Retention in Endoplasmic Reticulum 1 (RER1) Modulates Amyloid-β (Aβ) Production by Altering Trafficking of γ-Secretase and Amyloid Precursor Protein (APP)*

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Background: Aβ production is influenced by intracellular trafficking of secretases and amyloid precursor protein (APP).

Results: Retention in endoplasmic reticulum 1 (RER1) regulates the trafficking of γ-secretase and APP, thereby influences Aβ production.

Conclusion: RER1, an ER retention/retrieval factor for γ-secretase and APP, modulates Aβ production.

Significance: RER1 and its influence on γ-secretase and APP may be implicated for a safe strategy to target Aβ production.

The presence of neuritic plaques containing aggregated amyloid-β (Aβ) peptides in the brain parenchyma is a pathological hallmark of Alzheimer disease (AD). Aβ is generated by sequential cleavage of the amyloid β precursor protein (APP) by β- and γ-secretase, respectively. As APP processing to Aβ requires transport through the secretory pathway, trafficking of the substrate and access to the secretases are key factors that can influence Aβ production (Thinakaran, G., and Koo, E. H. (2008) Amyloid precursor protein trafficking, processing, and function. J. Biol. Chem. 283, 29615–29619). Here, we report that retention in endoplasmic reticulum 1 (RER1) associates with γ-secretase in early secretory compartments and regulates the intracellular trafficking of γ-secretase. RER1 overexpression decreases both γ-secretase localization on the cell surface and Aβ secretion and conversely RER1 knockdown increases the level of cell surface γ-secretase and increases Aβ secretion. Furthermore, we find that increased RER1 levels decrease mature APP and increase immature APP, resulting in less surface accumulation of APP. These data show that RER1 influences the trafficking and localization of both γ-secretase and APP, thereby regulating the production and secretion of Aβ peptides.

Alzheimer disease (AD)3 is traditionally characterized by two distinct proteinopathies, accumulation of senile plaques containing ~4 kDa amyloid β protein (Aβ) and neurofibrillary tangles containing the microtubule-associated protein Tau (2). Aβ is a normally secreted peptide derived from the larger β-amyloid precursor protein (APP) by the concerted and sequential action of two proteases, β- and γ-secretases. Although normally secreted as a soluble peptide, Aβ aggregates and accumulates in the brain to form classic amyloid deposits that form the core of neuritic plaques as well as other assemblies including a variety of soluble oligomers (3–5). The amyloid cascade hypothesis posits that Aβ accumulation is the primary disease initiator, and is strongly supported by molecular genetic studies of familial AD revealing that mutations in APP, presenilin (PS) 1, and PS2 genes accelerate Aβ production and studies show the sequential development of AD pathologies in the human (6, 7). On the basis of this hypothesis, modulating Aβ production has been a major focus of therapeutic development for AD (7, 8).

APP processing to Aβ is highly complex and modulated by many different factors (1, 9). APP is cleaved by β-secretase at the N terminus of Aβ to generate a large secreted derivative (sAPPβ) and a membrane-bound APP C-terminal fragment (CTFβ). Subsequent cleavage of CTFβ by γ-secretase results in the production of Aβ. Alternatively, APP can be cleaved within the Aβ sequence by α-secretase, which generates a secreted derivative (sAPPα) and its corresponding C-terminal fragment (CTFα). As β-secretase cleavage is essential for Aβ production and α-secretase precludes Aβ formation, β- and α-secretase cleavage pathways are often referred to as amyloidogenic and nonamyloidogenic, respectively. γ-Secretase is an intramembrane-cleaving protease complex that cleaves its substrates within their transmembrane domains (10, 11). It contains the presenilins (PS1 or PS2) as its catalytic core and three accessory proteins, nicastrin (NCT), APH-1 and PEN-2 (12). Although it has been widely held that a complex composed of all four subunits is required for γ-secretase activity, there is evidence that PS can be catalytically active in the absence of associated subunits under artificial in vitro circumstances (13). Other subunits of the complex, NCT, APH-1, and PEN-2 have been known to play roles in its trafficking and maturation. It has been...
suggested that NCT may be critical for substrate recognition (14, 15), although some evidence suggests that NCT may also have a more indirect role in regulation of the localization and activity of the complex (16). Steady-state accumulation of each component of the complex is coordinately regulated and, in large part, is dependent on the expression of the other members of the complex (17–19).

One focus of research in APP processing has been to determine the subcellular sites of $\beta$-secretase production and whether differential subcellular localization alters cleavage and contributes to increased risk for AD. Despite predominant localization of PS and other $\gamma$-secretase subunits to early compartments including the ER and ER to Golgi intermediate compartment (20, 21), many $\gamma$-secretase substrates reside in late secretory compartments and at the plasma membrane where only a small fraction of $\gamma$-secretase is detected (22–24). APP is synthesized in the endoplasmic reticulum (ER), post-translationally modified by N- and O-linked glycosylation, and transported through the secretory pathway to the cell surface. Various subcellular sites for amyloidogenic APP proteolytic processing have been reported. $\beta$-Secretase cleavage has been localized to the trans-Golgi network and to the endosomal/lysosomal system following APP endocytosis (25–27). On the other hand, $\alpha$-secretase-mediated shedding appears to occur predominantly on the cell surface (28).

$\gamma$-Secretase components are also trafficked through the trans-Golgi network to the plasma membrane. Our interest in the cellular factor(s) responsible for ER retrieval of $\gamma$-secretase developed in the course of studies using ANPP (APH1, NCT, PS1, PEN2) cell lines, which stably overexpress all four $\gamma$-secretase components and accumulate active $\gamma$-secretase to high levels (18, 20, 29, 30). Many $\gamma$-secretase substrates reside in late secretory compartments including the plasma membrane and early endosomes (26, 31, 32). Indeed, ectodomain shedding, which is an essential step for subsequent $\gamma$-secretase cleavage of many substrates, is believed to occur in the trans-Golgi, on the cell surface or endosomes (33, 34). Therefore, assembled $\gamma$-secretase complexes present in the ER require transport to the late compartments of the secretory pathway, to process substrates.

One possible candidate for ER retention of $\gamma$-secretase is RER1 (Retention in endoplasmic reticulum 1) protein involved in the ER retrieval for selected membrane proteins in yeast (35–38). RER1 was first identified in yeast by screening for defective mutants in the ER retention of Sec12p (36). Yeast Rer1 is required for the ER localization of Sec12p, Sed4p, Mns1p, Sec71p, and Sec63p (35, 37, 38). Yeast Rer1 binds to these proteins and carries them back to the ER via the COPI vesicles (38, 39). Human RER1 is an integral membrane protein that is 196 amino acids long and has four putative transmembrane domains that are predicted to form an M-shaped topology with both N and C termini facing the cytosol. It complements the RER1 deletion mutant of Saccharomyces cerevisiae (40), indicating that human RER1 also has a similar function to yeast Rer1 as an ER retrieval receptor.

Two groups have previously identified RER1 as a binding partner of different $\gamma$-secretase subunits, NCT (41) and PEN2 (42). Both studies showed that RER1 could affect $\gamma$-secretase assembly by regulating retention or retrieval of $\gamma$-secretase subunits. In the present study, the role of RER1 in modulation of $\gamma$-secretase activity and APP trafficking was investigated. We show that RER1 is associated with the $\gamma$-secretase complex and regulates the intracellular localization and trafficking of $\gamma$-secretase. Furthermore, we find that increasing RER1 expression decreases APP maturation, suggesting a potential mechanism by which RER1 may influence the trafficking of APP. Our data extend the previous reports of the effects of RER1 on $\gamma$-secretase but reveal a dual role for RER1 in regulating trafficking through secretory pathways of APP and $\gamma$-secretase to regulate the intramembrane proteolysis.

EXPERIMENTAL PROCEDURES

DNA Constructs and Antibodies—Human RER1 cDNA was isolated by RT-PCR using total mRNA from human kidney and cloned in a mammalian expression vector, pAG3Zeo (43). To facilitate biochemical detection, we appended a Myc-epitope tag to the carboxyl terminus of RER1. The sequences of the RER1-Myc cDNA were verified by DNA sequencing. To generate polyclonal antibodies against RER1 (R75 and R76), we generated a chimeric cDNA encoding a fusion protein containing the soluble domain of RER1 in the hydrophilic loop between transmembrane domains 2 and 3 (amino acids 89–120) fused to glutathione S-transferase (GST). Bacterially expressed fusion proteins were purified and used to immunize New Zealand White rabbits, as previously described (44).

The antibodies used in this study were PS1nt antibody, a polyclonal antibody that recognizes residues 1–65 of PS1 (29, 30); PS1 loop antibody, a polyclonal antibody generated against amino acids 263–407 of the loop region of PS1 (45); P2-1, a monoclonal antibody specific for epitopes in the human APP ectodomain (46); NCT54, a polyclonal antibody generated against amino acids 242–546 of NCT (18); SP719, a polyclonal antibody that recognizes residues 1–65 of PS1 (29, 30); PS1 loop antibody, a polyclonal antibody generated against amino acids 263–407 of the loop region of PS1 (45); P2-1, a monoclonal antibody specific for epitopes in the human APP ectodomain (46); NCT54, a polyclonal antibody generated against amino acids 242–546 of NCT (18); SP719, a polyclonal antibody raised against amino acids 689–709 of NCT (47); CT11, a polyclonal antibody that recognizes the last 7 amino acids of APLP1 (48); 9E10, a monoclonal antibody against the Myc-epitope tag; 82E1, an $\beta$ N-terminal-end specific monoclonal antibody (IBL, Hamburg, Germany); A8717, a polyclonal antibody generated against amino acids 676–695 of APP695 (Sigma).

Cell Culture and Transfection—Human embryonic kidney 293 (HEK293) cells were cultured at 37 °C in a humidified 5% CO$_2$ incubator in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). To generate ANPPR stable cell lines, ANPP cells (18) were transfected with human RER1-Myc cDNA and pBlast (Invitrogen) using a calcium phosphate method (49). Cells were selected with blasticidin S at 3 $\mu$g/ml (Invigene, San Diego, CA) and screened by immunoblot with 9E10 antibody. For transient transfection, Lipofectamine 2000 (Invitrogen) was used in accordance with the manufacturer’s instructions.

Western Blot—Detergent lysates of cells were prepared using immunoprecipitation (IP) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate) supplemented with a protease inhibitor mixture (a mixture of 4–(2-aminoethyl)benzenesulfonyl fluoride, pepstatin A, E-64, bestatin, leupeptin, and aprotinin; Sigma) (50).
Lysates were subjected to Tris glycine, BisTris, 10–20% Tris-Tricine, or 16.5% Tris-Tricine SDS-PAGE and then transferred to a nitrocellulose membrane prior to incubation with selected antibodies. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) detection system (PerkinElmer Life Sciences).

**Co-immunoprecipitation (Co-IP)**—Subconfluent dishes of stable cell lines were washed twice with ice-cold phosphate-buffered saline and solubilized in CHAPS co-IP buffer (1% CHAPS (Calbiochem, San Diego, CA), 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA). Lysates were subject to centrifugation at 15,682 × g for 10 min at 4 °C, and the resulting supernatant was used for co-IP with the respective antibodies at 4 °C overnight. The immune complexes were collected with Protein A-conjugated agarose beads (Pierce, Rockford, IL) and eluted by incubation at 50 °C for 15 min or 100 °C for 5 min in SDS sample buffer. The resulting immunoprecipitates as well as detergent lysates corresponding to 5 or 2.8% of the volume used for co-IP were resolved by Tris glycine or 16.5% Tris-Tricine SDS-PAGE and analyzed by Western blot with the indicated antibodies.

**RNA Interference**—Cells were plated at 2 × 10⁵ cells/well on 6-well plates. Cells were transfected with double-stranded small interfering RNA using Oligofectamine and RNAiMax in accordance with the manufacturer’s instructions (Invitrogen). Small interfering RNA oligonucleotides were synthesized by Ambion (Austin, TX). The target sequences 5'-TATCAAGT-CTGGCTAGACA (Rii85) and 5'-GGCTGAGCTTTGTCTACTCAT (Rii146) were used for human RER1 siRNAs. 5'-TTTTTTTTTC-CCCCAAGGGGGG (nonspecific) were used for negative control (51).

**Surface Biotinylation and Endoglycosidase H Treatment**—Cells were grown to 80% confluence in a 60-mm tissue culture dish, washed twice with ice-cold PBS-CM (1 mM CaCl₂ and 1 mM MgSO₄ in PBS), and incubated in 2 ml of ice-cold 0.5 mg/ml of sulfoconcanimidobiotin (Pierce) in a borate buffer (10 mM sodium borate, pH 9.0, 154 mM NaCl, 12 mM KCl, 2.25 mM CaCl₂) for 30 min on ice. The cells were washed four times with PBS-CM containing 50 mM NH₄Cl for quenching. Using the method described above, cells were lysed, and each lysate was divided in two, adjusted to 0.25% SDS, boiled for 10 min, and incubated with 50 μl of streptavidin-agarose beads (Pierce) at 4 °C overnight. The captured proteins were washed twice with IP buffer and an aliquot of each sample was treated with 500 units of endoglycosidase H (EndoH) (New England Biolabs, Ipswich, MA) in IP buffer containing protease inhibitors, 50 mM sodium citrate, pH 5.5, and 0.25% SDS at 37 °C for 4 h. The precipitates were washed and eluted by boiling in Laemmli SDS sample buffer. Five percent of the lysates used for precipitation were also treated with 500 units of EndoH or 500 units of peptide:N-glycosidase F (New England Biolabs) in IP buffer containing protease inhibitors, 50 mM sodium phosphate, pH 7.5, and 0.25% SDS. The precipitates and lysates were resolved by 5.5% SDS-PAGE and probed with NCT54 antibody.

**In Vivo Photoaffinity Cross-linking with L-852,505**—Cells were incubated in 2 μM L-852,505 (tetr-butyl (14S,17S,20R, 22R,23S)-14-(4-benzoylbenzyl)-20-benzyl-22-hydroxy-17-isobutyl-5,13,16,19-tetraoxo-1-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)-24-phenyl-6,12,15,18-tetraazatetracosan-23-yl-carbamate), a photolabeled transition state γ-secretase inhibitor (52, 53), at 37 °C for 1 h and then irradiated with 365 nm UV using Stratalinker (Stratagene) at room temperature for 90 min. Using the method described above, cells were lysed, and detergent lysates of the cells were incubated with streptavidin-agarose beads at 4 °C overnight. The precipitates and lysates were resolved by 12% SDS-PAGE and probed with PS1 loop antibody.

**Laser Confocal Microscopy**—Cells grown on poly-l-lysine-coated glass coverslips were washed with PBS-CM (PBS with 1 mM CaCl₂ and MgCl₂) and incubated with affinity-purified NCT54 antibody (18) and FITC-conjugated Vicia villosa lectin (VVL; Vector Laboratories Inc., Burlingame, CA) (54) at 10 °C for 45 min without fixation or detergent permeabilization. After cells were washed with PBS-CM, they were fixed in 4% paraformaldehyde in PBS for 10 min and incubated with Alexa 647-labeled anti-rabbit secondary antibody (Invitrogen) in PBS containing 1% BSA for 1 h. Cells were washed with PBS containing 0.2% Tween 20 and then mounted onto glass slides with VECTASHIELD mounting medium (Vector Laboratories Inc.). Images were acquired using the Leica TCS SP2 A0BS Spectral Confocal Microscope (Leica, Bannockburn, IL). Identical exposure times and power settings were used for the acquisition of both NCT and VVL images. For the BODIPY-FL L-685,458 labeling, cells were live-stained in 0.25 μM BODIPY-FL L-685,458 at 37 °C for 30 min, followed by fixation in 4% paraformaldehyde and permeabilization with 0.2% Triton X-100. Images were acquired on Olympus IX81-DSU Confocal Microscope.

**Pulse-Chase Experiments**—Cells at 80% confluence were incubated in methionine-free DMEM for 45 min and then metabolically labeled in methionine-free DMEM supplemented with 250 μCi/ml of [35S]methionine (PerkinElmer) and 1% diazylated FBS (Invitrogen) for 10 min. After labeling, cells were washed once and incubated for the indicated time periods in DMEM with 1% dialyzed FBS and 0.5 mM 1-methionine (Sigma). The conditioned medium was collected, and labeled cells were harvested in IP buffer, as described above. Using the aforementioned method, IP was carried out, as described above. After separation by Tris glycine SDS-PAGE, radioactive bands were visualized and quantified by phosphorimaging (Typhoon, Amersham Biosciences).

**Aβ ELISAs**—After coating Immulon 4HBX flat bottom 96-well plates (Thermo Scientific, Waltham, MA) with monoclonal antibodies (55) specific to Aβ40 (13.1.1, human Aβ35–40 specific), Aβ42 (2.1.3, human Aβ35–42 specific), or total Aβ (AB5, human Aβ1–16 specific), the cell culture medium, cell lysates, and Aβ peptides standard were added. Horseradish peroxidase (HRP)-conjugated 4G8 (Covance, Princeton, NJ) or AB5 antibodies were used as detection antibodies. Tetramethylbenzidine substrate was added and then 0.7% phosphoric acid stop solution was added to stop the reaction. The value was read at OD 450 nm and expressed in picomolar/milliliters.

**Immunoprecipitation followed by Mass Spectrometry (IP/MS)**—Magnetic sheep anti-mouse IgG beads (Invitrogen) were incubated with 4.5 μg of 4G8 (Covance) antibody for 30
min at room temperature with constant shaking. The beads were then washed and incubated with each extract, which were diluted appropriately. All sample incubations were in the presence of 0.1% Triton X-100. Bound beads were washed sequentially with 0.1 and 0.05% octyl glucoside followed by water. Samples were eluted using 0.1% trifluoroacetic acid. Samples were mixed in equal volume with α-cyano-4-hydroxycinnamic acid (15 mg/ml) in 60% acetonitrile, 40% MeOH, and 1 μl was spotted onto a 96-spot micro SCOUT plate (Bruker Daltonics GmbH, Germany) and analyzed with a Bruker microflex Series (Bruker) mass spectrometer.

Aβ Western Blotting—Detergent lysates were resolved by 4–12% Bis-Tris gel and the transferred membranes were boiled for 5 min. After blocking in Starting Block (Thermo Scientific) and incubation overnight with primary antibody (82E1, IBL) antibody conjugated to HRP (Jackson ImmunoResearch, West Grove, PA). Chemiluminescence signal (West Femto Chemiluminescent Substrate (Thermo Scientific)) was visualized with a FujiFilm system.

RESULTS

Establishment of ANPPR Cell Lines—We generated several ANPPR (APH1, pCT, pS1, pEN2, tRER1) cell lines from the previously described ANPP cell line (18). Multiple independent clones of ANPPR cells that stably overexpress Myc-tagged RER1 along with four γ-secretase subunits were selected. RER1-Myc was detected as a ~23-kDa protein as predicted (40) (Fig. 1A, lanes 2 and 3). Compared with the parental ANPP line (Fig. 1A, lane 1), expression levels of γ-secretase subunits were not altered in the ANPPR lines, indicating that total levels of γ-secretase in ANPPR cells are compatible with ANPP cells. Considering the nature of γ-secretase that each of the components serves to stabilize each other during a γ-secretase complex maturation process and that PS1 NTF and CTF are present only in the mature active form of the γ-secretase complex, the results indicate that RER1 does not affect the total levels of γ-secretase. To further test whether the total level of “active” γ-secretase changes by RER1, we treated ANPP and ANPPR cells with L-852,505 (Fig. 1B), a photoreactive, biotinylated derivative of L-685,458. Upon photoactivation at 350 nm, this compound has been shown to be cross-linked to PS1 CTF (52, 53). The active γ-secretase was recovered with streptavidin-conjugated agarose beads. Western blot analysis of the total cell extracts (Fig. 1B, lanes 1 and 2) and the captured “active” γ-secretase (Fig. 1B, lanes 3 and 4) using PS1 loop antibody revealed that RER1 overexpression did not change the total levels of active γ-secretase. Moreover, when we labeled ANPP cells with L-852,505 after RER1 siRNA transfection, knockdown of RER1 expression also did not affect the total levels of active γ-secretase (Fig. 1C, lanes 4–6), suggesting that RER1 has no detectable effect on total levels of active γ-secretase.

Interaction between RER1 and γ-Secretase Complex—We performed co-IP studies of RER1 and γ-secretase components in ANPPR cells. Using conditions that preserve γ-secretase activity and do not dissociate the complex, ~5% of the Myc-tagged RER1 was co-immunoprecipitated with PEN2 (Fig. 2A, lane 3), whereas an unrelated antibody did not co-immunoprecipitate Myc-tagged RER1 (Fig. 2A, lane 2). Co-IP of RER1 with NCT (Fig. 2A, lane 5) or with PS1 (Fig. 2A, lane 4) were also...
observed albeit to a lesser extent than with PEN2. The levels of RER1 co-immunoprecipitated with PEN2, NCT, or PS1 are much smaller than those of APH1-Myc in the same sample (Fig. 2A). Using newly generated polyclonal antibodies against RER1 (R75 and R76), we immunoprecipitated endogenous RER1 in addition to RER1-Myc (Fig. 2B, second panel), and RER1 decreases the surface active γ-secretase. Identical exposure time and power setting were used for the acquisition of images. The scale bar on the image is 20 μm.

**Effects of RER1 Overexpression on the Trafficking of γ-Secretase—**
To examine whether overexpression of RER1 affects the surface localization of γ-secretase, we performed cell surface biotinylation experiments coupled with EndoH digestion. NCT undergoes complex N-glycosylation in the late Golgi and sialylation in the trans-Golgi, generating EndoH-resistant, “mature” NCT (56). Western blot analysis of NCT in total cell extracts revealed mature and immature NCT (Fig. 3A, lanes 1–3). EndoH treatment reduced the molecular mass of both mature and immature bands by ~5 kDa (EndoH resistant) and ~40 kDa (EndoH sensitive), respectively (Fig. 3A, lanes 4–6) and peptide:N-glycosidase F treatments removed all N-linked oligosaccharide chains (Fig. 3A, lanes 7–9). Having confirmed the identity of the immature and mature forms of NCT in these cells, we observed that: 1) the levels of mature NCT were not significantly different between ANPP and ANPPR lines (Fig. 3A, lanes 1–3), and 2) that EndoH-resistant NCT were reduced in two independent ANPPR lines (R12 and R21) (Fig. 3A, lanes 4–6). In surface biotinylation studies, a much lower amount of total mature (Fig. 3A, lanes 10–12), as well as EndoH-resistant (Fig. 3A, lanes 13–15) NCT was detected in both ANPPR12 and -21 lines, compared with parental ANPP cells (Fig. 3A, lanes 11 and 12 versus 10, lanes 14 and 15 versus 13) even though the level of total NCT after peptide:N-glycosidase F digestion is comparable (Fig. 3A, lanes 7–9).

To compare the kinetics of NCT maturation between these cells, we performed pulse-chase experiments. It showed that the synthetic rate of NCT is not significantly different between these lines (Fig. 3B, lanes 1, 5, and 9), but that the levels of mature NCT after a 2- or 4-h chase period are significantly reduced in ANPPR lines (Fig. 3B, lanes 7 and 11 versus 3, lanes 8 and 12 versus 4; quantified in Fig. 3C). These findings indicate that RER1 increases the retention time of NCT in the early secretory compartments and thereby reduces the maturation rate of NCT. The difference in levels of mature glycosylation of NCT is most likely due to altered intracellular trafficking of NCT.
RER1 Modulates Aβ Production

To confirm the decreased levels of cell surface NCT in ANPPR cells, we stained unfixed, live cells with an anti-NCT antibody and FITC-VVL at 10 °C for 45 min. Surface NCT immunoreactivities were markedly decreased in ANPPR cell lines (Fig. 3D, a–c). In contrast, surface labeling by FITC-VVL was not significantly different (Fig. 3D, d–f), indicating that RER1 has no major effects on the overall surface localization of glycoproteins. To further investigate the localization of active, γ-secretase, we stained live cells using an active BODIPY-FL derivative of a highly potent, transition state analog inhibitor of γ-secretase, L-685,458. When we stained ANPP cells transfected with BODIPY-FL-L-685,458 compound, BODIPY fluorescence was predominantly observed on the plasma membrane (Fig. 3E, a). In both ANPPR12 and -21 lines, consistent with the results of NCT live staining, BODIPY fluorescence on the surface was significantly decreased, compared with the parental ANPP line (Fig. 3E, b and c), indicating that RER1 expression decreased the surface localization of active γ-secretase. These results indicate that RER1 overexpression reduces both the complex glycosylation of NCT in late Golgi and the cell surface levels of NCT and γ-secretase.

Effects of RER1 Depletion on the Trafficking of γ-Secretase—To verify the role of endogenous RER1 in the trafficking of NCT and γ-secretase, we synthesized two distinct siRNAs against RER1 (Ri85 and Ri146) and transfected cells with varying concentrations of these siRNAs. Transfection of ANPPR cells with these siRNAs efficiently suppressed the expression of both the transfected RER1-Myc and endogenous RER1 in a dose-dependent manner. RER1 was down-regulated by 80–90% at 72 h after transfection with 100 ng (7.5 pmol) of Ri85 and Ri146 siRNAs (Fig. 4A, lanes 2, 6, and 10). We next transfected ANPP cells with RER1 siRNAs and performed surface biotinylation/EndoH digestion experiments. In whole cell lysates, the levels of total mature NCT were not significantly different between cells (Fig. 4B, lanes 1–3). However, the level of EndoH-resistant, fully mature NCT was increased by RER1 siRNA (Fig. 4B, lanes 4–6). In addition, surface biotinylation studies revealed significantly higher levels of mature NCT (Fig. 4B, lanes 10–12) and EndoH-resistant NCT (Fig. 4B, lanes 13–15) in RER1-depleted RNAi-treated cells. Pulse-chase experiments demonstrated that the level of mature NCT was consistently increased in cells transfected with RER1 siRNA (Fig. 4C, lanes 4 and 8, quantified in D). These data indicate that endogenous RER1 is involved in the retention of NCT and possibly the whole γ-secretase complex in early compartments of the secretory pathway.

FIGURE 4. RER1 depletion accelerates the trafficking of γ-secretase. A, ANPPR cells were transiently transfected with siRNAs (Ri85 or Ri146) against RER1; both siRNAs dramatically decreased both exogenous and endogenous RER1 protein levels. B, RER1 depletion increases the maturation of NCT. siRNA-transfected ANPP cells were surface-biotinylated and biotinylated surface proteins were captured with streptavidin-agarose. Total levels of surface NCT (lanes 10–12), as well as endoglycosidase H-resistant NCT (EndoH, lanes 13–15) increase by RER1 knockdown. N, nonspecific siRNA; 85, Ri85; 146, Ri146. C, pulse-chase experiment of NCT in siRNA-transfected ANPP cells. ANPP cells were transfected with 100 ng of Ri85 and Ri146 siRNA for 72 h. Cells were pulse-labeled with [35S]methionine for 10 min, and chased for 0 (lanes 1, 2, 4, and 5) and 4 h (lanes 3 and 7), or 4 h (lanes 4 and 8). Detergent lysates were immunoprecipitated with NCT54 antibody and resolved by 5.5% SDS-PAGE. D, levels of mature NCT were quantified by phosphoimaging, normalized to the synthesis level of NCT at a 10-min pulse (lanes 1 and 5). The data were expressed as the percentage ratio relative to the maturation rate of NCT in ANPP cells transfected with nonspecific siRNA at the 4-h chase time point (lane 4). Average (± S.E.) of two separate experiments was shown. E, RER1 depletion increases levels of surface NCT. siRNA-transfected ANPP cells were stained with affinity-purified NCT antibody (a–c) and FITC-conjugated V. villosa lectin (VVL; d–f). F, RER1 depletion increases the levels of plasma membrane γ-secretase. Cells were stained live with 0.25 μM BODIPY-FL L-685,458 to directly visualize active γ-secretase. Identiﬁcal exposure time and power settings were used for the acquisition of both NCT and VVL images. The scale bar on the image is 20 μm.
RER1 Modulates Aβ Production

FIGURE 5. RER1 decreases extracellular Aβ. A. ANPP cells were transiently transfected with negative control, RER1, or GMP25. Conditioned medium were used for sandwich ELISA. B, mass spectrometric (MS) analysis following immunoprecipitation (IP) of Aβ from RER1 overexpressing cells. Wild type APP expressing Chinese hamster ovary (CHO) cells were transiently transfected with negative control (a), RER1 (b), or GMP25 (c). Conditioned medium were collected and immunoprecipitated with 4G8 antibody. Representative MS spectra are shown. Peaks corresponding to Aβ peptides have been labeled according to m/z. NT, nontransfected; N.C, negative control Error bars show mean ± S.E. *** p < 0.005.

results, the surface levels of active γ-secretase complex labeled with BODIPY-FL L-685,458 were markedly increased in cells by RER1 depletion (Fig. 4F, a–c). Collectively, these results indicate that RER1 is involved in the trafficking of γ-secretase and therefore regulates the surface levels of the protease.

Effects of RER1 on the Aβ Production—To examine the effects of RER1 on APP processing, we measured Aβ40, Aβ42, and total Aβ levels by ELISA of conditioned media (Fig. 5A) from ANPP cells overexpressing RER1 and APP. RER1 overexpression decreased secreted levels of Aβ40 by 78% and Aβ42 by 75% in cells expressing wild type APP (APPwt). The RER1 effects on secreted Aβ levels were highly similar in cells expressing a FAD-linked APP mutant (APPswe) (decreased Aβ40 by 82% and Aβ42 by 86%). In contrast, the overexpression of GMP25 (hp24α2), a member of the p24 protein family that localizes in COPI- and COPII-coated vesicles and is involved in vesicular transport processes at the ER-Golgi interface (57), did not affect the levels of secreted Aβ compared with the negative control (Fig. 5A). Consistent with the results by Aβ ELISA, we observed the markedly decreased secretion of Aβ by Western blotting (data not shown). Although RER1 significantly decreased the levels of secreted Aβ, our mass spectrometric analysis showed no change in the profiles of Aβ peptides (Fig. 5B). Aβ Western blotting using detergent lysates revealed that there was no significant change in the levels of intracellular Aβ monomer. However, a protein fragment with a higher molecular mass (Mr ~ 8,000) than the Aβ monomer was detected (Fig. 5C), suggesting that Aβ oligomers or longer forms of Aβ peptide might be produced due to RER1 co-expression (58).

Effects of RER1 on the Maturation and Trafficking of APP—To determine whether the levels of secreted APP (sAPP) also parallel decreased secretion of Aβ, we analyzed sAPP in conditioned medium by Western blotting. We observed a significant decrease in the levels of sAPP secreted by cells co-transfected with RER1 and APP (Fig. 6A, top panels). Considering the earlier reported roles for RER1 in regulating γ-secretase assembly (41, 42), it seems insufficient to explain our observation on the decreased levels of sAPP release, which is dependent on cleavage by α- or β-secretase. In addition, the Western blot analysis revealed that the steady-state levels of mature APP were significantly decreased and immature APP increased by RER1 overexpression (Fig. 6A, bottom panels), indicating that RER1 might be involved in APP maturation and trafficking.

To further test this possibility, we performed a pulse labeling experiment with [35S]methionine. Newly synthesized immature APP showed delayed maturation in RER1 co-expressing cells (Fig. 6B, top panel). In this experiment, RER1 overexpression therefore markedly decreased the ratio of mature APP: immature APP, compared with the negative control. Consistent with the decreased levels of mature APP, the levels of sAPP in medium were also significantly decreased by RER1 overexpression (Fig. 6B, bottom panel). Other than sAPP, SDS-PAGE analysis of conditioned medium after a 4-h continuous labeling showed no detectable change in the levels of secreted polypeptides (Fig. 6B, bottom panel; low molecular weight part not shown). Our live staining results revealed that APP levels on the cell surface were significantly decreased in cells co-expressing with RER1 (Fig. 6C), whereas RER1 did not decrease the levels of total APP (Fig. 6, A, bottom panels, and B, top panel), suggesting that RER1 has a role in regulation of the maturation and trafficking of APP but not in regulation of its expression. Levels of overall glycoproteins on the cell surface were not affected by RER1 (Fig. 6C, c and d), indicating that RER1 does not have a major effect on the secretory pathway in the cells.

We observed significantly decreased levels of the APP-CTFα (Fig. 6A, bottom panels) indicating that the impaired APP maturation and trafficking by RER1 resulted in decreased accessibility of APP to α-secretase (Fig. 6A, bottom panels). RER1 overexpression had less effect on APP-CTFβ production (Figs. 5C and 6A, bottom panels). Notably, several additional APP-CTFs
RER1 Modulates Aβ Production

![Diagram A](image1.png)

![Diagram B](image2.png)

![Diagram C](image3.png)

FIGURE 6. RER1 decreases APP maturation and alters APP processing. A, the levels of secreted APP N-terminal fragments were significantly decreased by RER1 overexpression. Conditioned media were collected 36 h post-transfection and dissolved in SDS-PAGE (top panel). RER1 overexpression also induced the additional production of APP-CTFs (asterisks in bottom panel). B, RER1 decreases mature APP and increases immature APP. Cells were labeled with [35S]methionine for 4 h and APP was immunoprecipitated by P2–1 antibody (top panel). Label is incorporated into a 110–120-kDa immature precursor protein, which is chased into a 145–150-kDa band representing mature APP. RER1 decreased the levels of sAPP in medium but did not change overall levels of the secreted proteins. Straight media were used (bottom panel). NT, nontransfected; N.C., negative control. C, RER1 retards the trafficking of APP. RER1 and APP were overexpressed in ANPP cells and cells were stained live with P2-1 antibody against APP (a and b) and FITC-conjugated V. villosa lectin (VVL; c and d). After fixing, cells were incubated with Alexa 647-labeled anti-rabbit IgG antibody for 1 h. Images were acquired on Olympus IX81-DSU confocal microscope. Identical exposure time and power settings were used for the acquisition of both APP and VVL images. The scale bar on the image is 20 μm.

were observed in RER1 co-expressing cells (asterisks in Fig. 6A, bottom panels). These RER1 induced APP-CTFs were not altered by either β-secretase (BACE1) or γ-secretase inhibitors (Fig. 7, A and B), indicating that they are not either products or substrates of the two activities. Aβ ELISA results show that treatments of secretase inhibitors blocked Aβ production efficiently (Fig. 7C). These data support a novel intracellular degradation pathway for APP mediated by RER1.

**RER1 Depletion Increases Extracellular Aβ and APP Maturation and Trafficking**—Transfection with RER1 siRNA attenuated RER1 expression in H4 human neuroglioma cells stably expressing APPwt (Fig. 8B, bottom panel). As shown above, suppression of endogenous RER1 resulted in acceleration of γ-secretase maturation and trafficking. To assess whether endogenous RER1 regulates APP trafficking, Aβ levels were measured after RER1 depletion. In multiple experiments, siRNA knockdown of RER1 expression significantly increased secretion of total Aβ by 40–68%, Aβ40 by 43–66%, and Aβ42 by 45–109% (Fig. 8A). RER1 depletion also increased levels of mature APP, indicating that its depletion accelerated the maturation and trafficking of APP (Fig. 8B, top panel). RER1 depletion did slightly increase the levels of immature APP; therefore, we cannot rule out a possibility that RER1 also influenced APP stability or synthesis. APP-CTFβ and APP-CTFα were not increased after RER1 depletion (Fig. 8B, second and third panels). Because the effects of RER1 RNAi knockdown and RER1 overexpression were opposite from each other, we did not conduct additional controls for nonspecific RNAi effects, as off-target RNAi would typically result in reductions (i.e., lower Aβ secretion) rather than induction of a biological process.

**DISCUSSION**

We have found that RER1 regulates the trafficking of γ-secretase and APP in early secretory compartments. Co-IP experiments show that RER1 associates with γ-secretase and APP in early secretory compartments. Co-IP experiments show that RER1 associates with γ-secretase and APP in early secretory compartments. Collectively, these data show that RER1 can modulate the production and secretion of Aβ peptides by controlling the trafficking of both the substrate and protease in early secretory compartments.

Previous studies have suggested that RER1 competes with APH1 for NCT binding and negatively regulates the assembly of γ-secretase (41); or that RER1 binds to PEN2 (42). However,
we find that RER1 co-immunoprecipitates with the γ-secretase complex, as opposed to selectively binding individual disassociated components of the complex. APH1 consistently co-immunoprecipitated with both stably overexpressed and endogenous RER1, a result that is difficult to explain if RER1 competes with APH1 for NCT binding in the assembling γ-secretase complex as suggested by (41). Moreover, manipulation of RER1 levels has no effect on the assembly of the γ-secretase complex or the total level of γ-secretase. Instead, both levels of fully mature NCT and active γ-secretase are regulated by RER1 expression. Notwithstanding these differences, these results are consistent with the major conclusions of the two previous studies (41, 42) in that we find RER1 associates with the immature γ-secretase complex in early secretory compartments and decreases γ-secretase cleavage.

Our previous studies showed that active γ-secretase is assembled in the ER and transported to the late compartments of the secretory pathway where it accesses substrates (20). Because Aβ is predominantly generated in the late secretory and endosomal pathways (31, 59, 60), positive and negative control of the transport of APP and γ-secretase in the ER and Golgi impacts the efficiency of Aβ production. Studies using brefeldin A, which causes a rapid redistribution of the Golgi apparatus into the ER (61), have shown that immature APP accumulates, cell surface APP decreases, and Aβ secretion decreases as a result of collapse of the Golgi network (62–64). BACE1 overexpression studies also suggested that Aβ production is highly dependent on the specific subcellular localization of APP and BACE1 (65). Additional data has also demonstrated
that cytosolic adaptors such as members of Mint/X11, Re65, Dab, and the JIP family of proteins and cargo proteins, such as P23/TMP21, regulate Aβ production by regulating APP trafficking and γ-secretase processing (66–68). FAD-linked PS1 mutants also have been shown to affect intracellular trafficking of the γ-secretase complex (60, 69). Thus, our current findings support the idea that γ-secretase cleavage and Aβ peptide production are tightly regulated by subcellular localization and trafficking of the complex and substrate. To explore the effects of RER1 on γ-secretase cleavage of Notch 1, we have transiently overexpressed an ectodomain-truncated Notch 1, NotchΔE, in ANPP and ANPPR cells. Both NotchΔE and the Notch intracellular domain were decreased in cells overexpressing RER1 (data not shown); thus, additional studies will be needed to determine how RER1 alters trafficking and γ-secretase cleavage of other substrates.

Interaction between APP and RER1 was not observed in our co-IP experiments, suggesting that RER1 affects APP trafficking and γ-secretase activity via distinct mechanisms. As RER1 recruits its substrate to COPII vesicles and transports it back to the ER from the cis-Golgi (38), a likely explanation for these results is that RER1 binds directly to γ-secretase and retrieves it through a retrograde transport system but affects APP trafficking by indirectly altering retrograde vesicle transport. Alternatively, RER1 may recruit proteins involved in the anterograde transport of APP from Golgi to the ER and indirectly delay APP maturation and trafficking. It is also possible that a direct association of APP and RER1 was disrupted by the lysis conditions used. Notably, our data showed no significant difference in overall surface glycoprotein levels or overall protein secretion used. Notably, our data showed no significant difference in overall surface glycoprotein levels or overall protein secretion.

Based on the previous studies of RER1 function and our analysis of its influence on APP and γ-secretase transport, RER1 appears to be a key ER retrieval factor of APP and γ-secretase, although by apparently distinct mechanisms. As altered Aβ levels are tightly linked to the development of AD and lowering Aβ is a prime target for prophylactic therapy, future study of the RER1 and its influence on γ-secretase and APP trafficking may reveal novel connections to AD or possibly novel factors that can safely be targeted to alter Aβ production.

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