrows) of amorphous bodies. Scale bars are 1 μm in (a) and 0.5 μm in (b).

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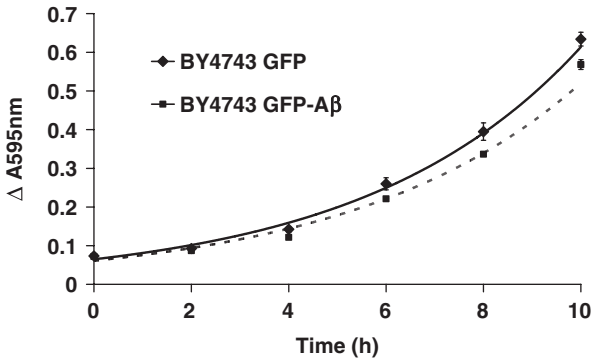

Fig. 5. Growth of W303-1a yeast with GFP, Aβ–GFP and GFP–Aβ. Mean and SE are shown ($n = 3$).

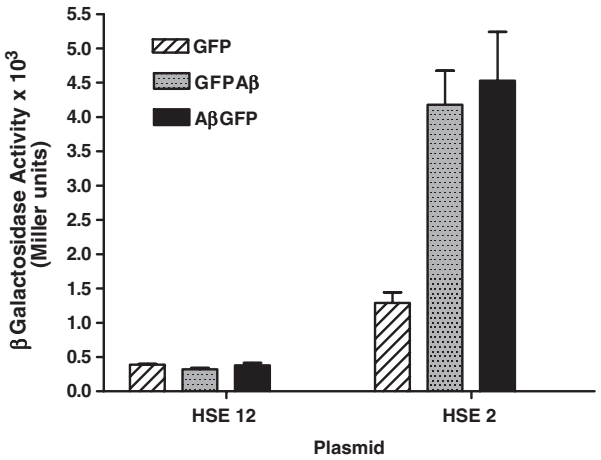

Fig. 6. HSR induced by Aβ in BY4743 yeast cells. β-Galactosidase levels are derived from three to 13 replicates. HSE12 has the mutant HSE, whereas HSE2 contains the wild-type HSE.

Aβ–GFP fusion protein exhibited no fluorescence, due to misfolding or aggregation of the Aβ–GFP fusion protein (Wurth *et al.*, 2002). We conclude that yeast contains an environment permitting correct, or at least sufficient, folding of Aβ with GFP for fluorescence to be observed. Also using yeast, Liebman and colleagues have observed that Aβ fused to a reporter enzyme, MRF, could cause loss of function of the reporter, depending on the degree of oligomerization induced by Aβ (Bagriantsev & Liebman, 2006).

When Aβ was fused to the N-terminus or C-terminus of GFP, the fusion protein had properties vastly different to those of GFP. Only a small proportion of GFP–Aβ or Aβ–GFP appeared in the cytoplasm; the majority (*c.* 95%) appeared in the pellet fraction after centrifugation. Furthermore, fluorescence microscopy revealed that Aβ caused both Aβ/GFP fusions to localize in punctate patches identified as lipid particles, and this result was further supported by immunoelectron microscopy. The very low abundance of similar amorphous structures in cells producing just GFP suggested that the structures were unique to cells producing Aβ–GFP and that the generation of these bodies was specifically induced by the presence of Aβ. The localization of the Aβ fusion protein into lipid particles is consistent with reports that Aβ is localized in lipid rafts (Lee *et al.*, 1998; Morishima-Kawashima & Ihara, 1998). Indeed, major players in the amyloid pathway, such as APP (Ehehalt *et al.*, 2003), APP-cleaving enzymes [β-secretase (Riddell *et al.*, 2001) and γ-secretase (Vetrivel *et al.*, 2004)] and presenilins (PS-1) (Parkin *et al.*, 1999), have also been found to be either located in the lipid rafts or interacting with raft components such as cholesterol (Wahrle *et al.*, 2002; Ehehalt *et al.*, 2003) and gangliosides (Hattori *et al.*, 2006). *In vitro* studies also report Aβ binding to lipoprotein and lipid particles (Koudinov *et al.*, 1998; Wilson *et al.*, 2006). Moreover, there is a profound correlation between AD and lipids, especially lipid rafts (reviewed in Simons & Ehehalt, 2002; Michel & Bakovic, 2007), cholesterol (reviewed in Wolozin, 2001; Yanagisawa, 2002; Yanagisawa & Matsuzaki, 2002; Puglielli *et al.*, 2003) and apolipoprotein E (Weisgraber & Mahley, 1996), in AD.

For each of the transformants producing Aβ fused to GFP, there was a 4% or 5% reduction in growth rates that was statistically significant. Therefore, it is concluded that these fusions impose a growth stress on cells. In addition, an HSR was found in both cell types. The HSR in yeast is triggered by the binding of the conformationally active heat shock transcription factor (HSF) to arrays of 5-bp sequences (nGAAn), called HSEs, present upstream of all heat shock genes (Xiao *et al.*, 1991; Wu, 1995; Hahn *et al.*, 2004). Cells producing GFP–Aβ and Aβ–GFP had an HSR that was threefold higher than that of cells with GFP. This HSR could arise from Aβ-inducing ROS and/or the presence of misfolded protein (Lee *et al.*, 2000; Trotter *et al.*, 2002). Currently, it is not clear whether Aβ causes GFP to misfold or the fusion to oligomerize; Aβ's lipid association makes such an analysis difficult. Similarly, our expression system was too insensitive to measure ROS production in these cells. Future efforts to distinguish these possibilities will involve the use of cells expressing Aβ with nonfluorescent protein fusion partners.

These studies may affect our understanding of Aβ biology in the context of AD. We have shown that even with a relatively low level of Aβ production, there is an effect on growth and a specific stress response. Therefore, it would appear that Aβ, at least when fused to GFP, is a toxic molecule that can stress yeast cells, and perhaps also neuronal cells. Yeast is an excellent model organism to investigate the stress response, and shows highly coordinated gene expression, involving a plethora of transcription factors, each of them performing a specific function to maintain homeostasis (Ruis & Schuller, 1995; Ikner & Shiozaki, 2005). The HSR is a critical cellular stress response that is highly conserved evolutionarily from yeast to humans (Sorger & Pelham, 1987). It manifests as a stress-induced,

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rapid and dramatic increase in the production of protein chaperones. Chaperones are known to facilitate proper folding or refolding of partially unfolded/misfolded proteins or to sequester damaged proteins for proteolytic digestion. The HSR is primarily modulated by the activity of HSF rather than by its concentration. In yeast, HSF is constitutively synthesized, trimerized and bound to promoters before stress (Guarente, 1983). Nearly 3% (165 genes) of the yeast genome can be targeted by HSF (Hahn *et al.*, 2004; Yamamoto *et al.*, 2005). This constellation comprises not only heat shock proteins (HSPs), but also genes involved in a wide range of cellular processes, including ubiquitination and proteolysis, vesicular transport, cell wall organization, small molecule transport, carbohydrate metabolism, energy generation, signal transduction and oxidative stress mechanisms (Hahn *et al.*, 2004; Yamamoto *et al.*, 2005). An analysis of genome-wide transcription in cells producing GFP/Aβ supports the notion that genes with HSEs become upregulated (S. Sankovich, H. George, P. Iliades, I. Macreadie & G. Kornfeld, unpublished data).

Does Aβ also induce an HSR in people with AD? Several studies have shown that HSPs are more abundant in brains of people with AD. HSP70, HSP72 and GRP78 (also known as HSPA5) levels were significantly increased in AD brains and in AD plaques (Hamos *et al.*, 1991; Smith *et al.*, 2005; Wilhelmus *et al.*, 2006).

The recognition that yeast responds to Aβ with an HSR further confirms that Aβ is a deleterious entity. To be more precise, it should be emphasized that this conclusion is extrapolated from studies of Aβ fused to GFP. It is possible that this observation in yeast opens a way to develop an assay to screen for potential therapeutic compounds that abrogate the stress caused by Aβ. It is also possible to study deletion mutants in yeast to identify which proteins involved in the HSR may be most protective in dealing with the damaging effects of Aβ. The further use of yeast in such experiments may lead to a new understanding of AD and new ways of reducing Aβ-induced damage.

Acknowledgements

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