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Erlin-2 is associated with active γ-secretase in brain and affects amyloid β-peptide production

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ABSTRACT

The transmembrane protease complex γ-secretase is responsible for the generation of the neurotoxic amyloid β-peptide (Aβ) from its precursor (APP). Aβ has a causative role in Alzheimer disease, and thus, γ-secretase is a therapeutic target. However, since there are more than 70 γ-secretase substrates besides APP, selective inhibition of APP processing is required. Recent data indicates the existence of several γ-secretase associated proteins (GSAPs) that affect the selection and processing of substrates. Here, we use a γ-secretase inhibitor for affinity purification of γ-secretase and associated proteins from microsomes and detergent resistant membranes (DRMs) prepared from rat or human brain. By tandem mass spectrometry we identified a novel brain GSAP; erlin-2. This protein was recently reported to reside in DRMs in the ER. A proximity ligation assay, as well as co-immunoprecipitation, confirmed the association of erlin-2 with γ-secretase. We found that a higher proportion of erlin-2 was associated with γ-secretase in DRMs than in soluble membranes. siRNA experiments indicated that reduced levels of erlin-2 resulted in a decreased Aβ production, whereas the effect on Notch processing was limited. In summary, we have found a novel brain GSAP, erlin-2, that resides in DRMs and affects Aβ production.

1. Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disorder and the most common cause of dementia. The pathological hallmarks in the AD brain are extracellular deposition of amyloid plaques composed of the amyloid β-peptide (Aβ), and intracellular neurofibrillary tangles (NFTs) made of hyperphosphorylated tau. Several studies implicate that Aβ aggregation is the initial insult in AD pathogenesis [1]. Aβ is generated from the Aβ precursor protein (APP) by sequential cleavages mediated by β-secretase and γ-secretase [2]. γ-Secretase cleavage results in Aβ peptides of different lengths; Aβ40 being most abundant, whereas Aβ42 and Aβ43 are more toxic and more amyloidogenic [3,4].

Inhibiting γ-secretase activity is thus a potential therapeutic target for treatment of AD. However, γ-secretase cleaves a number of other substrates, and γ-secretase inhibitors cause *in vivo* toxicity by blocking cleavage of the essential substrate Notch [5]. Thus, further studies on γ-secretase are imperative for development of drugs that selectively reduce Aβ production.

γ-Secretase is an aspartyl protease consisting of four proteins; presenilin (PS), nicastrin, Aph-1 and Pen-2. These proteins are necessary and sufficient to produce an active γ-secretase complex [2]. However, it is possible that other associated proteins affect γ-secretase activity. For instance, TMP21, CD147, proteins in the tetraspanin web and PION have been reported to associate with γ-secretase and modulate Aβ production without affecting Notch cleavage [6–9]. These studies were performed in cell-lines, and it is likely that other GSAPs exist in brain.

Abbreviations: Aβ, amyloid β-peptide; ACN, acetonitrile; AD, Alzheimer disease; AICD, APP intracellular domain; APP, Aβ precursor protein; FA, formic acid; GCB, γ-secretase inhibitor coupled to biotin via a cleavable linker; GSAPs, γ-secretase associated proteins; IP, immunoprecipitation; LC-MS/MS, liquid chromatography coupled online to tandem mass spectrometry; *m/z*, mass-to-charge ratio; NICD, notch intracellular domain; PS, presenilin; PS-CTF, presenilin C-terminal fragment; PS-NTF, presenilin N-terminal fragment; SA, streptavidin; WB, Western blot.

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In a previous study [10], we developed a method to identify GSAPs using a γ-secretase inhibitor with a cleavable biotin group (GCB) and nano liquid chromatography coupled on line to tandem mass spectrometry (LC-MS/MS). Subsequently, we performed large scale studies subjecting rat DRMs and synapses [11,12] as well as rat and human CHAPSO-solubilized microsomes (Teranishi et al., unpublished) to GCB purification. Here, we present the identity and further evaluation of one of the GSAPs, ER lipid raft-associated protein 2 (erlin-2). The interaction was confirmed by co-immunoprecipitation and by an *in situ* proximity ligation assay (PLA). siRNA-mediated knockdown of erlin-2 resulted in a decreased Aβ production with less effect on Notch processing.

2. Materials and methods

2.1. Antibodies

The following antibodies were used: PS1-NTF (529591; Calbiochem for WB), raised against amino acid residues 1–65 of human PS1 and (AF149, R&D system for PLA), raised against residues 1–80 of human PS1; PS1-CTF (MAB5232; Chemicon), raised against the loop (residues 263–378) of human PS1; nicastrin (N1660, Sigma) raised against C-terminal residues 693–709 of human nicastrin; Erlin-2 (Sigma, HPA002025) raised against the C-terminal residues 189–307 of human erlin-2; cleaved Notch1 (Val1744, Cell Signaling Technology, 2421).

2.2. Brain materials

Sprague–Dawley rats (B&K Universal) were sacrificed by carbon dioxide, and the brains were dissected to remove blood vessels and white matter. Sprague–Dawley rat brains (8–12 weeks old) were also purchased (Rockland Immunochemicals) and stored at −70 °C before use. Human brain material (frontal cortex from a non-AD case, 20 h postmortem time) was obtained from Huddinge Brain Bank. The use of brain materials in this study was approved by the Animal Research Ethical Committee of Southern Stockholm (S80-08, S149-08) and the Regional Ethical Committee in Stockholm (024-01, 2007/533-32).

2.3. Preparation of microsomes

Rat or human brain samples were homogenized in buffer A (20 mM Hepes, 50 mM KCl, 2 mM EGTA, pH 7.5) containing Complete protease inhibitor cocktail (Roche) with 25 strokes at 1500 rpm using a pestle-homogenizer. The samples were centrifuged at 1000*g* to remove nuclei and cell debris and at 10,000*g* to remove mitochondria. The supernatants were centrifuged at 100,000*g*, and the resulting microsomal pellets (P3) were resuspended in buffer A supplemented with 20% glycerol, and stored at −80 °C. All centrifugation steps were performed at 4 °C.

2.4. Preparation of DRMs

DRMs were prepared as described previously [13]. In brief, the microsomal fraction was incubated in 2.0% CHAPSO, adjusted to 45% sucrose and subjected to centrifugation in a discontinuous sucrose gradient. The fraction at the 5–35% sucrose interface (DRM fraction) was collected, diluted to 0.5% CHAPSO and used in the studies below.

2.5. Affinity pulldown of γ-secretase

Synthesis of GCB and affinity pulldown by GCB was described elsewhere [10]. In brief, microsomal proteins were solubilized in 1% (w/v) CHAPSO for 30 min at 4 °C and centrifuged at 100,000*g* for 30 min. The resulting supernatants (solubilized microsomes) were diluted in buffer A without CHAPSO to give a final detergent concentration of 0.5% (w/v) CHAPSO. The samples were incubated with SA-conjugated Sepharose beads (GE Healthcare) to remove endogenous biotinylated proteins. The supernatants were incubated in the presence or absence of 10 μM (50 μM for DRMs) L-685,458 (competing inhibitor) for 10 min at RT (30 min at 37 °C for DRMs), followed by incubation with 200 nM GCB for 30 min at RT (1 h at 37 °C for DRMs). The samples were incubated with SA-beads for 2 h at 4 °C, and washed 3 times with buffer A with 0.5% (1% for DRMs) CHAPSO at RT. The captured γ-secretase complex was eluted in buffer A containing 100 mM DTT and 0.5% CHAPSO, and SDS sample buffer (for WB), or 0.01% RapiGest (Waters) in 10 mM ammoniumbicarbonate supplemented with 10 mM DTT (for LC-MS).

2.6. Tryptic digestion and fractionation of peptides

The eluted proteins were digested by trypsin in digestion buffer (180 mM ammonium bicarbonate, 2 mM CaCl2 and 0.3% Rapigest) at 37 °C overnight. The digested samples were loaded onto ZipTips C18 (Millipore) according to the manufacturer's instructions. The samples were washed with 0.2% formic acid (FA) in water, eluted in 80% acetonitrile/0.2% FA, and dried in a vacuum centrifuge (Maxi dry lyo, Heto-Holten AIS).

2.7. Mass spectrometry

The digested samples were dissolved in 2% ACN/0.2% FA in water and analyzed on a 6330 HPLC chip-Ion Trap LC/MS system (Agilent Technologies), using a chip with a 150 mm × 75 μm analytical column and a 160 nL enrichment column, both packed with 5 μm Zorbax 300SB-C18. Samples were loaded at a flow rate of 2 μL min^{-1} in 2% ACN and 0.2% FA in water. Tryptic peptides were eluted into the mass spectrometer using a gradient of increasing mobile phase B at a flow rate of 200 nL min^{-1}. The gradient (mobile phase A: H_2O/0.2% FA; mobile phase B: ACN/0.2% FA) was from 3% to 26% B in 92 min, and from 26% to 36% B in 20 min. The capillary voltage was 1950 V and the drying gas flow was 4 L min^{-1} at 300 °C. Spectra were collected over an *m/z* range of 230–1800, and the five most intense ions were subjected to MS/MS. After two MS/MS spectra, the precursor was excluded for 30 s.

2.8. Protein identification by MASCOT Daemon software package

A list of the spectra was generated using the LC/MS Software Version 6.1 (Agilent Technologies) with an intensity threshold of 1000. The MASCOT Daemon software package (Matrix Science) was used for protein identification. The parameters used in the data base search were: database: NCBInr, Homo sapiens or Mammalia, maximum missed cleavages: 1; variable modification: methionine oxidization, protein mass tolerance: 2.0 Da, fragment ion mass tolerance: 0.3 Da, peptide charge state: 2^+, 3^+. Statistically significant protein hits found only in samples incubated in the absence of competing inhibitor were selected, and extracted ion chromatograms from the samples (±competing inhibitor) were compared.

2.9. SDS–PAGE and WB

Samples were boiled in Tricine sample buffer (Invitrogen) and separated by SDS–PAGE (10–20% Tricine gels, Invitrogen), transferred to PVDF membranes (Bio-Rad), probed with specific antibodies, and visualized by SuperSignal West Dura enhanced

chemiluminescence reagent (Pierce). For quantification, a Fuji LAS-3000 CCD camera was used.

2.10. Co-immunoprecipitations

Solubilized γ-secretase or DRMs were resuspended in buffer A containing 0.5% or 1.0% CHAPSO and complete protease inhibitor cocktail. The samples were incubated with end-over-end rotation for 20 min at 4 °C. The samples were pre-cleared with a 1:1 ratio of protein A/G Sepharose (GE Healthcare) for 30 min at 4 °C, and incubated with anti-erlin-2 or control rabbit IgG overnight at 4 °C, followed by incubation with protein A/G Sepharose for 1 h at 4 °C. After washing three times with buffer A containing 0.5% or 1.0% CHAPSO and complete protease inhibitor cocktail, the samples were eluted in 100 mM DTT in SDS sample buffer (Invitrogen).

2.11. Detection of erlin-2 and PS1 interaction using in situ PLA

The *in situ* PLA was carried out as previously described [14]. Briefly, BD3 cells (PS+) and BD8 cells (PS−) were grown in DMEM (high glucose, Invitrogen) supplemented with 10% FCS, 1 mM Sodium pyruvate, 0.1 mM 2-mercaptoethanol and 1× non-essential amino acid solution (Sigma–Aldrich). Around 10^4 cells per well were cultivated overnight on 8-well glass slide (NalgeNunc International), fixed in 3% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked in blocking reagent (Olink Bioscience). The cells were incubated overnight at 4 °C with primary antibodies (anti-erlin-2 antibody and anti-PS1 antibody (R&D system)) in antibody diluent (Olink Bioscience). After 3 × 5 min washes in TBS with 0.05% Tween 20 (0.05 mol/L Tris base, 9 g/L NaCl, pH 8.4, with 0.5 ml/L Tween 20), anti-Rabbit PLUS and anti-Goat Minus (Olink Bioscience) were diluted in antibody diluent and the detection was then accomplished using DII Det. Reag. Orange (Olink Bioscience) according to the manufacturer's instructions. Thereafter, cells were counterstained with Alexa Fluor 488 phalloidin (Invitrogen) and DAPI, respectively. Images of cells were acquired using an AxioplanII epifluorescence microscope (Zeiss), with 20× or 40×/oil Plan-Apocromat objective and analyzed using DuolinkImage Tool.

2.12. Labeling of mouse primary cortical neurons with GCB

Mouse primary cortical neurons were fixed in buffered 4% (v/v) paraformaldehyde, permeabilized with 0.2% Triton X-100 and blocked with Avidin/Biotin blocking Kit (Vector Laboratories, Inc.) and DAKO protein block serum-free. The neurons were pre-incubated with 50 μM L-685,458 for 5 min, followed by incubation with 500 nM GCB for 10 min, RT. Subsequently, the neurons were incubated with Streptavidin-Alexa Fluor 488 (Invitrogen, Molecular Probes Inc.) for 30 min at 37 °C. Incubation with erlin-2 antibody was performed at 4 °C overnight. After washing with PBS, the neurons were incubated with Alexa Fluor 546-conjugated anti-rabbit-IgG (Invitrogen) antibody diluted in 2% normal goat serum for 1 h at RT. All samples were visualized using an inverted laser scanning microscope (LSM 510 META, Zeiss).

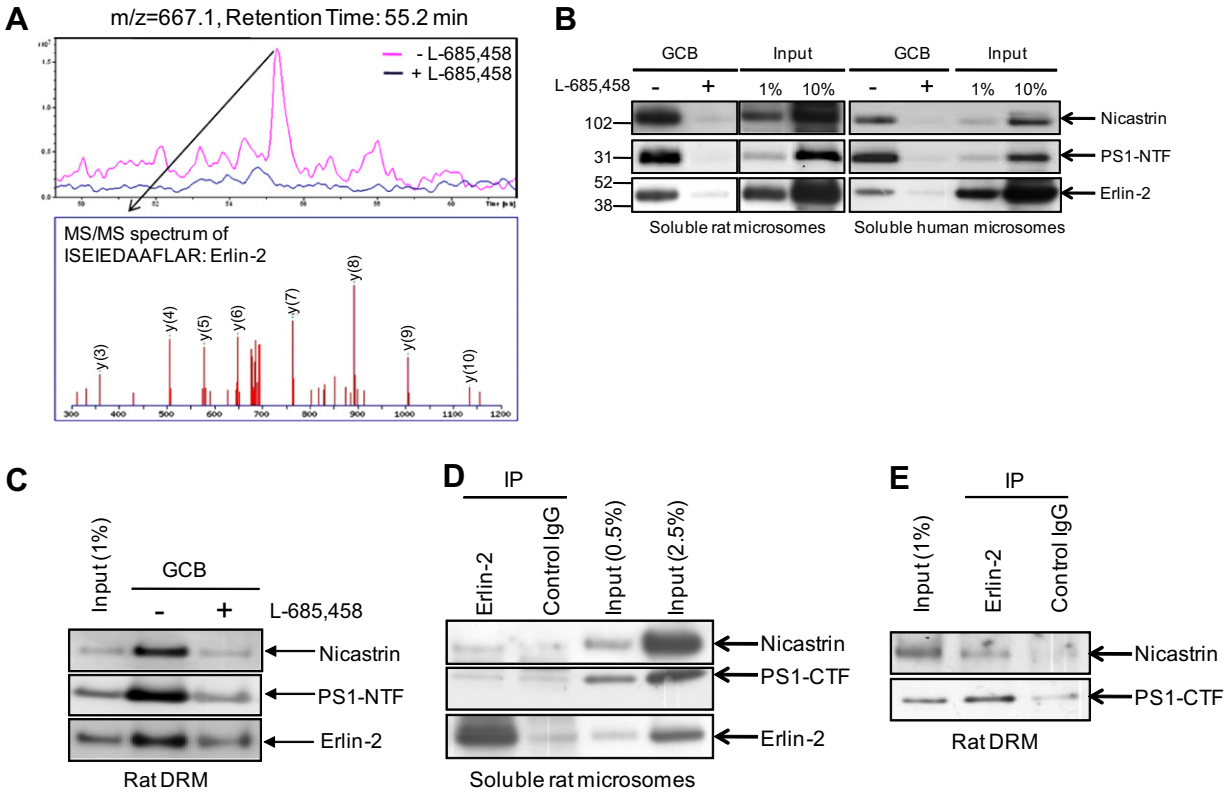

Fig. 1. Erlin-2 interacts with γ-secretase. (A) Identification of erlin-2 by LC-MS/MS analysis. Affinity-purified samples were analyzed by mass spectrometry. Upper panel: extracted ion chromatograms (*m*/*z* 667) in the absence (pink line), or presence (blue line) of the competing inhibitor, L-685,458. Lower panel: MS/MS spectrum of the peak at 55.2 min indicated the peptide sequence ISEIEDAAFLAR, which is found in erlin-2. (B, C) WB analysis of GCB affinity purified γ-secretase complexes from rat or human soluble microsomes (B), or rat DRMs (C). GCB affinity purified samples prepared in the absence or presence of 50 times excess of the competing inhibitor L-685,458 were loaded on a SDS polyacrylamide gel and assessed for co-purification of PS1-NTF, Nicastrin and erlin-2. (D and E) Co-immunoprecipitation of PS1-NTF and nicastrin with an erlin-2 antibody in rat soluble microsomes (D) or rat DRMs (E).

2.13. Cell lines, siRNA transfection and determination of Aβ and NICD levels

siRNA transfection was performed as previously described [12]. In brief, HEK cells expressing human APP (for Aβ determination) or HEK cells expressing human Notch-1 lacking the ectodomain (NotchΔE, for NICD determination) were transfected with 0.3, 3 or 9 nM of siRNA directed to erlin-2 (Qiagen, SI03127509) using Lipofectamine RNAiMAX (Invitrogen). After two days, the medium was replaced with OptiMEM and after an additional 24 h, secreted Aβ40 and Aβ42 levels were determined using a commercial sandwich ELISA (Wako). Three days after transfection, NICD levels in cell lysates were determined by WB, using a neo-epitope specific antibody. The Aβ and NICD levels were adjusted for cell viability using the WST-8 assay (MBL) and compared to the Aβ levels in cells treated with only Lipofectamine. Knock-down efficacy was determined by Western blotting using an anti-erlin-2 antibody. Statistical analyses were performed using One Sample *T*-test to test if the mean values were significantly different from 100% (Lipofectamine control).

3. Results and discussion

3.1. Identification of ER lipid raft-associated protein 2 as a GSAP in membranes from brain

We used GCB for affinity purification of γ-secretase and associated proteins from CHAPSO soluble microsomes prepared from rat as well as from human brain. Samples incubated in the presence of an excess of non-biotinylated inhibitor (L-685,458) served as control for non-specific binding. Western blot (WB) analysis with respect to the four known γ-secretase proteins showed high specificity and good recovery [10]. By using nano-scale liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) we identified several proteins that were absent or substantially

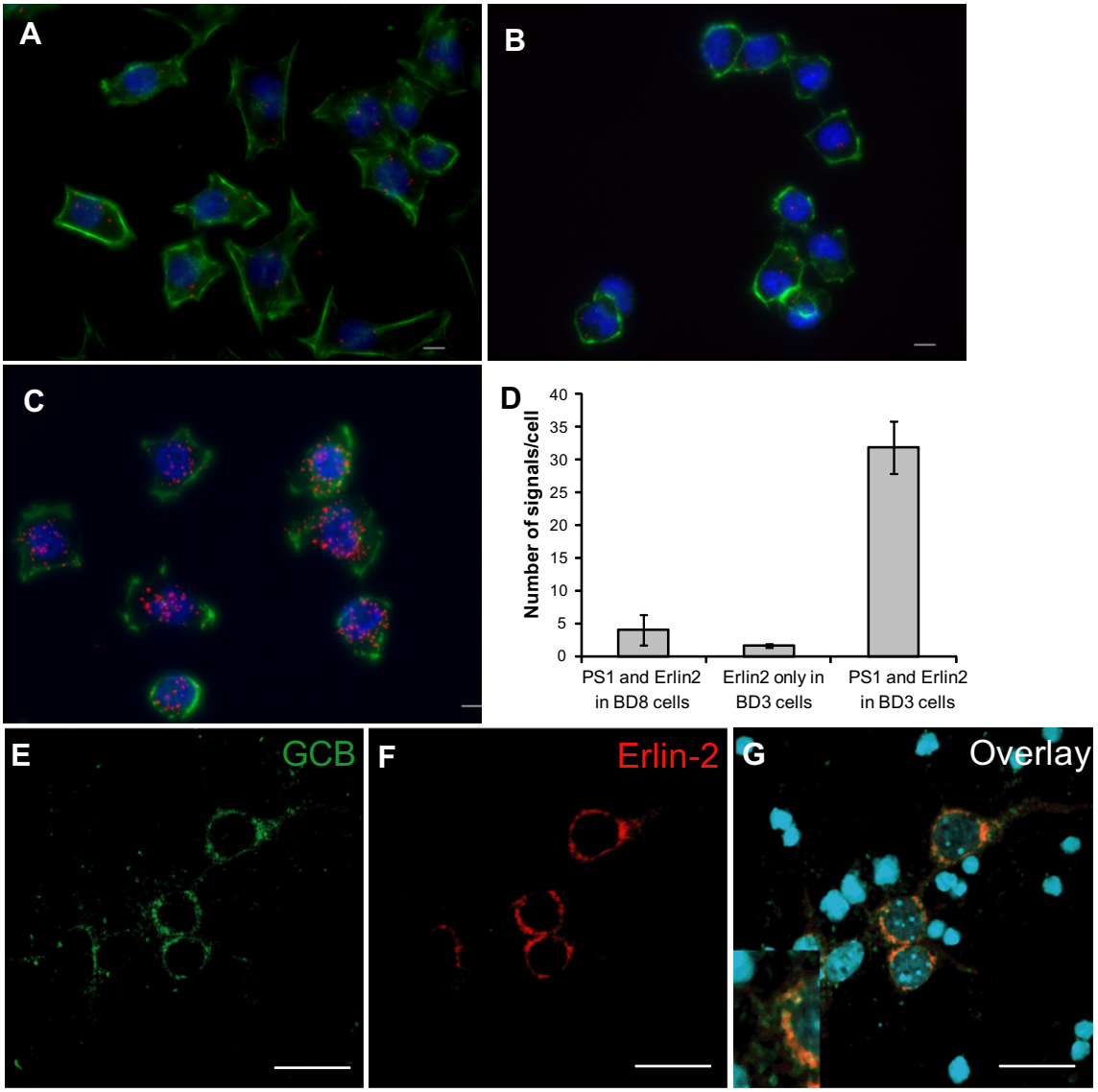

Fig. 2. Confirmation of interaction and co-localization of PS1 and erlin-2 in intact cells. (A–D) Detection of erlin-2 and presenilin1 interaction using *in situ* PLA. Red dots indicate interaction. The nuclei were stained with DAPI (blue), and the cytoskeleton was stained with phalloidin (green). (A) PLA using antibodies to erlin-2 and PS1 in BD8 cells, deficient of PS1 and used as a negative control. (B) PLA using only an antibody directed to erlin-2 in BD3 cells, expressing PS1. (C) PLA using antibodies to erlin-2 and PS1 in BD3 cells. (D) Quantification of the signals in A–C using the Duolink ImageTool. Graphs show mean number of signals/cell ± SD ($n = 3$). (E, F) GCB labeling (green, E) combined with erlin-2 immunocytochemistry (red, F) in mouse primary cortical neurons. The merged picture is shown in G. Scale bar = 10 μm.

decreased in the competed samples [10]. Based on chromatographic and mass spectrometric data (Fig. 1) we selected one of these, ER lipid raft-associated protein 2 (erlin-2), for further studies. Interestingly, erlin-2 was previously identified as a GSAP using tandem affinity purification (TAP) from mouse embryonic fibroblasts [8]. Our study confirms that erlin-2 is a GSAP, and shows that erlin-2 is associated with active γ-secretase in brain.

Erlin-1 and erlin-2 are members of the prohibitin domain containing family of proteins [15], which bind to themselves, cholesterol, and target proteins, and regulate the formation and function of large protein-cholesterol complexes [16]. Erlin-1 and erlin-2 are present in lipid raft-like domains in the endoplasmic reticulum (ER) membrane [17]. They form homo- and hetero-oligomers and are part of large multimeric complexes independent of their association to detergent resistant membranes (DRMs) [18]. An ER membrane complex including erlin recognizes inositol 1,4,5-trisphosphate receptors and participates in the ER-associated degradation [19]. Assembly of the γ-secretase complex occurs within the ER, and only fully assembled and functional γ-secretase complexes are transported to the plasma membrane [20,21]. It is thus possible that erlin binds to γ-secretase in the ER and regulates its maturation, trafficking and/or degradation.

3.2. Validation of the erlin-2/γ-secretase interaction

Affinity purified samples, prepared in the presence or absence of competing inhibitor, were subjected to WB analysis, which confirmed that erlin-2 co-purified with γ-secretase. In CHAPSO-soluble microsomes prepared from rat brain, around 1% of erlin-2 associated with γ-secretase, while the fraction of γ-secretase associated erlin-2 was clearly lower in microsomes from human brain (Fig. 1B). We have earlier shown that γ-secretase activity declines with post-mortem time in rat brain [13], possibly due to dissociation of the γ-secretase. Thus, it is not surprising that the degree of association is lower in post-mortem human brain.

Erlin-2 is a lipid raft associated protein, and γ-secretase is enriched in lipid rafts [13]. Hence, we prepared DRMs from rat brain, and used GCB to purify γ-secretase and associated proteins. Analysis by WB showed that clearly more than 1% of erlin-2 in DRMs was associated to γ-secretase (Fig. 1C). Thus, erlin-2 associates with γ-secretase to a larger extent in DRMs than in CHAPSO-soluble microsomes.

The interaction between erlin-2 and γ-secretase was further studied by immunoprecipitation using an erlin-2 antibody. WB analysis indicated that less than 0.5% of the total amount of nicastrin and presenilin associate with erlin-2 (Fig. 1D). Immunoprecipitation with the erlin-2 antibody from rat brain DRMs showed that around 1% of nicastrin and PS1 co-precipitated with erlin-2 (Fig. 1E) confirming a higher degree of interaction between erlin-2 and γ-secretase in DRMs.

3.3. Validation of the erlin-2/γ-secretase interaction and co-localization in intact cells

Since both GCB affinity purification and immunoprecipitation are based on homogenized samples and homogenization might create false interactions, we further confirmed the interaction between erlin-2 and PS1 using *in situ* PLA. In the *in situ* PLA, the interacting proteins are recognized by a set of PLA probes consisting of antibodies conjugated to DNA oligonucleotides. Once in proximity, the DNA oligonucleotides facilitate the formation of an amplifiable DNA molecule via enzymatic ligation [22]. The signals obtained from presenilin-containing BD3 cells (Fig. 2C) were compared with PS-deficient BD8 cells (Fig. 2A). The average number of signals obtained from BD3 cells was 8 times higher than in the BD8 control cells, and 20 times higher than the negative control without PS1 antibody (Fig. 2B). Thus, endogenous γ-secretase interacting with erlin-2 could be monitored in intact cells.

To determine the subcellular localization of active γ-secretase and erlin-2 and their potential co-localization, we labeled mouse primary cortical neurons with GCB and streptavidin-Alexa Fluor 488 (Fig. 2E) as well as with an anti-erlin-2 antibody and anti-rabbit IgG conjugated to Alexa Fluor 546 (Fig. 2F).The binding of GCB was specific since it was competed by an excess of the unlabeled inhibitor L-685,458 (data not shown). We found partial co-localization of γ-secretase with erlin-2, and both PLA and immunocytochemistry showed a peri-nuclear staining pattern, possibly reflecting ER (Fig. 2G).

3.4. The effect of erlin-2 on Aβ and NICD production

To investigate the effect of erlin-2 on Aβ levels, we treated HEK-APP cells with siRNA directed to erlin-2. The knockdown efficacy was confirmed by WB analysis (Fig. 3A). The treatment was well tolerated according to cell viability data (data not shown). Interestingly, quantification of Aβ by ELISA showed that treatment with erlin-2 siRNA dose dependently reduced the secretion of both Aβ40 and Aβ42 (Fig. 3C).

In clinical trials, inhibition of γ-secretase results in notch-related side effects. Therefore, we studied the effect of erlin-2 with respect to notch processing. HEK cells overexpressing NotchΔE,

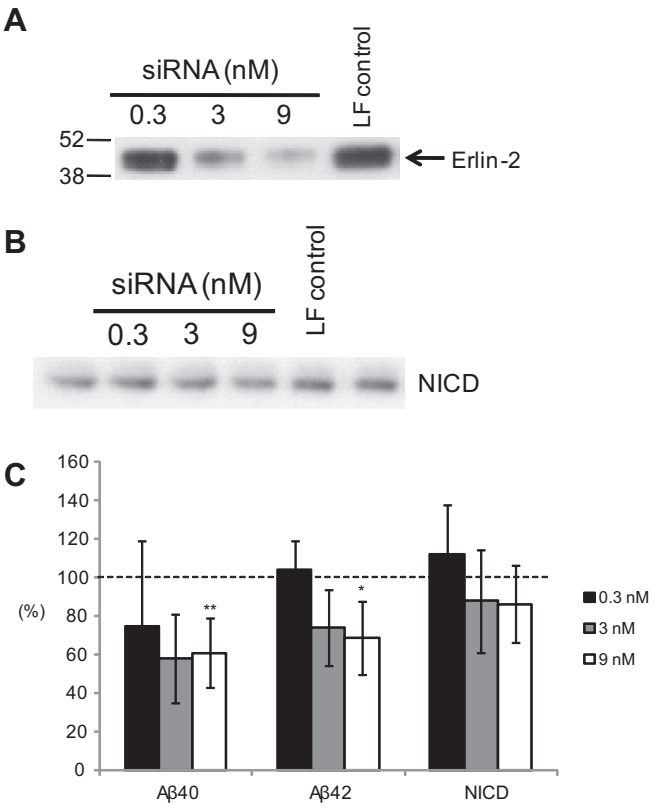

Fig. 3. Erlin-2 siRNA treatment significantly reduces Aβ, but not NICD, levels. (A) siRNA directed to erlin-2 was transfected into HEK-293 cells stably overexpressing APP695. Erlin-2 protein levels were efficiently reduced by siRNA treatment compared to cells treated with only lipofectamine (LF control). (B) siRNA directed to erlin-2 was transfected into HEK-293 cells stably overexpressing NotchΔE, and the NICD levels were analyzed by WB. (C) Secreted Aβ40 and Aβ42 levels from HEK-APP cells were quantified using ELISA and NICD levels in HEK-NotchΔE cells were quantified from the Western blots in (B) using a CCD-camera. All data were adjusted to cell survival data (WST-8) and expressed as percentage of the levels in cells treated with lipofectamine alone or untreated cells. Values are expressed as mean ± SD (n = 3–5), $p < 0.05$ (*), $p < 0.01$ (**).

which is the immediate substrate for γ-secretase, were transfected with siRNA directed to erlin-2. The levels of notch intracellular domain (NICD), which is generated upon cleavage by γ-secretase, were detected by WB using an antibody specific for the NICD neo-epitope. Importantly, the reduction in NICD levels was less pronounced (and not significant) than for Aβ (Fig. 3B, C).

To study the effect of overexpression of erlin-2 on Aβ production we tested transient overexpression of erlin-2 in HEK-APP cells. Overexpression of erlin-2 had no effect on Aβ production (data not shown), possibly due to the endogenous high expression of erlin-2 in many cell lines including HEK cells.

In summary, we show that erlin-2 interacts with γ-secretase in brain, and reduces Aβ production while the effect on notch processing is limited. Hence, interfering with the erlin-2/γ-secretase interaction might reduce Aβ production without affecting Notch processing, and thus be a pharmacological strategy for treatment of AD.

Acknowledgments

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