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In cerebrospinal fluid ER chaperones ERp57 and calreticulin bind β-amyloid

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Abstract

The β-amyloids (abetas) are the major components of the plaque observed in the brains of patients with Alzheimer's disease. The conundrum is that although they are produced in everyone during the posttranslational processing in the endoplasmic reticulum (ER) of the amyloid precursor protein (APP), deposits are only observed in the elderly. Our work suggests that normals have a carrier protein(s) keeping them in solution. Based on immunoblotting studies of cerebrospinal fluid (CSF) from normals, we find that the bulk of the abetas are bound to the ER chaperones, ERp57 and calreticulin, suggesting that these may be carrier proteins which prevent aggregation of the abetas and that the deposits are due to faulty ER posttranslational processing of APP with the failure to form this complex. If membrane protein synthesis is similarly affected, it could explain the neuronal dysfunction characteristic of Alzheimer's disease.

Keywords: Alzheimer's disease; Dementia; β-Amyloids; ERp57; Calreticulin; Posttranslational protein processing

Alzheimer's disease is characterized by dementia and the presence of plaque in the neocortex containing deposits of the abetas. The abetas are a series of 38–43 amino acid peptides produced during the normal, posttranslational processing of a 120 kDa protein, amyloid precursor protein (APP) [1–4]. Under physiological conditions free abetas readily aggregate to form the insoluble deposits which are a major component of the plaque seen in this disease [1]. Their low solubility suggests that plaque forms when the free, extracellular, abeta concentrations exceed their solubility constants. This implies that their concentrations should be higher in Alzheimer's patients than in healthy controls. This is consistent with the widely accepted hypothesis that plaque formation is due to increased abeta production. Such increases have been reported in patients who have rare genetic forms of early onset Alzheimer's disease and in the early disease seen in Down's syndrome [1,2,4,5]. The latter results from duplication of chromosome 21 which contains the gene for APP [4].

Since a portion of the CSF is derived from the extracellular fluid, the best approach to determining whether there is increased abeta production in Alzheimer's

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patients is to determine their CSF concentrations. Yet, when this has been done, it has been found that the total concentrations of abetas in the CSF of Alzheimer's patients were equal to or less than that in normals [4,6–9], indicating that factors other than the rate of abeta production lead to plaque formation. For example, one factor could be that Alzheimer's patients have a nucleation factor which enhances plaque deposition. This may be the basis for the increased plaque seen in individuals with at least one apolipoprotein E4 (apoE4) allele [4,10], since under oxidizing conditions apoE4 enhances abeta precipitation more than the other two isoforms do [11].

Alternatively, there may be binding proteins in the CSF which inhibit aggregation. If such is the case, then Alzheimer's disease could result from low levels of these carriers. Koudinov's laboratory has reported that apolipoprotein J, also known as clusterin, may be such a carrier [12,13].

Yet another group of proteins which may serve as carriers to keep the abetas in solution are the ER chaperones. These have been shown to form complexes with several secretory and plasma membrane proteins [14]. The first example of this was the finding that the β-subunit of prolyl-4-hydroxylase was the protein disulfide isomerase, ERp55 [14–16]. More recently, a related chaperone, ERp57, has also been shown to form stable complexes with several membrane and secretory proteins. These complexes form in the ER in association with other chaperones, such as calnexin and calreticulin, and cannot be reconstituted by admixture of the purified components [14].

In the current study, we have examined whether in the CSF of normals the abetas are bound to carrier proteins. We find that on immunoblotting all of the observable abetas appear to be bound to the ER chaperones, ERp57 and calreticulin. In light of the recent advances in the elucidation of the biochemical processes involved in the post-translational processing of nascent proteins in the ER, our data would suggest that the basic defect of Alzheimer's disease is decreased activity in one or more of the metabolic steps in this pathway. This resultant failure to form the complex with the abetas could then lead to the plaque deposits characteristic of this condition.

Materials and methods

Subjects. CSF samples from young individuals were clinical waste from the Laboratory Service of Regions Medical Center, St. Paul, MN. Since the individuals were not identified, this was deemed exempt research by the Institutional Review Boards of both Regions Medical Center and the University of Minnesota. Samples from normal, elderly subjects were obtained by the Anesthesia Service at the VA Medical Center, Minneapolis, during the administration of spinal anesthesia to patients undergoing transurethral resection of the prostate. Before receiving any medications, they signed a consent form which had been approved by the Institutional Review Board of the VA Medical Center.

The sources of antibodies used to identify the abetas and the ER chaperones in the CSF. Polyclonal antibodies to calreticulin, calnexin, ERp55, ERp57, ERp72, and synthetic abeta 1–43 were prepared in chickens as previously described [17]. Rabbit antibodies to BiP, ERp57, and calreticulin were purchased from StressGen (Vancouver, BC). Monoclonal anti-abeta antibodies specific for amino acids 1–17 (6E10) (Mab1–17) and 17–24 (4G8) (Mab17–24) were obtained from Senetek (Napa, CA) and to amino acids 1–42 (AMY-33) (Mab1–42a) from Zymed (South San Francisco, CA) and Sigma (Mab1–42b) (St. Louis, MO). Antigen specific, rabbit, polyclonal antibodies to abetas (R1282) were kindly provided by Dr. Dennis J. Selkoe (Harvard Medical School). The specificity of these antibodies for the abetas has been previously reported [18].

Immunoblotting procedures. We performed SDS–PAGE as previously described [15]. The protein bands were detected either by enhanced chemiluminescence (ECL) (Amersham Life Science, Piscataway, NJ) or by the alkaline phosphatase reaction (Bio Rad, Richmond, CA).

In studies in which the electrophoresis was run on native gels, the SDS and DTT were deleted and the CSF was applied to the gel without prior heating.

Isolation of the complex by affinity chromatography. We isolated the abeta–ERp57 complex by immunochromatography with chicken antibodies to ERp57 and abeta bound to CNBr-activated Sepharose. In these studies, the resins were suspended in NaCl (1 mole/L), mixed with 1 ml CSF; shaken overnight at 4 °C, and poured into a column. The columns were then washed with NaCl until the eluates had no absorbance at 280 nm. The bound proteins were eluted with glycine (0.1 mole/L, pH 9.0) in NaCl. The ERp57 and abetas in the eluates were identified by immunoblotting.

Results

Identification of abeta–ERp57 complex: when CSF from controls was examined by immunoblotting by SDS–PAGE, the major chaperone we observed was ERp57 (Fig. 1A). While purified ERp57 gives a single sharp band at $M_r = 57$ kDa, in the CSF it gave a doublet with M_r's of 57 and 62 kDa (Fig. 1A). We have previously reported a similar doublet in immunoblots of hepatic microsomes [19].

When the blots were incubated with the antigen specific, rabbit, polyclonal antibodies to abetas, R1282, there was only a single band at $M_r = 62$ kDa (Fig. 1B). We observed no bands at the M_r's which would be expected for the abeta monomers or their aggregates [2,4]. Since in this study the dye front was run only two-thirds of the way down the gels, it is unlikely that the abeta monomers or their aggregates were missed because they ran off the gel. Furthermore, it is unlikely that our failure to observe aggregates of the free abetas in the CSF was due to a methodological problem since, as noted below, we could detect them when we hydrolyzed the complex in base (Fig. 4, channel 9). Finally, when the blots were run without the first antibody, no reactive bands were observed. We have examined CSF samples from over 200 individuals and have consistently observed only this single anti-abeta immunoreactive band.

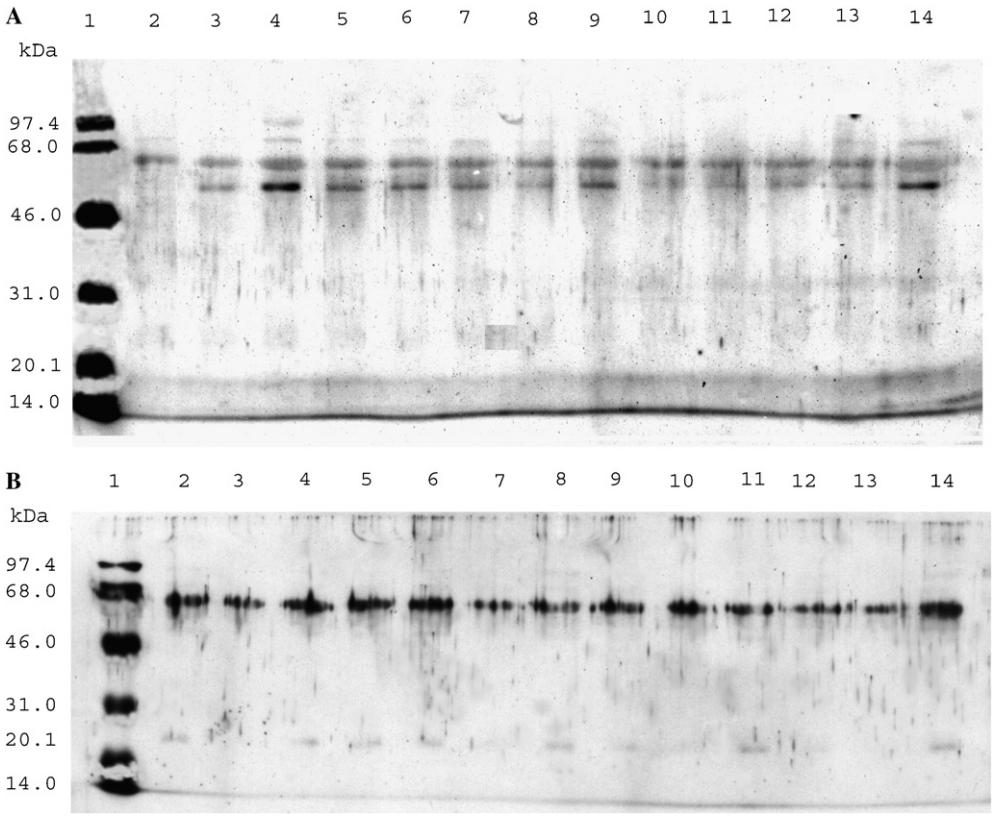

Fig. 1. Immunoblots with: (A) chicken polyclonal antibodies to ERp57 and (B) rabbit polyclonal antibodies to abeta (1282). Channel 2, purified abeta–ERp57 complex; channels 2–14, 1.25 ml of CSF from normal, humans, ages 6 months to 68 years. The bands were detected with the ECL assay.

The 62 kDa M_r bands, which we observed in both the abetas and ERp57 immunoblots, are approximately the M_r that would be predicted for a complex consisting of these two proteins.

The identity of the abeta band in the CSF has been confirmed with three monoclonal antibodies, Mab1–17, Mab1–42a, and Mab1–42b, and our chicken antibody. On the other hand, we could not detect the complex when we used the monoclonal antibodies, Mab17–24, even with very high concentrations of antibody ($\geqslant$1 mg/ml), suggesting that the binding of Mab17–24 to the abetas was sterically hindered. This hindrance may be due to either N-glycosylation at asparagine 27 or O-glycosylation at serine 26 of the abetas. The failure of Mab17–24 to detect the abetas in the complex is consistent with the observations first reported from High's laboratory indicating that ERp57 only binds to N-glycosylated proteins [20,21]. Unfortunately, for reasons which are unclear at the present time, *N*- and *O*-glycosidases (H- and G-) did not hydrolyze the complex, suggesting that these enzymes do not have access to the asparagine 27. On the other hand, as noted below, the complex could be hydrolyzed in base. Finally, in support of the existence of this complex, we have in preliminary studies isolated and tentatively identified it from tissue preparations (Erickson et al., MS in preparation).

In light of the failure of others to report that the bulk of the abetas appears to be bound to a carrier protein, it has been repeatedly suggested that our observations are an artifact of the antibodies we have used. Although this is a legitimate concern, we feel that it is very unlikely that our results are due to cross-reactivity with other proteins. In response to this criticism we have employed a number of antibodies, many of which have been widely used by other workers in published studies. In particular, it should be noted that it is extremely unlikely that the reaction of the monoclonal antibody, Mab1–17, is due to an artifact of the assay procedure. This antibody is reactive to an epitope in the first 17 amino acids of the abetas. In a search of the NCBI protein database, we have found that the sequence of this segment of the peptide is unique and highly conserved in vertebrates. Hence, there is very little likelihood that this antibody is nonspecifically cross-reacting with other proteins. Furthermore, as a result of its generally recognized specificity, this antibody has been widely used by many workers to identify the abetas in samples containing a heterogeneous collection of proteins, such as those found in plaque. If there were a problem with cross-reactivity with this antibody, it would raise the question of the validity of these other studies. Similarly, none of the antibodies

we have employed for the detection of either the abetas or ERp57 reacted with either keratin, a major contaminant of tissue preparations, or clusterin, a previously identified carrier protein [12,13].

Immunoblots of the abeta–ERp57 complex when run in a native gel

When we examined the CSF for other chaperones, the only one we observed was calreticulin. But on SDS–PAGE immunoblots this chaperone had a slightly different M_r from that observed for the abetas. On the other hand, work from Helenius' laboratory has suggested that calreticulin, rather than ERp57, binds to the N-glycosylation site of various proteins [22,23]. Hence, it could be the carrier protein. In line with their observations, when we performed immunoblots on native gels, we found that the complex had a $M_r = 130$ kDa which was reactive with antibodies to abetas, calreticulin and ERp57 (Fig. 2). This is the M_r which would be expected for this ternary complex. The failure to detect the calreticulin on SDS–PAGE of the complex would suggest that the ERp57 tightly binds to the N-glycosylation site and then the calreticulin binds to it.

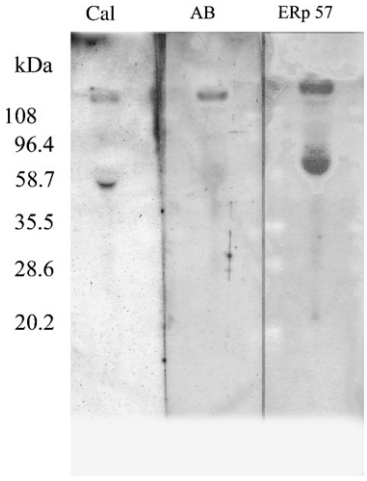

Fig. 2. Immunoblot of CSF run on a native Gel. Channel 1, anti-calreticulin; channel 2, anti-abeta; channel 3, anti-ERp57.

Immunochromatography of the complex

The identity of the complex was further verified by isolating it by immunochromatography. In this portion of the study, when the complex was bound to either the anti-ERp57 or anti-abeta antibody column and the bound proteins were eluted, the eluates from both columns showed a major immunoreactive band at $M_r = 62$ kDa (Figs. 3A and B). The observation that antibodies to either protein immobilized both abeta and ERp57 indicates that they were most likely present in the CSF as a complex.

Yet, when the complex was immunopurified and immunoblotted with anti-abeta antibodies, several extra bands were also observed (Fig. 3B, channel 3). These appear to be aggregates of the low concentrations of the free abetas that are present in the CSF. It is not surprising that the affinity resin concentrated them, since the polyclonal antibodies used to prepare the gel were developed against the synthetic peptide and would therefore be expected to have a higher affinity for the free peptides and their aggregates than for the complex. Furthermore, if the abetas in the complex are glycosylated at asparagine 27 or serine 26, then those antibodies in the polyclonal mixture directed to the epitopes around these two amino acids would only bind to the

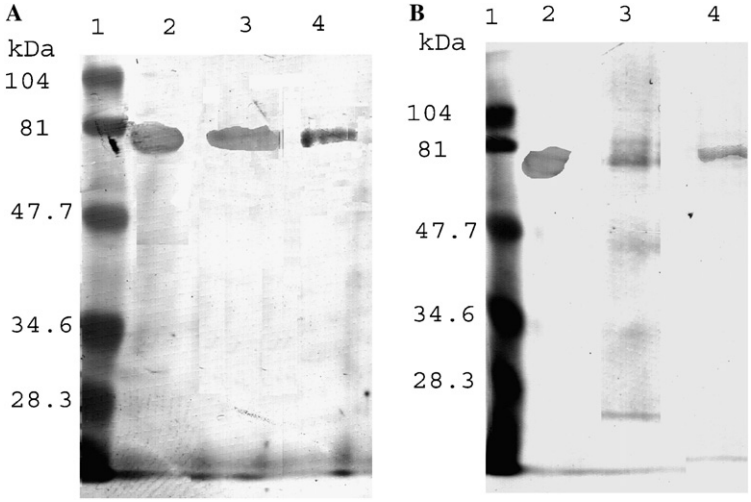

Fig. 3. Immunoblots with: (A) polyclonal antibodies to ERp57 and (B) to abeta. Channel 1, prestained standards; channels 2, CSF; channel 3, CSF separated by affinity chromatography with anti-abeta antibodies; channel 4, CSF separated by affinity chromatography with anti-ERp57 antibodies. The bands were detected with the alkaline phosphatase reaction.

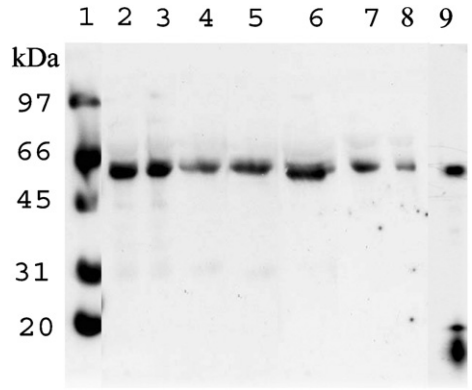

Fig. 4. ECL, immunoblots with rabbit polyclonal antibodies to abeta (1282) of normal human CSF after treatments to dissociate the abeta–ERp57 complex. Channel 1, molecular weight markers; channel 2, untreated CSF; channel 3, CSF treated with 70% formic acid; channel 4, CSF treated with 80% trifluoroacetic acid; channel 5, CSF treated with 6 M guanidine isothiocyanate; channel 6, CSF treated with 6 M urea; channel 7, CSF proteins which did not bind to a boronate column; channel 8, CSF proteins which bound to a boronate column; channel 9, treatment of the complex with glycine buffer, pH 9.0.

unglycosylated peptides and serve to concentrate them in the eluate.

Effect of various dissociation agents on the abeta–ERp57 complex

A number of workers have solubilized the abetas in plaque with either concentrated formic or trifluoroacetic acid and then characterized them by mass spectrometry [5,24–26]. In these studies, they have observed that the abetas in plaque are only present as the naked peptides. It is possible that they did not observe the complex in plaque because it may have been dissociated by the acid treatment. This is unlikely because the complex in the CSF was stable when exposed to either neat formic or trifluoroacetic acids under the same conditions of time and temperature as they employed (Fig. 4). Similarly the complex was unaffected by treatment with the chaotropic agents, guanidine isothiocyanate and urea. On the other hand, it did dissociate when treated with glycine buffer (pH 9.0) at 4 °C for several days (Fig. 4, channel 9). This high pH most likely hydrolyzed the carbohydrate bound to the glycosylation site. As noted above, after hydrolysis we did observe the free abeta aggregates in our immunoblots. This observation represents a positive control which supports our contention that the reason we did not observe the aggregates in the untreated CSF was that they are only a minor fraction of the total abetas present in the samples.

Finally, we found that the complex bound to a boronate column (Fig. 4, channel 8). Since this column specifically binds carbohydrates, this result would suggest, in line with the initial reports from High's laboratory [20,21], that the complex is glycosylated.

Discussion

We believe that the current study strongly suggests that in normal individuals the abetas in the CSF are present as a complex with the ER chaperones, ERp57 and calreticulin. Based on studies from other laboratories, it would appear that the abetas in this complex are N-glycosylated. But unlike the studies from Helenius' laboratory, our data suggest that the ERp57 is bound to the abetas and the calreticulin is bound to it.

On the other hand, a number of workers have reported that after solubilization of the abetas in plaque with concentrated formic or trifluoroacetic acids, it is recovered as the naked peptide [5,24–26]. One possible explanation for this difference between the abetas found in plaque and those found in the CSFs of normals is that during the solubilization of the plaque the abeta complex is dissociated by the acid treatment. This is unlikely, since we have found that, following the same protocols as were employed by these other workers, the complex in the CSF did not dissociate when exposed to these acids (Fig. 4). This is a critical observation, since it would strongly suggest that the abetas in plaque, unlike those found in CSF, are not present as a complex with the ERp57. It would further imply that the deposition of plaque is due to the failure of the cell to form the complex during the ER processing of the abetas.

Our immunoblot data lend further support to this concept. In this portion of the study, we found that the monoclonal antibody, Mab17–24, did not react with the complex. Most likely this lack of reactivity was due to steric hindrance around one of the possible glycosylation sites at either serine 26 or asparagine 27. Yet, this antibody is commonly used to detect abetas in brain plaque. It would only be possible for this antibody to detect the abetas in plaque if these peptides are not glycosylated.

Even though we have uniformly found that on routine immunoblotting the bulk of the abetas in the CSF are present as the complex, a number of investigators have raised the issue of why this complex has not been previously reported. One reason for this discrepancy between our studies and those of many others is that most workers who have determined the CSF abeta concentrations have used a variety of ELISAs [7–9]. Clearly, such assays cannot distinguish between the bound and free forms. But, four groups have published immunoblotting studies of the CSF and each has reported markedly different values for the M_r's of the abetas. Two of them, Ida et al. [6] and Koudinov and co-workers [12], did report the presence of a high M_r abeta band, but neither group characterized it. Koudinov's study was of particular interest since the band was detected with Mab1–17 (6E10) and had an $M_r = 62$ kDa. On the other hand, Seubert et al. [27] and Golabek et al. [28] failed to observe the high M_r complex in their immunoblots. The

reason for this discrepancy between their studies and ours may be due to differences in study design.

Similarly, it is possible that other workers may have observed the same band and did not publish their observations because they assumed that it was due either to high molecular weight aggregates of the abetas or immuno-cross-reactivity with some common contaminant, such as keratin. Even if they had considered the possibility that the abetas were bound to a carrier protein, because of its low concentration in the CSF and the large number of proteins with similar M_r's and chromatographic characteristics, it is unlikely that they would have been able to identify the carrier as ERp57 by standard proteomic procedures, such as 2-D PAGE followed by mass spectrometry, without extensive purification from a large volume of sample.

Finally, we feel that our observations may have broad implications for the study of Alzheimer's disease and possibly other maladies associated with aging. This is based on the premise that membrane proteins and the abetas undergo the same posttranslational processing in the ER. Since this is most likely the case, the observation that the abetas found in plaque are present as the naked peptide, while those found in normal CSF are present as a complex with ERp57 and calreticulin, would suggest that the dementia of Alzheimer's disease is due to a similar faulty, ER, posttranslational processing of neuronal, membrane proteins. This concept is at variance with the widely held view that it is due to the toxicity of the free peptides. This latter hypothesis is supported by animal studies in which it has been found that the injection of abeta aggregates into rat brains does lead to decreased cognitive function [31]. Furthermore, reports from population based and longitudinal studies have indicated that there is a rough correlation between the plaque scores and cognitive function at the time of death [32–34]. But also in these same studies the investigators have reported that up to 40% of the individuals with plaque had normal cognitive function at the time of death [32–34]. These observations would suggest that the presence of plaque is probably only a marker for the basic biochemical defect which leads to the dementia rather than a cause of the cognitive deficit.

In line with this concept it is generally held that the initiation, consolidation, and retrieval of memory is dependent upon the formation and maintenance of synapses [35,36]. This, in turn, requires the synthesis of functional, synaptic, membrane proteins. They and the abetas undergo the same posttranslational metabolic processing in the ER. If there is an age related decline in any of the steps in this pathway, then the membrane proteins, like the abetas in plaque, will not fold into their native configuration leading to decreases in their levels in the synaptic membranes. In support of this paradigm, it has been observed that in Alzheimer's disease there is a correlation between the loss of cognitive function and a decline in the content of at least one synaptic, membrane protein [35,37].

Second, faulty folding can also activate the ER surveillance systems that initiate the ER associated degradation process (ERAD) [38] and the unfolded protein response (UPR) [39–43]. The ERAD pathway identifies unfolded proteins and directs them to the translocon pore for ubiquitination and degradation by the proteosome [38,44,45]. Unlike the bulk of other proteins synthesized in the ER, the poorly folded abetas probably aggregate so rapidly that they become too bulky to be translocated through the pore for degradation [46]. Instead, they remain in the lumen of the ER and are eventually secreted into the extracellular space. If such is the case, then plaque is primarily a marker for the presence of the disease rather than the primary cause of the cognitive decline.

The central component of the UPR is the ER chaperone, BiP, also known as GRP78. During normal processing, BiP binds to and inactivates two protein kinases, PERK and IRE1, and a proform of a negative transcription factor, ATF6 [39–41,43]. On the other hand, in the presence of high concentrations of unfolded proteins in the lumen of the ER, the free concentration of BiP declines and by mass action it leaves the two kinases and the proform of ATF6. This activates them and leads to the inhibition of the transcription of the bulk of the proteins synthesized in the ER. But at the same time these same transcription factors initiate increases in the synthesis of the mRNAs for the chaperones, such as BiP, which can then catalyze the renaturation of the denatured proteins [39]. Hence, the failure to properly configure the membrane proteins leads to a decrease in their synthesis both through a loss of effective translation and a decrease in their transcription. The activation of UPR with decreased transcription of membrane proteins could be the basis for the observations of Callahan et al. [35] in which they reported that there were lower levels of the mRNA for synaptophysin in neuronal cells from Alzheimer's patients.

Finally, if the quantity of unfolded protein becomes too high, the UPR can activate yet another set of transcription factors leading to apoptosis [42,43]. Alternatively, decreases in the luminal concentration of BiP can lead to the leakage of toxic levels of Ca^{2+} across the pore of the translocon into the cytosol thereby initiating cell death through necrosis [47].

In summary, since synaptic function is crucial for the formation, maintenance, and retrieval of memory, our data would suggest that the central defect leading to the dementia of Alzheimer's disease is the faulty, posttranslational ER processing of synaptic, membrane proteins. The failure of the ER to properly modify and fold these proteins into their native configuration could lead first to the loss of synaptic function, as well as to the development of the plaque which is characteristic of this

condition. Eventually the activation of the UPR leads to cell death. Together these processes could lead to the dementia seen in individuals with Alzheimer's disease. We feel that if this paradigm is fully validated in future studies, it could lead to new modalities for the diagnosis and treatment of Alzheimer's disease as well as other physiological deficits associated with aging.

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