AT-1 is the ER membrane acetyl-CoA transporter and is essential for cell viability

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Summary
The transient or permanent modification of nascent proteins in the early secretory pathway is an essential cellular function that ensures correct folding and maturation of membrane and secreted proteins. We have recently described a new form of post-translational regulation of the membrane protein β-site APP cleaving enzyme 1 (BACE1) involving transient lysine acetylation in the lumen of the endoplasmic reticulum (ER). The essential components of this process are two ER-based acetyl-CoA:lysine acetyltransferases, ATase1 and ATase2, and a membrane transporter that translocates acetyl-CoA into the lumen of the ER. Here, we report the functional identification of acetyl-CoA transporter 1 (AT-1) as the ER membrane acetyl-CoA transporter. We show that AT-1 regulates the acetylation status of ER-transiting proteins, including the membrane proteins BACE1, low-density lipoprotein receptor and amyloid precursor protein (APP). Finally, we show that AT-1 is essential for cell viability as its downregulation results in widespread cell death and induction of features characteristic of autophagy.

Key words: AT-1, Acetyl-CoA, Lysine acetylation, Membrane transport, Autophagy, Neurodegeneration

Introduction
Our group has recently identified a new form of post-translational regulation of the membrane protein β-site APP cleaving enzyme 1 (BACE1), the rate-limiting enzyme for the generation of the Alzheimer’s disease (AD) amyloid β-peptide (Aβ) from its precursor, APP (Costantini et al., 2007). The process involves transient acetylation of the highly disordered N-terminal globular domain of nascent BACE1 in the lumen of the endoplasmic reticulum (ER) by two acetyl-CoA:lysine acetyltransferases, which we named ATase1 and ATase2 (Ko and Puglielli, 2009). The acetylated intermediates of BACE1 are able to reach the Golgi complex, where they are deacetylated by a Golgi-resident deacetylase (Costantini et al., 2007). By contrast, non-acetylated intermediates are retained and degraded in a post-ER compartment – most probably the ER Golgi intermediate compartment (ERGIC) – by a mechanism that requires the serine protease proprotein convertase subtilisin kexin type 9 (PCSK9, also known as NARC-1) (Jonas et al., 2008). As a result, changes in the expression levels and activity of ATase1 and/or ATase2 (Ko and Puglielli, 2009) as well as PCSK9 (Jonas et al., 2008) influence both BACE1 metabolism and Aβ generation, which are directly involved in the pathogenesis of AD.

In addition to the two acetyltransferases ATase1 and ATase2, the acetylation of nascent BACE1 requires active transport of the highly charged and membrane-impermeable acetyl-CoA from the cytosol into the lumen of the ER. Acetyl-CoA is the donor of the acetyl group in the reaction of lysine acetylation and represents an essential biochemical requirement for the above events (Costantini et al., 2007).

In 1997, Kanamori et al. reported the identification of a new protein (AT-1) that appeared to affect the acetylation of complex gangliosides in the secretory pathway (Kanamori et al., 1997). The authors were interested in the Golgi-resident acetyltransferase responsible for the O-acetylation of complex gangliosides but, surprisingly, the new protein displayed a possible ER localization in addition to features that were consistent with membrane transporters rather than acetyltransferases. These included the presence of multiple membrane-spanning domains and a leucine-zipper motif. Additionally, when the effects of AT-1 overexpression were assessed in semi-intact cells, only ~5% of radiolabeled acetyl-CoA was found to be incorporated in glycolipids, suggesting that AT-1 activity preferentially affects non-lipid constituents (Kanamori et al., 1997).

As a result, the authors proposed a possible role as ER membrane acetyl-CoA transporter and named the new protein acetyl-Coenzyme A transporter 1 (AT-1) (Hirabayashi et al., 2004; Kanamori et al., 1997). However, no functional proof was provided regarding the biochemical activity or properties of this protein. AT-1 has homologs in lower organisms, including Saccharomyces cerevisiae, Caenorhabditis elegans and Drosophila melanogaster (Hirabayashi et al., 2004), and is upregulated as result of ER-induced stress, suggesting a possible role during the unfolded protein response (UPR) (Shaffer et al., 2004). Recent work has also shown that AT-1 is upregulated in motor neurons of patients affected by sporadic amyotrophic lateral sclerosis (ALS) (Jiang et al., 2007) and mutated in patients affected by autosomal dominant spastic paraplegia-42 (SPG42) (Lin et al., 2008), suggesting an implication in neurodegenerative disorders.

Here, we report that AT-1 (also called solute carrier family 33 member 1, SLC33A1) is an ER membrane acetyl-CoA transporter. AT-1 regulates the acetylation of BACE1, LDLR, APP and other ER-based protein substrates, and is upregulated in the brain of late-onset (sporadic) AD patients. Importantly, we show that AT-1 is essential for cell viability because its downregulation results in widespread cell death and induction of features characteristic of autophagy. These studies point to a fundamental role of the ER-
based acetylation machinery in both physiological and pathological conditions.

**Results**

**AT-1 is the ER membrane acetyl-CoA transporter**

To assess whether AT-1 is responsible for the acetyl-CoA transport activity that we have identified and described in the ER membrane (Costantini et al., 2007), and to characterize its biochemical properties together with its disease-relevant functions, we generated several individual colonies of Chinese hamster ovary (CHO) cells that overexpress human AT-1 (Fig. 1A, B). Transgenic AT-1 displayed ER localization and was completely absent from Golgi fractions (Fig. 1C), which is consistent with our previous localization of the ER membrane acetyl-CoA transport activity (Costantini et al., 2007). Next, we assayed the acetyl-CoA transport activity in individual fractions from a subcellular fractionation gradient of control (non-transfected) cells. The assay was performed under native conditions and in the absence of detergents, which preserves biochemical properties as well as in vivo topographical orientation of the membranes (Carey and Hirschberg, 1981; Costantini et al., 2007; Ko and Puglielli, 2009; Puglielli et al., 1999a; Puglielli et al., 1999b). Fig. 1D shows that the acetyl-CoA transport activity was only observed in fractions corresponding to the ER and that the distribution pattern of AT-1 overlapped with the endogenous acetyl-CoA membrane transport activity. Additionally, when we compared the acetyl-CoA membrane transport activity of similar ER fractions generated from control and AT-1 overexpressing cells, we found a significant increase in the rate of acetyl-CoA translocation following AT-1 overexpression (Fig. 1E).

The above results demonstrate that AT-1 is restricted to the ER membrane where it overlaps with the endogenous acetyl-CoA transport activity observed in native membranes. They also indicate that overexpression of AT-1 results in increased translocation of acetyl-CoA into the ER lumen. These results are consistent with our previous report showing that ER- but not Golgi-membranes possess acetyl-CoA transport activity (Costantini et al., 2007). However, they do not provide definitive evidence that AT-1 is solely responsible for acetyl-CoA translocation across the ER membrane.

To prove that AT-1 is the ER membrane acetyl-CoA transporter, we purified transgenic Myc-tagged AT-1 from stably transfected cells (see Fig. 1B) by using an anti-Myc antibody covalently attached to aldehyde-activated agarose beads (anti-Myc column). Affinity-purified AT-1 was then reconstituted into artificial liposomes prior to biochemical assessment of transport activity. Reconstituted proteoliposomes displayed very high acetyl-CoA transport activity, which was absent in liposomes alone or in non-reconstituted proteoliposomes (Fig. 1F). Finally, activity was also absent when reconstitution was performed with the elution product of the anti-Myc column from control (non-transfected) cells (Fig. 1F; reconstitution without AT-1). The above results clearly indicate that AT-1 alone is sufficient for transport activity into an artificial liposomal system and support the conclusion that AT-1 is the ER membrane acetyl-CoA transporter.

Previous work with nucleotide–sugar transporters of the Golgi membrane, which are required for Golgi-based glycosylation of glycoproteins and glycolipids, has shown that they act as antiporters (Berninsone and Hirschberg, 2000; Hirschberg et al., 1998). As a result, the import of the nucleotide–sugar complex is inhibited with high specificity by the nucleotide moiety. To test whether this was also the case for AT-1, we assessed the ability of CoA and other non-related molecules to inhibit translocation of acetyl-CoA into the lumen of purified ER vesicles. Fig. 2A, B shows that only CoA was able to reduce acetyl-CoA translocation across the ER membrane in a dose-dependent fashion. Importantly, no effect was observed when ER vesicles were incubated together with acetyl-CoA and ATP, UDP-galactose or Na-acetate (Fig. 2B). ATP is actively transported into the ER and Golgi lumen by two specific membrane transporters, whereas UDP-galactose is transported into the Golgi lumen by a Golgi-based membrane transporter (Berninsone and Hirschberg, 2000; Hirschberg et al., 1998). Therefore, the fact that neither was able to interfere with the translocation of acetyl-CoA indicates that the inhibition shown in
Fig. 2A is specific for CoA. Finally, the inability of Na-acetate, the sodium salt of acetic acid, to inhibit acetyl-CoA transport suggests that the CoA moiety of acetyl-CoA serves as the recognition signal for AT-1.

Downregulation of AT-1 causes ER expansion and autophagic cell death

To further analyze its function in vivo, we downregulated AT-1 by using small interfering RNA (siRNA) targeted against AT-1. Although reduced levels of AT-1 mRNA were already evident 2 days after siRNA treatment (Fig. 3A), robust changes in the protein levels could only be observed after 6 days of treatment, suggesting a long half-life of the transporter (Fig. 3B). Surprisingly, the downregulation of AT-1 was accompanied by widespread cell death as assessed by conventional field microscopy (Fig. 3C), calcein incorporation into living cells (Fig. 3C), and direct counting of cells stained with a live-dead assay (Fig. 3D,E). Such an effect was not observed when cultures were treated with non-silencing siRNA (Fig. 3C–E) or with other non-related siRNAs previously used (Costantini et al., 2006; Jonas et al., 2008; Ko and Puglielli, 2007). These results should be viewed together with the fact that downregulation of AT-1 in zebrafish causes developmental defects and death (Lin et al., 2008). Additionally, apparent haploinsufficiency of AT-1 is associated with autosomal dominant SPG42 (Lin et al., 2008), a human disease that is caused by degeneration of motor neurons in the corticospinal tract. When taken together, the above results clearly point to a fundamental role of AT-1 in the physiology of the cell.

To further advance our understanding of the mechanisms of cell death, we used transmission electron microscopy. Fig. 4A shows a collection of cellular features that are reminiscent of different stages of autophagic cell death (see panels a–d). In rare cases, the autophagic features coexisted with apoptosis-like chromatin condensation, suggesting activation of both autophagy- and apoptosis-dependent pathways (Fig. 4A; panels e and f). However, it must be noted that we were unable to detect caspase activation or poly(ADP-ribose) polymerase (PARP) cleavage (data not shown). This might reflect the fact that the vast majority of dying cells did not display apoptosis-like features when analyzed by electron microscopy. The activation of autophagic cell death was also associated with the upregulation of the autophagic markers beclin, LC3 and ATG5 (Fig. 4B). The presence of enlarged ER tubular structures was among the earliest events that we could detect following siRNA-mediated downregulation of AT-1 (Fig. 4C). This was often accompanied by a few autophagosomes (Fig. 4C), which
became very abundant in later stages (Fig. 4D). The autophagosomes were, at least initially, in close proximity to the ER, most probably because the ER membrane itself appears to be involved in the generation of the autophagic membrane (Axe et al., 2008). Finally, no morphological alterations of other organelles were observed at this stage, suggesting that the autophagy is triggered by ER-dependent events.

AT-1 regulates the acetylation status of several ER-based proteins
The results described above suggest that the cell needs a constant supply of acetyl-CoA into the lumen of the ER. This is most probably owing to the fact that acetyl-CoA serves as the donor of the acetyl group for the ε-amino acetylation of lysine residues on ER-transiting and, possibly, ER-resident proteins. Our previous results indicate that increased translocation of acetyl-CoA across the ER membrane favors the lysine acetylation of nascent BACE1 in the lumen of the ER, thereby resulting in increased cellular levels of BACE1 (Costantini et al., 2007). Consistently, BACE1_Gln – a gain-of-acetylation mutant form of BACE1 where the lysine residues that are acetylated in the ER lumen (K126, K275, K279, K285, K299, K300, K307) are mutated to glutamine to mimic the effect of lysine acetylation (Masumoto et al., 2005) – displayed resistance to PCSK9 degradation (Jonas et al., 2008), rapid translocation along the secretory pathway and a longer half-life (Costantini et al., 2007; Jonas et al., 2008).

To test whether AT-1 can regulate BACE1 metabolism, we analyzed the levels of endogenous BACE1 in CHO cells that
overexpress AT-1. Fig. 5A,B shows that BACE1 levels were greatly increased (~3.5-fold) in AT-1 overexpressing cells when compared with control (non-transfected) cells; this was also paralleled by increased levels of the APP C-terminal fragment C99 (Fig. 5A,B) and 

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\text{A}_{B}^{\beta}\text{total}\text{:A}_{\beta}^{\gamma}\text{ratio (data not shown). Results are the average + s.d. (n=3).} \quad ^{\text{2}F<0.005}
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APP is a substrate of the ER-based acetylation machinery

We have previously reported that the non-acetylated intermediates of both BACE1 and LDLR are disposed of by PCSK9 (Jonas et al., 2008). A recent report indicates that overexpression of APP in human neuroglioma (H4) cells reduces PCSK9-mediated disposal of LDLR (Abisambra et al., 2009). These preliminary results
indicate functional competition between different substrates. In fact, transgenic APP (when overexpressed) can theoretically compete with endogenous LDLR, skewing PCSK9 towards APP (rather than LDLR) non-acetylated intermediates. However, for this to be the case, APP would need to be a substrate of the ER-based acetylation machinery. The presence of an acetylated band migrating with the apparent molecular mass of immature APP (Fig. 6E, indicated by the symbol <) would be consistent with such a possibility. To test this hypothesis, we immunoprecipitated APP from a total-cell lysate and analyzed whether APP can be detected with an antibody against acetylated lysine. Only the immature form of APP was found acetylated (Fig. 7A, right panel). Importantly, the upper two bands, which correspond to the mature species of APP (Fig. 7A, left panel), were not acetylated, mimicking the behavior previously described for both BACE1 and LDLR (Costantini et al., 2007; Jonas et al., 2008; Ko and Puglielli, 2009). Next, we assessed whether overexpression of AT-1 increased the levels of the ER-based nascent form of APP. Fig. 7B clearly shows increased levels of immature APP in ER vesicles purified from AT-1 overexpressing cells. To further prove that APP is a substrate of the ER-based acetylation machinery, a protein extract of highly purified ER vesicles was immunoprecipitated with an antibody against acetylated lysine and then analyzed by immunoblotting with an anti-APP antibody. Fig. 7C shows the presence of the immature form of APP in the immunoprecipitate, which supports an acetylated status. Similar results were obtained with LDLR (Fig. 7D) and BACE1 (Fig. 7E), for which the acetylation status has already been proven (Costantini et al., 2007; Jonas et al., 2008; Ko and Puglielli, 2009). Finally, we analyzed whether APP levels are affected by the genetic disruption of \( \text{Pcsk9} \). In fact, the non-acetylated intermediates of both BACE1 and LDLR – currently the only two known substrates of the ER-based acetylation machinery – are disposed of by the serine protease PCSK9. As a result, their levels are increased in \( \text{Pcsk9}^{-/-} \) mice (Jonas et al., 2008; Rashid et al., 2005). Increased levels of APP were found in the neocortex of \( \text{Pcsk9}^{-/-} \) mice, thus supporting our hypothesis (Fig. 7F).

Therefore, the above results indicate that APP represents a substrate of the ER-based acetylation machinery. When taken together, the results support the conclusion that the ER-based acetylation of the \( \varepsilon \)-amino group of lysine residues is not limited to BACE1 or LDLR (Figs 6, 7). Future identification and characterization of all the different substrates will help define the impact of the ER-based lysine acetylation to the biology of the cell.
AT-1 is tightly regulated by the lipid second messenger ceramide

We have previously shown that the lipid second messenger ceramide can stimulate the acetylation of nascent BACE1 by increasing both the maximum velocity ($V_{\text{max}}$) of acetyl-CoA translocation across the ER membrane (Costantini et al., 2007) and the expression levels of the acetyltransferases ATase1 and ATase2 (Ko and Puglielli, 2009). To assess whether the increased import of acetyl-CoA in the lumen of the ER (Costantini et al., 2007) is a result of increased expression of AT-1, we treated CHO cells, H4 cells and mouse primary neurons with 10$\mu$M ceramide, following a protocol that has already been described in detail (Costantini et al., 2007; Ko and Puglielli, 2009; Puglielli et al., 2003). Treatment resulted in a marked increase in the mRNA levels of $AT-1$, as detected by quantitative real-time PCR (Fig. 8A–C). Previous work has shown that ceramide levels in the mouse brain increase spontaneously during aging (Costantini et al., 2005) and are higher in $p44^{+/+}$ transgenic mice (Costantini et al., 2006), a progeroid animal model that resembles an accelerated form of aging (Maier et al., 2004) and develops premature cognitive deficits as well as an accelerated form of AD-like neuropathology (Pehar et al., 2010). Consistently, quantitative real-time determination of $AT-1$ mRNA levels in the brain of $p44^{+/+}$ transgenic animals revealed a ~2.5-fold increase when compared with both non-transgenic and heterozygous $p44^{+/–}$ mice (Fig. 8D). It is already known that the progeroid phenotype caused by the overexpression of p44 (also called DNp53) is only evident in the homozygous animals (Maier et al., 2004). As a result, heterozygous $p44^{+/–}$ mice have normal life-spans (Maier et al., 2004) and normal levels of ceramide and BACE1 (Costantini et al., 2006). We have previously shown that the levels of ceramide on the cell surface of mouse primary neurons increase spontaneously as the culture ages (Costantini et al., 2006). As such, we decided to assess the levels of $AT-1$ in both ‘young’ (day 3) and ‘old’ (day 24) neuronal cultures, following an experimental strategy that we have already described (Costantini et al., 2006). Both real-time mRNA quantitation and western blotting showed that old neurons had increased levels of $AT-1$ (Fig. 8E).

When taken together, the above results indicate that expression levels of AT-1 are tightly regulated by the second messenger ceramide; they also explain our previous finding that ceramide regulates acetyl-CoA import into the ER lumen (Costantini et al., 2007) and the increased acetylation pattern of ER-based proteins shown in Fig. 6D. The fact that the same results were obtained following spontaneous upregulation (Fig. 8D,E) and exogenous administration (Fig. 8A–C) of ceramide strengthen our conclusions. Finally, similar results were obtained with cell lines, primary neurons and brain tissue, clearly indicating that the above events are not limited to a specific cell type or to ex vivo experimental conditions.

Previous work has also shown that late-onset (sporadic) AD patients display increased levels of ceramide (approximately...
the post-translational events that occur in the early secretory pathway are crucial for the correct assembly of membrane and secreted proteins. As such, the widespread cell death associated with the downregulation of AT-1 in cultured cells as well as the neuronal degeneration caused by apparent h猿loinsufficiency of AT-1 in autosomal SPG42 is easily conceivable. The preferential association between changes in AT-1 levels and/or activity and neuronal disorders might be due to the constant need of neuronal cells to synthesize and transport membrane and/or secreted proteins, making them more susceptible to disruption of the secretory pathway. However, AT-1 is also upregulated during the differentiation of B-cells into immunoglobulin-secreting plasma
cells, a process that involves dramatic reorganization and expansion of the ER and activation of ER stress, with the sole purpose of synthesizing and secreting a large quantity of correctly folded immunoglobulin (Shaffer et al., 2004). ER stress, which can be initiated by overloading the secretory machinery and/or accumulating unfolded or misfolded proteins, typically activates the UPR to remove toxic aggregates. However, when insufficient, the UPR can also lead to cell death. As such, a defect in acetyl-CoA transport into the ER lumen would impede acetylation of nascent proteins that require this form of modification and result in their abnormal accumulation in the secretory pathway with consequent cellular toxicity. In this regard, it is important to point out that we are currently dissecting the ER stress signaling machinery responsible for the functional regulation of AT-1. Future work in this area might help understand the precise role of lysine acetylation of nascent membrane and/or secreted proteins as part of the UPR.

Autophagy is a general process that allows degradation and/or turnover of subcellular components (He and Klionsky, 2009). In addition to starvation and growth-factor deprivation, autophagy is activated during ER stress to dispose of large quantities of expanded ER that result from the abnormal accumulation of unfolded proteins (He and Klionsky, 2009). Evidence in yeast and mammalian cells indicates that autophagy is essential in maintaining ER homeostasis under the above conditions (Bernales et al., 2006; Ding et al., 2007; Ogata et al., 2006). Emerging data indicate that the ER membrane itself is involved in the generation of the autophagic membrane (Axe et al., 2008), thus explaining the close proximity of emerging autophagosomes to the expanded and enlarged ER structures. The dramatic activation of autophagy upon downregulation of AT-1 points to a crucial role of the ER-based acetylation machinery in maintaining ER homeostasis. Our previous work with BACE1 loss-of-acetylation and gain-of-acetylation mutants indicates that the transient acetylation of nascent BACE1 is essential for correct maturation of the nascent protein (Costantini et al., 2007). The fact that many proteins are acetylated in the ER (Fig. 6) indicates that the above events are not limited to BACE1. As such, global failure of the machinery could impede the maturation of nascent membrane and/or secreted proteins that undergo transient acetylation of the ε-amino group of lysine residues, with consequent activation of the UPR, expansion of the ER and activation of autophagy.

**Materials and Methods**

**Plasmid constructs**

Human AT-1 (NM_004733) cDNA was obtained from Origene (SC117182) and cloned into pcDNA3.1A/Myc/His (Invitrogen) and pcDNA3.1V5/His/TOPO (Invitrogen).

**Animals**

Non-transgenic and homozygous Pcsk9−/− mice (B6;129S6-Pcsk9tm1Jdh; stock no. 005993) were from The Jackson Laboratory. p44+/+ and p44–/– mice have been used as control. siRNA was applied every 2 days during the day experiments. Hippocampal neurons were isolated from embryonic day 15 (E15) rats and cultured in vitro for 3 or 4 days as previously described (Costantini et al., 2005; Costantini et al., 2006). Cells were harvested and analyzed by western blot or real-time PCR, as described below. For ceramide treatment, primary neurons were cultured at a density of 32,000 cells/cm²; after 3 days, cultures were treated with 10 μM C6 ceramide (Matreya) for 16 hours. An identical treatment was used for CHO and H4 cells. Total RNA was isolated using the RNeasy-4PCR kit (Ambion-Invitrogen). Cell death was assessed with the Live/Dead Cell Viability/Cytotoxicity Kit (Invitrogen) and by direct cell counting.

**Antibodies and western blotting**

Protein electrophoresis was performed as described before (Costantini et al., 2005; Costantini et al., 2006; Jonas et al., 2008; Ko and Puglielli, 2009) on a NuPAGE system using 4–12% Bis-Tris gels (Invitrogen). The following primary antibodies were used: rabbit anti-β-Catenin (Cell Signaling, 3772S), anti-β-Actin (Cell Signaling, 8457), anti-Calnexin (Abcam ab11352, polyclonal), anti-c-MyC (Sigma C3956, polyclonal), anti-Early Endosomal Antigen 1 (EEA1) (BD Transduction Laboratories, 640156, monoclonal), anti-ERGIC 53 (Sigma E1031, polyclonal), anti-GAPDH (GE Healthcare). Chemiluminescent detection was performed with Lumiglo (KPL) or ECL Plus (GE Healthcare). In the experiments involving Pcsk9−/− mice, samples were imaged and quantified with the Li-Cor Odyssey Infrared Imaging System (LI-COR Biosciences) as described before (Jonas et al., 2008).

**Real-time PCR**

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen), unless otherwise specified. Mouse livers were homogenized in TRIzol and RNA was purified on a Qiagen RNeasy column. To determine AT-1 mRNA levels, total RNA was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Quantitative PCRs were carried out in a LightCycler480 Real-time PCR System (Roche) using LightCycler480 SYBR Green I Master Mix (Roche). The cycling parameters were as follows: 95°C, 10 seconds; 55°C, 10 seconds; 72°C, 15 seconds, for a maximum of 40 cycles. Controls without reverse transcription were included in each assay. PCR primers specific to each gene were as follows: AT1-mouse, 5’-TACGGAGAACCTGTCGCTGT-3’ and 5’-ATAGC-39 (95 bp); actin-mouse, 5’-CAGAAGAAGCTGAGCTGTTG-3’ and 5’-GACGAGCTGAGCTGTTG-3’ (97 bp); ATF4-mouse, 5’-ATGGCTGACACAGCGGTGAG-3’ and 5’-GACCTGCTGGGACG-39 (333 bp); glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-human, 5’-TTGCTCAAGTCTTCTGTTCTG-39 and 5’-TCAACAGTGGTTGACTGG-39 (225 bp). AT-1 mRNA levels were normalized against actin or GAPDH levels and are expressed as percent of control. The mRNA of AD patients and age-matched controls was a generous gift of Craig Atwood (University of Wisconsin-Madison, Madison, WI, USA).

**Acetyl-CoA membrane transport**

Sonication was performed as described (Costantini et al., 2007; Jonas et al., 2008; Ko and Puglielli, 2009). Individual fractions were immediately centrifuged at 100,000 g for 15 minutes in an air-driven ultracentrifuge (Airfuge; Beckman), and pellets were resuspended in isatinic/cryogenic buffer (0.25 M sucrose and 10 mM Tris/HCl pH 7.4). Acetyl-CoA transport into intact native vesicles was performed as described in detail (Costantini et al., 2007). In the case of competition assays, purified native ER fractions were incubated in the presence of 10 μM [3H] acetyl-CoA and increasing concentrations of CoA (Sigma), (ATP) (Sigma), UDP-galactose (MP Biomedicals) and sodium acetate (Sigma) in isotonic/cryogenic buffer. Reactions were run as a typical membrane transport assay (Costantini et al., 2007).

**In vitro reconstitution**

For the reconstitution of AT-1 activity into artificial liposomes, transgenic AT-1 was purified from stably transfected cells with the Myc-Tag IP/Cis-IP Kit (Pierce) as suggested by the manufacturer and already described in our previous work (Costantini et al., 2007). Briefly, cell lysates from CHO cells stably transfected with AT-1 were loaded onto the above anti-Myc immobilized column, washed and eluted by lowering the pH to 2.0. The pH was immediately neutralized by adding 10 μl of neutralizing buffer (1 M Tris pH 9.5) per 200 μl of elution buffer. 18 μl of the elution was gently mixed with 50 μl of artificial liposomes and reconstituted with five cycles of freeze-thaw in an acetone-dry ice bath, as described before (Mandon et al., 1994; Puglielli and Hirschberg, 1999; Puglielli et al., 1999a; Puglielli et al., 1999b). Liposomes were made of a mixture of phosphatidylcholine:phosphatidylethanolamine:

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phosphatidylethanolamine and phosphatidylserine (55:30:15). Phosphatidylethanolamine and phosphatidylserine were added to mimic more closely the natural lipid environment of the ER membrane (Yungans et al., 1970). In fact, previous work with the ER membrane ATP transporter suggests that, in contrast to Golgi membrane transporters (Mondon et al., 1994; Puglielli and Hirschberg, 1999; Puglielli et al., 1999a), ER membrane transporters are not efficiently reconstituted into simple phosphatidylcholine artificial liposomes (Guillem and Hirschberg, 1995). Reconstituted proteoliposomes were assayed for acetyl-CoA transport immediately after the last freeze-thaw cycle. As control, transport was also assayed in liposomes alone (in the absence of AT-1) and in mixtures of liposomes plus AT-1 without freeze-thaw-mediated reconstitution. Finally, as additional control, a cell extract from control (non-transfected) cells was also loaded onto the above anti-Myc column and the elution volume was reconstituted into artificial liposomes.

**Trypsin digestion**

Purified ER vesicles were incubated for 60 minutes at 25°C with trypsin (Sigma) at a final concentration of 1 µg of protease per µg of ER or Golgi proteins. Digestion was halted by the addition of anti-trypsin specific inhibitor (Sigma) and by lowering the temperature to 0–4°C. The anti-trypsin inhibitor was used at the final concentration of 1 µg of inhibitor per µg of protease. Trypsin digestion was performed in the presence or absence of 0.05% Triton X-100.

**Electron microscopy**

Transmission electron microscopy was performed at the Electron Microscopy Facility of the William S. Middleton Memorial Veterans Hospital (Madison, WI) and University of Wisconsin-Madison, as described before (Oberley et al., 2008). All supplies and reagents were from Electron Microscopy Sciences (Hatfield, PA). Sections were observed using a transmission electron microscope (Hitachi H-600) operated at 75 kV.

**Aβ determination**

For Aβ determination in the conditioned medium, CHO cells were plated in six-well Petri dishes. When 80–90% confluent, cells were washed in PBS and incubated in 1 ml of fresh medium for 48 hours. Secreted Aβ was determined as described before by using a standard sandwich enzyme-linked immunosorbent assay (ELISA) system designed for the detection of rodent Aβ by using a standard sandwich enzyme-linked immunosorbent assay (ELISA) system.

**Statistical analysis**

Results are always expressed as mean of the indicated number of determinations. Unless specified, the data were analyzed by ANOVA and Student’s t-test comparison, using GraphPad InStat3 software. Statistical significance was reached at P<0.05.

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