Endoplasmic reticulum chaperones inhibit the production of amyloid- β peptides

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A β (amyloid- β peptides) generated by proteolysis of APP (β -amyloid precursor protein), play an important role in the pathogenesis of AD (Alzheimer's disease). ER (endoplasmic reticulum) chaperones, such as GRP78 (glucose-regulated protein 78), make a major contribution to protein quality control in the ER. In the present study, we examined the effect of overexpression of various ER chaperones on the production of A β in cultured cells, which produce a mutant type of APP (APPsw). Overexpression of GRP78 or inhibition of its basal expression, decreased and increased respectively the level of A β 40 and A β 42 in conditioned medium. Co-expression of GRP78's co-chaperones ERdj3 or ERdj4 stimulated this inhibitory effect of GRP78. In the case of the other ER chaperones, overexpression of some (150 kDa oxygen-regulated protein and calnexin) but not others (GRP94 and calreticulin) suppressed the production of A β . These results indicate that certain ER chaperones are effective suppressors of

A β production and that non-toxic inducers of ER chaperones may be therapeutically beneficial for AD treatment. GRP78 was co-immunoprecipitated with APP and overexpression of GRP78 inhibited the maturation of APP, suggesting that GRP78 binds directly to APP and inhibits its maturation, resulting in suppression of the proteolysis of APP. On the other hand, overproduction of APPsw or addition of synthetic A β 42 caused up-regulation of the mRNA of various ER chaperones in cells. Furthermore, in the cortex and hippocampus of transgenic mice expressing APPsw, the mRNA of some ER chaperones was up-regulated in comparison with wild-type mice. We consider that this up-regulation is a cellular protective response against A β .

Key words: amyloid- β peptides (A β), β -amyloid precursor protein (APP), endoplasmic reticulum (ER) chaperone, glucose-regulated protein 78 (GRP78), protein maturation.

INTRODUCTION

AD (Alzheimer's disease) is the leading cause of adult onset dementia, with a dramatic increase in the incidence of AD apparent in our rapidly aging society. AD is pathologically characterized by the accumulation of tangles and senile plaques. Senile plaques are composed of the A β (amyloid- β peptides), A β 40 and A β 42 [1,2]. A β is generated by secretase-dependent proteolysis of APP (β -amyloid precursor protein). Prior to proteolysis, APP undergoes modification [for example, by N-glycosylation in the ER (endoplasmic reticulum) and O-glycosylation in the Golgi apparatus]. In order to generate A β 40 and A β 42, APP is first cleaved by β -secretase and then by γ -secretase. For the cleavage of APP, β -secretase competes with α -secretase, which produces non-amyloidogenic peptides [3,4]. γ -Secretase is an aspartyl protease complex composed of four core components, including PS1 (presenilin 1) and PS2 [5]. The early onset familial form of AD (FAD) is linked to three genes, APP, PS1 and PS2 [5,6], strongly suggesting that the production of A β is a key factor in the pathogenesis of AD. Therefore, cellular factors that suppress the generation of A β provide important drug targets for the treatment of AD.

Proteins, including APP, first translocate into the ER where they undergo modification. N-glycosylation of APP in the ER is essential for the generation of A β [4]. The ER is also proposed

to be important for A β -induced apoptosis of neuronal cells; for example, a potential intracellular target of A β in mediating apoptosis, ERAB (ER-associated $\alpha\beta$ -binding protein), is an ER membrane protein [7,8]. Accumulation of unfolded protein in the ER induces the ER stress response, a process involving three types of ER transmembrane protein: IRE1 (protein-kinase and site-specific endoribonuclease), PERK (protein kinase R-like ER kinase) and ATF 6 (activating transcription factor 6) [9–11]. ER stressors phosphorylate PERK, which in turn phosphorylates eIF2 α (eukaryotic initiation factor-2 α), leading to activation of ATF4 expression (ATF4 pathway) [12,13]. ER stressors also cause cleavage of p90^{ATF6} into p50^{ATF6}, which translocates to the nucleus (ATF6 pathway) [11]. Both ATF4 and p50^{ATF6} specifically activate transcription of ER stress response-related genes, including those genes that encode ER chaperones. A close relationship between the ER stress response and A β has been suggested; mutations in the PS1 or PS2 genes increase cellular sensitivity to ER stressors by suppressing the activation of IRE1, PERK and ATF6 [14–18]. These observations suggest that the ER is an important cellular compartment for the pathogenesis of AD.

ER chaperones, such as GRP78 (glucose-regulated protein 78), GRP94, ORP150 (150 kDa oxygen-regulated protein), CRT (calreticulin) and CNX (calnexin), contribute greatly to protein quality control in the ER by assisting the refolding of unfolded proteins [19–21]. Therefore, it is reasonable to speculate that ER

Abbreviations used: A β , amyloid- β peptides; AD, Alzheimer's disease; AICD, β -amyloid precursor protein intracellular domain; APP, β -amyloid precursor protein; APPsw, Swedish mutant of APP; APPwt, wild-type APP; ATF6, activating transcription factor 6; CNX, calnexin; Co-IP, co-immunoprecipitation; CRT, calreticulin; CTF α , C-terminal fragment α ; DAPT, N-[N-(3,5-difluorophenyl)-L-alanyl]-S-phenylglycine *t*-butyl ester; DMEM, Dulbecco's modified Eagle's medium; EDEM, endoplasmic reticulum degradation-enhancing α -mannosidase I-like protein; eIF2 α , eukaryotic initiation factor-2 α ; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERdj4 Δ J, J domain-deleted ERdj4; FAD, familial AD; GRP, glucose-regulated protein; HEK-293, human embryonic kidney 293; HSP, heat shock protein; imAPP, immature APP; IRE1, protein-kinase and site-specific endoribonuclease; mAPP, mature APP; ORP150, 150 kDa oxygen-regulated protein; PERK, protein kinase R-like ER kinase; PS1, presenilin 1; RT, reverse transcriptase; sELISA, sandwich ELISA; siRNA, small interfering RNA.

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chaperones affect the generation of A β and the pathogenesis of AD. In fact, some ER chaperones have been shown to physically interact with APP, and overexpression of GRP78 in cells decreases the level of both mature APP and secreted A β [22,23]. Furthermore, the accumulation of GRP78 in senile plaques, the up-regulation of ER chaperones in the brains of AD patients and the co-localization of ER chaperones with A β have all been reported [24–26]. In the present study, we systematically examined the effect of overexpression of various ER chaperones and found that some, but not all, suppress the generation of A β *in vitro*. We propose that this suppression is due to inhibition of the secretase-dependent proteolytic processing of APP through direct interaction between ER chaperones and APP, resulting in the inhibition of APP maturation. Furthermore, we found that ER chaperones are up-regulated not only in cultured neuronal cells overproducing mutant forms of APP or treated with synthetic A β 42, but also in the cortex and hippocampus of transgenic mice expressing mutant APP.

MATERIALS AND METHODS

Cell culture and overexpression of ER chaperones

HEK-293 (human embryonic kidney 293) or SH-SY5Y cells were cultured in DMEM (Dulbecco's modified Eagle's medium) or DMEM/Ham's-F12 medium respectively, supplemented with 10 % (v/v) fetal bovine serum, 100 units/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere of 95 % air with 5 % CO₂ at 37°C. SH-SY5Y cells expressing APP^{sw} (Swedish mutant of APP) or APP^{wt} (wild-type APP) were described previously [27]. For transient expression of each gene, cells were seeded 24 h before the transfection in 24-well plates at a density of 1.5×10^5 cells/well. The transfection was carried out using LipofectamineTM 2000 (Invitrogen) according to the manufacturer's instructions. Cells were used for experiments after a 24 h recovery period. Transfection efficiency was determined in parallel plates by transfection of the pEGFP-N1 control vector. Transfection efficiency was greater than 90 % in all experiments. The stable transfectants expressing each gene were selected by immunoblotting or real-time RT (reverse transcriptase)-PCR analyses. Positive clones were maintained in the presence of 800 μ g/ml G418, 100 μ g/ml zeocin or 200 μ g/ml hygromycin.

Immunoblotting analysis

Whole cell extracts were prepared as described previously [28]. For detection of CTF α (C-terminal fragment α) and CTF β , membrane fractions were prepared as described previously [29]. The protein concentration of samples was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories), according to the manufacturer's instructions. Samples were applied to 7 % (for APP), 8 % (for GRP78), 10 % (for actin), 12 % (for His-tagged ERdj3), 15 % (for Myc-tagged ERdj4) or 16.5 % (for CTF α and CTF β ; v/v) polyacrylamide gels and subjected to SDS/PAGE, after which proteins were immunoblotted with respective antibodies.

sELISA (sandwich ELISA) assay for A β

Cells were cultured for 24 h and the conditioned medium was subjected to sELISA using three types of specific monoclonal antibody, as described previously [30].

Co-IP (co-immunoprecipitation) assay

Co-IP was carried out as described previously [31], with some modifications. Cells were harvested, lysed with buffer (10 mM

Hepes/KOH, pH 7.4, 150 mM NaCl and 0.5 % Triton X-100) and centrifuged at 20000 g. The antibody against the C-terminal fragment of APP was added to the supernatant, and the sample incubated for 12 h at 4°C with rotation. Dynabeads[®] coated with Protein A were then added and the samples were incubated for 2 h at 4°C and rotated, after which they were centrifuged at 20000 g. The beads were then washed four times with the same buffer and the proteins were eluted by boiling the beads in SDS sample buffer [62.5 mM Tris/HCl, pH 6.8, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol, 10 % (v/v) glycerol and 0.001 % Bromophenol Blue].

Real-time RT-PCR analysis

Total RNA was extracted from cells and mouse brain using an RNeasy kit according to the manufacturer's protocols (Qiagen). Samples were reverse-transcribed using a first-strand cDNA synthesis kit according to the manufacturer's instructions (Amersham Biosciences). Synthesized cDNA was subjected to real-time RT-PCR using SYBR[®] Green PCR Master Mix (Applied Biosystems) and analysed with ABI PRISM 7500 Sequence Detection software according to the manufacturer's instructions (Applied Biosystems). Real-time cycle conditions were 2 min at 50°C, followed by 10 min at 90°C and then 45 cycles at each of 95°C for 30 s and 63°C for 60 s. Specificity was confirmed by electrophoretic analysis of the reaction products and by inclusion of template- or RT-free controls. To normalize the amount of total RNA present in each reaction, the actin gene was used as an internal standard.

Statistical analysis

All values are expressed as the means $\pm$ S.D. Student's *t* test for unpaired results was used for the evaluation of differences between the two groups. Differences were considered to be significant for *P* < 0.05.

RESULTS

ER chaperones inhibit the generation of A β

We based our investigations on HEK-293 cells that stably express APP with the double mutations, K651N/M652L, known as the 'Swedish' mutations (APP^{sw}) [30]. These mutations elevate cellular and secreted levels of A β [32]. The effect of ER chaperones on the generation of A β 40 and A β 42 was monitored by determining the amount of these peptides in conditioned medium by sELISA after transient transfection of the cells with expression plasmid for each ER chaperone. Transient overexpression of GRP78 (Figure 1A) caused a decrease in the level of A β 40 and A β 42 (Figures 1B and 1C) suggesting that GRP78 inhibits the production of A β . This was confirmed using siRNA (small interfering RNA). Transfection of siRNA against GRP78 not only caused a decrease in the background cellular expression of GRP78 (Figure 1D), but also led to a weak increase in the level of A β 40 and A β 42 in the conditioned medium (Figures 1E and 1F). The weakness of the induction may be due to other ER chaperones compensating for the function of GRP78 under the experimental conditions (see Supplementary Figure S2 at <http://www.BiochemJ.org/bj/402/bj4020581add.htm>).

GRP78 belongs to the HSP70 (heat shock protein 70) family of proteins for which co-chaperones have also been identified [19]. For example, HSP40 binds to HSP70, stimulating its ATPase and chaperone activities [33]. Various co-chaperones have been suggested for GRP78, among which ERdj3 and ERdj4 have been

Figure 1 Inhibition of A β generation by transient expression of GRP78

HEK-293 cells expressing APPsw were transiently transfected either with expression plasmid for GRP78 or control vector (**A–C**), or with siRNA against GRP78 (siGRP78) or non-silencing (ns) siRNA (**D–F**), after which they were cultured for 24 h. Whole cell extracts (10 μ g protein) were analysed by immunoblotting with an antibody against GRP78 (**A**) or actin (**D**). To detect GRP78 in the control sample, a relatively longer exposure was used in (**D**) than in (**A**). The amount of A β 40 and A β 42 in the conditioned medium was determined by sELISA and expressed relative to the control. Results are means $\pm$ S.D. ($n=3$). ***, $P < 0.001$; *, $P < 0.05$ (**B, C, E and F**).

shown to bind to GRP78, enhancing its ATPase and chaperone activities [34,35]. Here we examined the effect of overexpression of ERdj3 and ERdj4, or their co-expression with GRP78, on A β generation. Overexpression of ERdj3 or ERdj4 was confirmed by immunoblotting (see Supplementary Figure S1 at <http://www.BiochemJ.org/bj/402/bj4020581add.htm>) and real-time RT-PCR (results not shown) and found that the level of overexpression of GRP78 (or ERdj3 and ERdj4) was not affected by simultaneous overexpression of ERdj3 and ERdj4 (or GRP78; see Supplementary Figure S1). As illustrated in Figures 2(A) and 2(B), transfection of an expression plasmid for ERdj3 decreased the level of both A β 40 and A β 42. Furthermore, co-transfection of expression plasmids for both GRP78 and ERdj3 produced an even greater decrease (Figures 2A and 2B). Although the transfection of an expression plasmid for ERdj4 significantly decreased the level of A β 40 but not A β 42 (Figures 2C and 2D), co-transfection of expression plasmids for both GRP78 and ERdj4 caused a clear decrease in both peptides; co-overexpression of both GRP78 and ERdj4 decreased the level of A β 42 to about 30 % of the control level (Figure 2D). These results show that ERdj3 and ERdj4 stimulate the inhibitory effect of GRP78 on the generation of A β and suggest that this effect of GRP78 involves its ATPase and chaperone activities. The slight inhibitory effect of overexpression of ERdj3 or ERdj4 alone on the generation of A β may be due to the activation of endogenous GRP78 by these co-chaperones.

The J domain of the HSP40 family of proteins is responsible for their interaction with the HSP70 family of proteins [33]. It has been shown that the J domain of ERdj4 is essential for its interaction with GRP78 [35]. As shown in Figures 2(E) and 2(F), and in comparison with the results obtained with the wild-type

Figure 2 Stimulation by ERdj3 or ERdj4 of inhibitory effect of GRP78 on A β generation

HEK-293 cells expressing APPsw were transiently transfected with expression plasmid for GRP78 (**A–F**), ERdj3 (**A and B**), ERdj4 (**C and D**), ERdj4 Δ J (**E and F**) and/or control vector (**A–F**), with total DNA amounts fixed at 1 μ g, and cultured for 24 h. The amount of A β 40 and A β 42 in the conditioned medium was determined and expressed as described in the legend for Figure 1. Results are means $\pm$ S.D. ($n=3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$. n.s., not significant.

ERdj4 (Figures 2C and 2D), transfection of an expression plasmid for ERdj4 Δ J (J domain-deleted ERdj4) had less activity in the stimulation of the effect of GRP78 on A β generation. A similar level of overexpression between wild-type ERdj4 and ERdj4 Δ J was confirmed by immunoblotting (see Supplementary Figure S1) and real-time RT-PCR (results not shown). These findings suggest that the inhibitory effect of ERdj4 (and maybe ERdj3) on A β production seems to be achieved via its interaction with GRP78.

We also examined the effect of ER chaperones other than GRP78 (ORP150, GRP94, CNX and CRT). Overexpression of

Figure 3 Inhibition of A β generation in clones stably expressing ER chaperones

HEK-293 clones expressing APPsw and the indicated ER chaperones were cultured for 24 h. The amount of A β 40 and A β 42 in the conditioned medium was determined and expressed as described in the legend for Figure 1. Results are means $\pm$ S.D. ($n=3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

each ER chaperone was confirmed by immunoblotting and/or real-time RT-PCR analyses (results not shown). The results revealed that these ER chaperones can be classified into three groups. As well as GRP78, overexpression of ORP150 decreased the level of both A β 40 and A β 42. Overexpression of CNX decreased the level of A β 42 but not that of A β 40. On the other hand, the expression of GRP94 and CRT had no effect on the level of either A β 40 or A β 42 (see Supplementary Figure S2). Thus, while the suppression of A β production is not specific to GRP78, neither is it a general feature of all ER chaperones.

Mechanism for inhibitory effect of GRP78 on A β generation

In order to examine the molecular mechanism governing the inhibitory effect of GRP78 on A β production, we produced HEK-293 cells that stably expressed not only APPsw, but also GRP78, His-tagged ERdj3 and/or Myc-tagged ERdj4. Expression of each ER chaperone was confirmed by immunoblotting (which are shown in Figures 5A and 5C). First, we examined the production of A β in each clone. As shown in Figure 3, the level of both A β 40 and A β 42 in the conditioned medium was decreased for the GRP78-overexpressing clone. A further decrease was observed for clones overexpressing GRP78 and ERdj3 or ERdj4, which was consistent with the results obtained from our transient-expression experiments (Figure 2). However, the difference in the effects of ERdj4 and ERdj3, as illustrated in Figure 2, was not observed in stable-expression experiments (Figure 3).

Figure 4 Co-IP of GRP78 with APP

Whole cell extracts prepared from HEK-293 clones expressing APPsw and the indicated ER chaperones were precipitated with or without antibody against the CTF of APP. Whole cell extracts (WCE) and the precipitates (PPT) with or without the antibody were analysed by immunoblotting with antibodies against GRP78 or APP.

Using these clones, the physical interaction of GRP78 with APP was estimated by co-IP. The mature (N- and O-glycosylated) and immature (N-glycosylated alone) forms of APP (mAPP and imAPP respectively) can be separated by SDS/PAGE on the basis of their molecular masses [36]. As shown in Figure 4, GRP78 was co-precipitated with APP in a manner that was dependent on the antibody against APP, which showed that GRP78 physically interacted with APP. Furthermore, co-expression of either ERdj3 or ERdj4 slightly stimulated this interaction (Figure 4).

We then examined the maturation of APP, an essential step in the production of A β that occurred in ER. As shown in Figures 5(A) and 5(B), the amount of mAPP relative to imAPP was decreased in the GRP78-overexpressing clone, with a further decrease being observed in the clone expressing both GRP78 and ERdj3. Similar results were obtained with ERdj4 (Figures 5C and 5D). These results suggest that GRP78 inhibits the maturation of APP and that this action is stimulated by its co-chaperones.

We also performed pulse-chase labelling experiments. Proteins were pulse-labelled with [35 S]methionine, chased with excess amounts of cold methionine, precipitated with antibody against APP and then examined by autoradiography. Compared with the controls, the conversion of labelled imAPP to mAPP and the disappearance of labelled imAPP and mAPP were retarded in clones expressing GRP78/ERdj3 or GRP78/ERdj4 (see Supplementary Figure S3 at <http://www.BiochemJ.org/bj/402/bj4020581add.htm>). These results show that the maturation of APP was inhibited, whereas the half-life of APP was prolonged, by the overexpression of GRP78/ERdj3 or GRP78/ERdj4.

Based on the results described above, we speculated that the inhibition of A β production by ER chaperones is due to the inhibition of secretase-dependent APP proteolysis as a result of inhibition of APP maturation. In order to assess this, we attempted to detect the CTFs of APP that are generated by α -, β - and γ -secretase (CTF α , CTF β and CTF γ respectively). CTF γ could not be detected using our experimental conditions (results not shown). However, as shown in Figure 6, the amount of CTF α and CTF β was decreased in the GRP78-overexpressing clone, with a further decrease being observed in clones overexpressing GRP78/ERdj3 or GRP78/ERdj4. Based on these results we consider that proteolysis of APP by α - and β -secretases is inhibited in cells overexpressing these ER chaperones.

Figure 5 Effect of ER chaperones on the maturation of APP

HEK-293 clones expressing APPsw and the indicated ER chaperones were cultured for 24 h. Whole cell extracts (10 μ g protein) were analysed by immunoblotting with antibodies against GRP78, the CTF of APP, the His-tag (for ERdj3), the Myc-tag (for ERdj4) and actin (**A** and **C**). The band intensity ratio (mAPP/imAPP) was determined (**B** and **D**). Similar results were obtained in independent experiments.

Figure 6 Effect of ER chaperones on the amount of CTF α and CTF β in cells

HEK-293 clones expressing APPsw and the indicated ER chaperones were cultured for 24 h. Membrane fractions (20 μ g protein) were analysed by immunoblotting with antibodies against APP and the CTF α and CTF β of APP in response to GRP78 and ERdj3 (**A**) or ERdj4 (**C**). The band intensity was determined and expressed relative to the control (**B** and **D**). Similar results were obtained in independent experiments.

Unfolded or misfolded proteins in the ER are degraded by a system, called ERAD (ER associated degradation), which is mediated by the proteasome system [9]. Therefore, it is possible that ERAD is involved in the inhibitory effect of GRP78 on A β production. To address this issue, we examined the effect of a proteasome inhibitor (lactacystin) on A β production. As shown in Supplementary Figure S4 (<http://www.BiochemJ.org/bj/402/bj4020581add.htm>), lactacystin did not affect the level of A β production in either the presence or absence of GRP78-over-expression, excluding a possibility described above.

Up-regulation of mRNA of ER chaperones by A β

It is well known that A β is toxic to neuronal cells both *in vitro* and *in vivo*, and this toxicity seems to play an important role in the pathogenesis of AD [37]. The results described above suggest that ER chaperones protect cells against A β by decreasing the amount of secreted A β . Therefore, it is reasonable to speculate that cells up-regulate ER chaperones in response to A β in order to protect themselves. To test this idea, we first compared the mRNA expression of various ER chaperones between APPsw-overexpressing, APPwt-overexpressing and control neuroblastoma (SH-SY5Y) cells [27]. As shown in Figure 7(A), the mRNAs of all of the ER chaperones tested were up-regulated in APPsw-overexpressing cells but not in APPwt-overexpressing cells. We also showed that GRP78 was weakly induced by over-expression of APPsw but not APPwt (see Supplementary Figure S5 at <http://www.BiochemJ.org/bj/402/bj4020581add.htm>). An inhibitor of γ -secretase, DAPT {N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phenylglycine *t*-butyl ester}, attenuated this up-regulation (Figure 7A), strongly suggesting that γ -secretase-dependent proteolytic fragments of APP (such as A β), but not APP itself, are responsible for this upregulation. As shown in Figure 7(B), the level of A β in the culture medium was much higher in APPsw-overexpressing cells than APPwt-overexpressing cells, and treatment of cells with DAPT caused the decrease in the level of A β . We therefore examined the effect of adding synthetic A β 42 to the conditioned medium on mRNA expression of various ER chaperones (Figure 7C). In all cases there was a dose-dependent upregulation of the ER chaperone mRNA. A β 42 at a concentration of 0.1 μ M or 1 μ M did not affect the cell viability; however, treatment of cells for 48 h with 10 μ M A β 42 caused apoptosis in 5–10% of the cells (results not shown).

Both the ATF4 and ATF6 pathways are involved in the up-regulation of ER chaperones by ER stressors [11–13]. In the present study, we used siRNAs against ATF4 and ATF6 to examine the contribution of these transcription factors to A β 42-dependent up-regulation of ER chaperones. As shown in Figures 8(A) and 8(B), transfection of a given siRNA suppressed the expression of its target gene, but not the other gene, regardless of the presence or absence of A β 42. A β 42-dependent up-regulation of GRP78 mRNA was partially suppressed by siRNA against either ATF4 or ATF6 (Figure 8C). Similar results were obtained for A β 42-dependent up-regulation of mRNA of other ER chaperones (Figures 8D–8I). None of the transfections illustrated in Figure 8 affected the baseline cell viability (results not shown). These results suggest that both the ATF4 and ATF6 pathways are involved in the up-regulation of ER chaperones by A β 42.

In order to test the *in vivo* relevance of the upregulation of ER chaperones by expression of APPsw in neuronal cells, we compared the mRNA expression of various ER chaperones in the brains (cortex and hippocampus) of transgenic mice expressing APPsw (APP23) and wild-type mice. The cortex and hippocampus were chosen for investigation as these are the main areas involved in senile plaque formation [38,39]. As shown in Figure 9,

Figure 7 Up-regulation of mRNA of various ER chaperones by expression of APPsw or addition of synthetic A β 42

SH-SY5Y clones expressing APPwt, APPsw and vector control were cultured for 48 h in the presence or absence of 1 μ M DAPT (**A** and **B**). SH-SY5Y cells were cultured for 48 h in the presence of the indicated concentrations of A β 42 (**C**). Total RNA was extracted and subjected to real-time RT-PCR using specific primers for each chaperone. Values were normalized to actin gene expression and expressed relative to the control (**A** and **C**). The amount of A β 40 and A β 42 in the conditioned medium was determined and expressed as described in the legend for Figure 1(B). Results are means $\pm$ S.D. ($n=3$). ***, $P < 0.001$; **, $P < 0.01$.

the mRNA level of some, but not all, of the tested ER chaperones were significantly up-regulated in APP23 mice (at 6-months old) compared with wild-type controls. The increase in the amount of A β and development of senile plaques were reported in APP23 mice of this age [40]. Among the ER chaperones, both GRP78 and ORP150 were significantly up-regulated at the mRNA level in both the cortex and hippocampus of APP23 mice (Figure 9). These findings suggest that the *in vitro* results obtained in the present study are functionally significant, reflecting the *in vivo* relevance of our cell culture studies.

DISCUSSION

In the present study, we have shown that, consistent with previous results [22], overexpression of GRP78 in cells decreases the amount of A β 40 and A β 42 in conditioned medium. Furthermore, we found that some, but not all, of the ER chaperones have a similar activity. Expression of ORP150 decreased the level of both A β 40 and A β 42 more significantly than GRP78, whereas expression of CNX decreased the level of A β 42 alone; neither CRT nor GRP94 had any effect. At present, it is unclear what underlies these differences. Given that CNX and CRT have similar biochemical activities and are thought to play similar roles in cells [21], their differing effect on A β 42 production is of particular interest. One possibility is that their different cellular locations are responsible for their differing effects. Similar to APP, CNX locates in the ER membrane, whereas CRT is an ER-soluble protein [21]. This difference in location is believed to underlie the differing contributions of these proteins to ERAD; CNX, but not CRT, binds to EDEM (ER degradation-enhancing α -mannosidase I-like protein; an important protein for ERAD), resulting in the stimulation of ERAD [41].

In order to uncover the mechanism responsible for the decrease in the level of secreted A β following overexpression of ER chaperones, we performed several experiments. Since the level of CTF α and CTF β also decreased in cells expressing ER chaperones, the decrease in the level of secreted A β seems to be due to inhibition of secretase-dependent proteolytic processing of APP. Co-expression of ERdj3 or ERdj4 but not ERdj4 Δ J stimulated the GRP78-dependent inhibition of A β production. We also showed that GRP78 was co-immunoprecipitated with APP. Furthermore, overexpression of GRP78/ERdj3 or GRP78/ERdj4 inhibited the maturation of APP in cells. These results suggest that GRP78 binds directly to APP, inhibiting its maturation, which results in the suppression of secretase-dependent proteolytic processing of APP. We consider that the interaction of GRP78 with APP inhibits the translocation of APP from the ER to the Golgi apparatus, where maturation of APP is completed [4].

We also found that overproduction of APPsw in cells causes up-regulation of the mRNAs of various ER chaperones. Given that this upregulation is diminished by treatment of cells with an inhibitor of γ -secretase (DAPT), and that addition of synthetic A β 42 to the conditioned medium also caused upregulation of the mRNA of various ER chaperones, A β but not APP itself seems to be responsible for this up-regulation. However, in experiments designed to examine the effect of overproduction of APPsw in cells, the concentration of A β 42 in the conditioned medium was about 0.3 nM, this being much lower than the concentration of synthetic A β 42 required for up-regulation of ER chaperones (about 100 nM). There are three possibilities to explain this discrepancy: (i) endogenous A β 42 is more active than the synthetic form in terms of up-regulating ER chaperones; (ii) endogenous A β 42 acts in cells before being secreted into the conditioned medium; and (iii) although a previous study has shown that A β 42 is more neurotoxic than A β 40 [42], it is possible that A β 40 but not A β 42 is responsible for the up-regulation of ER chaperones. Another potential discrepancy lies in the findings of Kadowaki et al. [43], who reported that A β 42 (up to 40 μ M) did not up-regulate GRP78 in PC12 cells, based on immunoblotting and RT-PCR (not real-time RT-PCR) experiments. However, this difference may be due to variations in the cell types and experimental methods used.

In terms of the mechanism underlying the up-regulation of ER chaperones by A β , we found that siRNA against either ATF4 or ATF6 partially suppressed this effect, indicating the involvement of both the PERK/eIF2 α /ATF4 and ATF6 pathways. Another ER transmembrane protein, IRE1, may also be involved. However,

Figure 8 Effect of siRNA for ATF4 or ATF6 on the A β 42-dependent up-regulation of ER chaperone mRNA

SH-SY5Y cells transfected with siRNA against ATF4 (siATF4), ATF6 (siATF6) and/or non-silencing (ns) siRNA (the total amount of siRNA was fixed at 5 μ g) were incubated with or without 10 μ M A β 42 for 48 h. Total RNA was extracted and subjected to real-time RT-PCR by use of specific primers for each gene. Values were analysed and expressed as described in the legend for Figure 7(A). Results are means $\pm$ S.D. (n = 3). ***, P < 0.001; **, P < 0.01; *, P < 0.05.

since none of the siRNAs against IRE1 that were tested in the present study significantly suppressed the target gene (results not shown), we could not test the contribution of the IRE1-pathway to A β 42-dependent up-regulation of ER chaperones. In considering the mechanism upstream of activation of ER transmembrane proteins by A β , we believe that an increase in intracellular Ca²⁺ plays an important role. It is well known that changes in cellular Ca²⁺ levels can induce the ER stress response [44,45]. It has also been reported that addition of A β to neuronal cells leads to a rise in the concentration of intracellular Ca²⁺ by stimulating the influx of extracellular Ca²⁺ and efflux of ER Ca²⁺ [46,47]. It was found recently that AICD (β APP intracellular domain) stimulates the

transcription of some genes [48]. Therefore, it is also possible that AICD is involved in the up-regulation of ER chaperones by A β .

In the cortex and hippocampus of transgenic mice expressing APPsw, some ER chaperones were also up-regulated, suggesting that A β exerts an effect both *in vitro* and *in vivo*. It is therefore possible that this *in vivo* up-regulation contributes to the protection of neurons by inhibiting the production of A β , which was also observed *in vitro*. Furthermore, as described above, the accumulation of GRP78 in senile plaques, the up-regulation of ER chaperones in the brain of AD patients and the co-localization of ER chaperones and A β have all been reported [24,25], highlighting

Figure 9 Expression of mRNA of various ER chaperones in cortex and hippocampus in transgenic mice expressing APPsw

Cortex and hippocampus were taken from transgenic mice expressing APPsw (APP23) and wild-type mice at 6-months old. Total RNA was extracted and subjected to real-time RT-PCR using specific primers for each chaperone. Values were analysed and expressed as described in the legend for Figure 7(A). Results are means $\pm$ S.D. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

the possible protective role of ER chaperones against A β and AD. Therefore, we propose that non-toxic inducers of ER chaperones may be therapeutically beneficial for AD. It is well known that ER chaperones impair the aggregation of protein in the ER [19]. It has also been demonstrated that A β aggregates intracellularly, and that this aggregation plays an important role in the pathogenesis of AD [49], although the precise location at which this aggregation occurs has not yet been determined. If that site is the ER, non-toxic inducers of ER chaperones may prove valuable in the treatment of AD, not only by decreasing the level of A β , but also by impairing the aggregation of A β in cells. Both previous studies [22] and the present one have examined the effect of ER chaperones on the level of A β in APP-overexpressing cells. Similar analysis in wild-type cells may be important to estimate the role of ER chaperones in the production of A β and the treatment of AD.

When we were preparing this manuscript, a related article was published by Kudo et al. [50], which showed that thapsigargin or tunicamycin, both of which induce ER chaperones, inhibit the maturation of APP and retained APP in the early compartment of the secretory pathway (such as the ER) when they decreased the amount of secreted A β .

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