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Ferritin light chain interacts with PEN-2 and affects γ -secretase activity

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Abstract

Alzheimer's disease (AD) is primarily caused by overproduction/deposition of β -amyloid ($A\beta$) in the brain. Dysregulation of iron in the brain also contributes to AD. Although iron affects β -amyloid precursor protein (APP) expression and $A\beta$ deposition, detailed role of iron in AD requires further elucidation. $A\beta$ is produced by sequential proteolytic cleavages of APP by β -secretase and γ -secretase. The γ -secretase complex comprises presenilins (PS1 or PS2), Nicastrin, APH-1, and PEN-2. Herein, we find that PEN-2 can interact with ferritin light chain (FTL), an important component of the iron storage protein ferritin. In addition, we show that overexpression of FTL increases the protein levels of PEN-2 and PS1 amino-terminal fragment (NTF) and promotes γ -secretase activity for more production of $A\beta$ and Notch intracellular domain (NICD). Furthermore, iron treatments increase the levels of FTL, PEN-2 and PS1 NTF and promote γ -secretase-mediated NICD production. Moreover, downregulation of FTL decreases the levels of PEN-2 and PS1 NTF. Together, our results suggest that iron can increase γ -secretase activity through promoting the level of FTL that interacts with and stabilizes PEN-2, providing a new molecular link between iron, PEN-2/ γ -secretase and $A\beta$ generation in AD.

Keywords

γ -secretase; Alzheimer's disease; ferritin light chain; iron; PEN-2

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1. Introduction

A major pathological hallmark of Alzheimer's disease (AD) is the formation of senile plaques in the brain [25, 29], whose major components are β -amyloid ($A\beta$) peptides that are derived from β -amyloid precursor protein (APP) through sequential cleavages by β - and γ -secretases [37, 38]. Accumulating evidence demonstrates that $A\beta$ is neurotoxic and can trigger a cascade of neurodegenerative steps ending in neuronal loss, suggesting that overproduction/accumulation of $A\beta$ in vulnerable brain regions is a primary cause of AD [19, 37, 38]. Therefore, a dysregulation of proteins involved in $A\beta$ generation may increase $A\beta$ levels and lead to disease pathogenesis.

γ -secretase cleaves many type I transmembrane proteins including APP and Notch [3, 30]. γ -secretase is composed of four core subunits as presenilin 1 or 2 (PS1 or PS2), Nicastrin, APH-1, and PEN-2 [11, 15]. Nascent PS1/2 undergo endoproteolytic cleavage with the resulting amino-terminal fragment (NTF) and carboxyl-terminal fragment (CTF) forming a PS heterodimer that is believed to exert the γ -secretase enzymatic activity [5]. PEN-2 is involved in the endoproteolysis of PSs [17, 18, 21, 31, 36]: the combination of PS1 and PEN-2 was necessary and sufficient to induce PS endoproteolysis and γ -secretase-like activity [1]. By interacting with PSs and PEN-2, Nicastrin and APH-1 help on assembly, stabilization, and trafficking of the entire functional γ -secretase complex [32].

Iron is an essential cofactor for many proteins that are involved in the normal function of neuronal tissue, such as the non-haem iron enzyme tyrosine hydroxylase required for dopamine synthesis [16, 35]. Iron is also important for ryanodine receptor-mediated calcium release after NMDA receptor stimulation, which in turn promotes ERK1/2 activation, an essential step of sustained hippocampal long-term potentiation [24]. However, increasing evidence suggests that iron accumulation in the brain can cause central nervous system disorders including AD [35]. Iron is found to have a direct impact on $A\beta$ plaque formation through its effects on APP metabolism [4, 27]. Iron also promotes both $A\beta$ deposition and oxidative stress-induced toxicity [22], as well as the aggregation of hyperphosphorylated tau which is the major constituent of neurofibrillary tangles [33]. Cellular iron is stored in a dynamic fashion by ferritin that protects cells from iron-dependent radical damage and allows the release of iron according to cellular demand [13]. Ferritin consists of ferritin heavy chain (FTH) and ferritin light chain (FTL). FTH has a di-iron binding site and confers ferroxidase activity. FTL has no enzymatic activity but accelerates the transfer of iron from the ferroxidase center to the iron core and stabilizes the ferritin complex structure [2, 6]. There is an age-related increase in ferritin expression which correlates with the increase in iron [8, 9, 34]. Ferritin levels are further increased in AD and a robust ferritin immunoreaction accompanies senile plaques and many blood vessels in the AD brain tissue [9, 26]. However, the exact involvement of ferritin dysregulation in AD and the underlying mechanism have yet to be elucidated.

2. Materials and Methods

2.1. Yeast two-hybrid screening and β -galactosidase activity assay

Yeast two-hybrid screening was performed with the Matchmaker GAL4 Two-Hybrid System 3 kit (Clontech Company) using a bait plasmid pGBKT7-PEN-2 containing the intracellular loop domain (amino acids 34–65) sequence of PEN-2. Plasmid DNAs isolated from positive clones were subjected to sequencing and blasting of the GenBank database for identification.

For β -galactosidase activity assay, the pCAT2-A7 plasmid containing the FTL sequence, as well as its control plasmid pGADT7, were co-expressed with pGBKT7 (control) or

pGBKT7-PEN-2 in yeasts. Yeast lysates were incubated with the ONPG substrate at 30°C until color appeared. Na₂CO₃ was applied to stop the reaction and readings at A420nm were taken to measure β -galactosidase activity.

2.2. Cell culture, plasmids and transfection

HEK293 cells and HEK293 cells stably expressing human APP Swedish mutations (HEK293Swe) were cultured in DMEM (Hyclone) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco), in the absence and presence of 400 μ g/mL G418 (Sigma), respectively.

Full-length FTL protein coding sequence was PCR-amplified from a human fetal brain cDNA library and subcloned into the pcDNA3.1/Myc-His vector (Invitrogen) at *Bam*HI and *Xho*I sites. The HA-PEN-2 [36] and the Notch N Δ E [28] plasmids have been reported.

Transient transfection was performed by using TurboFect *in vitro* Transfection Reagent (Fermentas) according to the manufacturer's protocol.

2.3. Antibodies

Antibodies used in the study include anti-myc antibody (9E10), anti-HA antibody, anti-PEN-2 antibody and anti-FTL antibody from Santa Cruz Biotechnology; anti- α -tubulin antibody from Sigma; and anti-A β antibody (6E10) from Covance. Anti-PS1 NTF antibody (Ab14) was developed in our laboratory.

2.4. Co-immunoprecipitation

Equal protein amounts of cell lysates were incubated with normal IgG or indicated antibodies, together with rProtein A sepharose (GE) at 4°C overnight. Immunoprecipitated proteins were analyzed by Western blot with indicated antibodies.

2.5. RNA interference

The FTL shRNA sequences used in this study were as follows:

FTL shRNA 1 (si-f1) = 5'-ACGCCATGAAAGCTGCCAT-3';

FTL shRNA 2 (si-f2) = 5'-GATGAGTGGGGTAAAACCC-3'.

The shRNA sequences were inserted into a pLVTHM vector. A pLVTHM vector containing a scrambled shRNA sequence was used as control. Vectors were transfected into cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol.

2.6. RNA extraction, reverse transcription and quantitative-PCR

Total RNA was extracted using the TRIzol Reagent (Invitrogen). Reverse transcription was performed using ReverTra Ace qPCR RT Kit (TOYOBO). Equal amounts of cDNA from each group of samples were used for quantitative-PCR with primers for FTL (Forward: 5'-GCGATGATGTGGCTCTG-3', Reverse: 5'-AGGGCATGAAGATCCAA-3'), for PEN-2 (Forward: 5'-GCAAACGTCCATAACTGAAAGTAGCT-3', Reverse: 5'-CCGGCACAGGTTCAATTTCTCCTCA-3') and for glyceraldehyde 3-phosphate dehydrogenase (Forward: 5'-GAGTCCACTGGCGTCTTCAC-3', Reverse: 5'-TGAGTCCTTCCACGATACCAA-3').

2.7. Statistical analysis

Two-tailed Student's t test was used for statistical analyses.

3. Results

3.1. FTL is identified as a PEN-2-interacting protein

PEN-2 has two transmembrane domains with both amino- and carboxyl- termini facing the lumen of the endoplasmic reticulum [10]. To identify intracellular proteins that interact with PEN-2, we carried out yeast two-hybrid assays to screen a human fetal cDNA library, using the PEN-2 intracellular loop domain (amino acids 34–65) as the bait. We identified a positive clone containing a partial cDNA sequence of FTL. When FTL and PEN-2 were co-expressed in yeast, the β -galactosidase activity was significantly increased, indicating an interaction between the two (Fig. 1A). In addition, we co-transfected myc-tagged full-length FTL and HA-tagged full length PEN-2 into HEK293 cells and carried out co-immunoprecipitation study. An anti-FTL antibody pulled down overexpressed PEN-2, whereas an anti-PEN-2 antibody pulled down overexpressed FTL (Fig. 1B), confirming the interaction between FTL and PEN-2 in mammalian cells.

3.2. Overexpression of FTL promotes PEN-2 protein level and γ -secretase activity

To determine the effect of FTL on PEN-2, we overexpressed FTL in HEK293 cells stably expressing human APP Swedish mutations (HEK293Swe) and found that the protein level (Fig. 2A) but not the mRNA level (Fig. 2B) of PEN-2 was significantly increased. Since PEN-2 mediates endoproteolytic cleavage of PS1 into an amino-terminal fragment (NTF) and a carboxyl-terminal fragment (CTF) that keep associated [1, 31], we determined whether FTL can affect PS1 endoproteolytic cleavage and found that the level of PS1 NTF was increased upon FTL overexpression (Fig. 2A). Overexpression of FTL in HEK293 cells also increased the protein levels of PEN-2 and PS1 NTF (Supplementary Fig. 1 online), excluding the possibility that oxidative stress induced by A β in HEK293Swe cells could participate in FTL-mediated PEN-2 and PS1 NTF level change.

Notch and APP are two major substrates of γ -secretase. In cells overexpressing Notch N Δ E that lacks the ectodomain of Notch1 and can be processed in a ligand-independent manner by γ -secretase to produce NICD [28], we found that overexpression of FTL promoted the γ -cleavage of Notch N Δ E for NICD production (Fig. 2C). Furthermore, in HEK293Swe cells, overexpression of FTL dramatically increased the levels of secreted A β (Fig. 2D). Together, these results indicate that overexpression of FTL can elevate PEN-2 protein level and PS1 endoproteolytic cleavage for increasing γ -secretase activity and NICD and A β generation.

3.3. Iron stimulates FTL expression and increases PEN-2 level and γ -secretase activity

Because the expression of FTL is known to be regulated by iron [16] and accumulation of iron in the brain has been associated with AD pathology, we investigated whether iron overload has impact on PEN-2 expression and γ -secretase activity. We found that treatment with 10^{-4} mol/L FeCl₂ induced dramatic increase of FTL level without obvious cell death in HEK293Swe cells and used this concentration in our study. We found that along with the increase of FTL level, the protein levels of PEN-2 and PS1 NTF were markedly increased (Fig. 3A). In addition, the production of NICD was increased upon iron treatments (Fig. 3B), indicating an elevation of the γ -secretase activity.

3.4. Downregulation of FTL reduces the levels of PEN-2 and PS1 NTF

Next, we downregulated FTL in HEK293Swe cells by shRNA transfection and confirmed the reduction of FTL mRNA levels (Fig. 4A). Because basal endogenous FTL protein level is low and hard to be detected, we treated cells with iron during shRNA transfection and showed that FTL protein levels were indeed downregulated (Fig. 4B). Upon downregulation of FTL, both the levels of PEN-2 and PS1 NTF were decreased (Fig. 4B).

4. Discussion

Dysregulation of iron homeostasis and iron-associated proteins has been found in multiple diseases, including neurodegenerative disorders [13, 16, 26, 35]. Abnormally high concentrations of iron in affected brain areas have been observed in neuroferritinopathy, Parkinson's disease, Huntington's disease, and AD [16]. Accumulation of iron in AD brain, particularly in neurons that are associated with neuritic plaques [14] and neurofibrillary tangles [20, 23], is found primarily complexed with ferritin, the essential iron storage protein; and ferritin levels are increased in AD brains [8, 9, 13, 34]. An iron response element found in the APP mRNA sequence has been proposed to mediate iron-dependent regulation of APP synthesis [27]. However, high levels of iron inhibits APP maturation and causes an elevated secretion of APP ectodomain fragment without blocking formation of immature APP, suggesting that iron acts on the level of α -secretase activity [4, 27]. Iron has also been found to promote both A β deposition and oxidative stress-induced toxicity [22], and can induce aggregation of hyperphosphorylated tau, the major constituent of neurofibrillary tangles [33]. On the other hand, APP has been proposed as a ferroxidase that oxidizes Fe²⁺ to Fe³⁺ and facilitates the efflux of iron out of the cell; and suppression of APP in neurons induces marked iron retention that can be inhibited by zinc [12]. When cellular iron levels are high, translation of APP as well as ferritin and ferroportin is increased to ensure the safe storage and efflux of iron, while the iron importer transferring receptor mRNA is degraded to stop iron import [26]. Thus, disruption in iron homeostasis in the brain upon alterations of iron regulatory proteins may increase the vulnerability of cells to oxidative stress that is involved in the onset, progression and pathology of AD [7, 39].

In this study, we demonstrate that FTL, a subunit of ferritin, can interact with PEN-2. Overexpression of FTL increases the protein but not mRNA levels of PEN-2, suggesting that FTL affects PEN-2 protein levels through their interaction, possibly by disturbing PEN-2 degradation. PEN-2, which is an indispensable component of the γ -secretase complex, not only contributes to PS1 endoproteolytic cleavage, but also stabilizes the γ -secretase complex. Herein we find that overexpression of FTL also increases the level of PS1 NTF and γ -secretase activity for more production of A β and NICD; while downregulation of FTL decreases the levels of PEN-2 and PS1 NTF. In addition, iron stimulation elevates the levels of FTL, PEN-2 and PS1 NTF and promotes γ -secretase-mediated NICD generation. Together, our results demonstrate that iron can increase γ -secretase activity through promoting the level of FTL that interacts with and stabilizes PEN-2, providing a molecular link between iron, PEN-2/ γ -secretase and A β generation in AD, and will be important for elucidating the participation of iron in AD pathogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

A β	β -amyloid
AD	Alzheimer's disease

APP	β -amyloid precursor protein
CTF	carboxyl-terminal fragment
FTH	ferritin heavy chain
FTL	ferritin light chain
HEK293Swe	HEK293 cells stably expressing human APP Swedish mutations
NICD	Notch intracellular domain
NTF	amino-terminal fragment
PS1 or PS2	presenilin 1 or 2

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Highlights

- ▶ PEN-2 interacts with FTL.
- ▶ Overexpression of FTL promotes PEN-2 level and γ -secretase activity.
- ▶ Iron stimulates FTL expression and increases PEN-2 level and γ -secretase activity.

Fig. 1.

FTL interacts with PEN-2. (A) pGBKT7-PEN-2 and pACT2-FTL plasmids were co-expressed in yeast. Cell lysates were subjected to β -galactosidase activity assay. N=3, *** p <0.001. (B) Myc-tagged FTL and HA-tagged PEN-2 were co-transfected into HEK293 cells. Equal amounts of cell lysates were used for immunoprecipitation (IP) with anti-FTL antibody, anti-PEN-2 antibody or control IgG, followed by Western blot (WB) with anti-myc antibody or anti-HA antibody to detect FTL or PEN-2, respectively. Twenty percent of cell lysates were used as input. * Nonspecific bands.

Fig. 2.

Overexpression of FTL increases PEN-2 protein level and γ -secretase activity. (A) HEK293Swe cells were transfected with FTL (+) or control (-) plasmids. PEN-2 and PS1 NTF protein levels were analyzed by Western blot. α -tubulin was used as loading control. PEN-2 and PS1 NTF levels were quantified by densitometry. $N=3$, * $p<0.05$, ** $p<0.01$. (B) HEK293Swe cells transfected with FTL or control (cont) plasmids were collected 24 h after transfection. Total RNA was extracted, reverse transcribed, and subjected to quantitative-PCR. PEN-2 mRNA levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase and compared to control. $N=3$, N.S. $p>0.05$. (C) HEK293Swe cells were first transfected with Notch NΔE. After splitting equally, cells were transfected with FTL (+) or

control (–) plasmids. Notch NΔE and NICD were analyzed by Western blot. NICD levels were quantified by densitometry. N=3, ** $p<0.01$. (D) HEK293Swe cells were transfected with different amounts (0, 1, 3μg) of FTL cDNA. Aβ in conditioned media were precipitated and analyzed by Western blot. Aβ levels were quantified by densitometry. N=3, * $p<0.05$.

Fig. 3.

Iron stimulates FTL expression and increases PEN-2 levels and γ -secretase activity. (A) HEK293Swe cells treated with 10^{-4} mol/L FeCl_2 were collected 24 h after treatments. Equal amounts of cell lysates were analyzed for FTL, PEN-2, and PS1 NTF by Western blot. PEN-2 and PS1 NTF levels were quantified by densitometry. $N=3$, $*p<0.05$. (B) HEK293Swe cells were first transfected with Notch NΔE. After splitting equally, cells were treated with 10^{-4} mol/L FeCl_2 for 24 h. Notch NΔE and NICD were analyzed by Western blot. The levels of NICD were quantified by densitometry. $N=3$, $*p<0.05$.

Fig. 4.

Downregulation of FTL reduces PEN-2 and PS1 NTF levels. (A) HEK293Swe cells transfected with two FTL shRNAs (si-f1 and si-f2) and a scrambled control shRNA (sc) were collected 72 h after treatments. Total RNA was extracted, reverse transcribed, and subjected to quantitative-PCR. FTL mRNA levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase and compared to control. N=3, ** p <0.01. (B) HEK293Swe cells were transfected with two FTL shRNAs (si-f1 and si-f2) and a sc shRNA for 48 h. Cells were then treated with 10⁻⁴ mol/L FeCl₂ for 24 h. Equal amounts of cell lysates were analyzed for FTL, PEN-2, and PS1 NTF by Western blot. FTL, PEN-2, and PS1 NTF levels were quantified by densitometry. N=3, * p <0.05, ** p <0.01.