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The intracellular domain of amyloid precursor protein interacts with flotillin-1, a lipid raft protein

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Abstract

Amyloid β (Aβ) is a pathological hallmark of Alzheimer's disease (AD). It is derived from the amyloid precursor protein (APP) by two sequential proteolytic cleavages, which also generate the APP intracellular domain (AICD). The precise cellular function(s) of AICD still remain obscure. To elucidate the roles of AICD in the development of AD, a yeast two-hybrid system was used to screen a human brain cDNA library for proteins interacting directly with AICD. One of the potential AICD-interacting proteins identified from our screening result is a lipid raft-associated protein, flotillin-1. The interaction was confirmed by glutathione *S*-transferase pull-down and coimmunoprecipitation studies. Since lipid raft has been suggested to play an important role in signal transduction as well as the pathogenic development of neurodegenerative diseases, it is proposed that flotillin-1 may recruit APP to lipid rafts and therefore participate in the localization and processing of APP.

Keywords: Lipid rafts; Alzheimer's disease; AICD (APP intracellular domain); Flotillin

Alzheimer's disease (AD), especially the late-onset AD, is a progressive neurodegenerative disorder often associated with elderliness. It is characterized by insoluble extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) [1]. The major constituent of amyloid plaque, the Aβ peptide, is derived from the amyloid precursor protein (APP) by two sequential proteolytic cleavages. In addition to Aβ, the aforementioned proteolytic processing also generates the APP intracellular domain (AICD), the extreme C-terminus of APP composed of 57–59 amino acid residues. Recent evidences suggest that AICD forms complexes with certain PTB domain-containing proteins such as Fe65 and X11, which, in turn, interact with other proteins (e.g., Tip60 and transcription factor CP2/LSF/LBP1 in the case of Fe65), and may play a signaling role analogues to that of Notch or its intracellular domain (NICD) [2–8]. The precise cellular function of AICD, however, still remains obscure.

To elucidate the roles of AICD in the pathogenic pathway of Alzheimer's disease, a yeast two-hybrid system was used to identify its interacting partners. Our preliminary screening result revealed that a lipid raft-associated protein, flotillin-1, was a potential AICD-interacting protein. Lipid rafts (or the so-called caveolae-like microdomains) are liquid-ordered membranous microdomains enriched in cholesterol, glycolipid, and sphingolipid. It is generally believed that lipid rafts are involved in many cellular processes such as membrane sorting, trafficking, and signal transduction, and have been implicated in the pathogenesis of a number of human diseases, including Alzheimer's disease (reviewed in [9–14]). It has been proposed that the amyloidogenic processing of APP occurs predominantly in lipid rafts [15,16]. This hypothesis is supported by the observations that both γ-secretase complex and BACE are concentrated in lipid rafts, in which Aβ and a small fraction of cellular APP are also present [15,17–26].

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Flotillin-1 has been used as a lipid raft marker protein by many researchers since its first isolation from the Triton X-100-insoluble membrane fraction [27]. In addition, it has been demonstrated that flotillin-1 is able to form hetero-oligomeric complex with caveolins, the major protein components of caveolae, and the expression of flotillin-1 could drive the formation of lipid rafts in cultured cells [28]. Since the accumulation of flotillins has been correlated with the progression of Alzheimer pathology [29,16], whether flotillins are participating in the partitioning of the macromolecules involved in the processing of APP into lipid rafts is becoming an increasingly important issue. Recent studies have shown that caveolin-1 and -3 are physically associated with APP, suggesting that caveolins may play an important role in the proteolysis of APP [30,31]. Although a large body of experimental work had indicated the presence of APP in lipid raft (as previously mentioned), there was no direct evidence that flotillin-1, just like caveolin-1 or -3, interacts with APP. In this study, the interaction of AICD with flotillin-1 was confirmed by glutathione *S*-transferase (GST) pull-down and coimmunoprecipitation experiments. We also narrow the interacting region of flotillin-1 to amino acid residues 189–282. Our results support the hypothesis that flotillin-1 may recruit APP to lipid rafts and therefore be involved in the localization and processing of APP.

Materials and methods

Yeast two-hybrid screening. The yeast two-hybrid screening was conducted using Matchmaker GAL4 Two-Hybrid System 3 (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions. DNA fragment containing the coding sequence of the intracellular domain of amyloid precursor protein (APP) (i.e., the C-terminal 59 amino acids of APP) was PCR amplified from a human brain cDNA library (Clontech) using primers designed based on a GenBank sequence (GenBank Accession No. NM_201414) (forward primer: 5′-GGAATTCATAGCGACAGTGATCGTC-3′, reverse primer: 5′-CGGGATCCTAGTTCTGCATCTGC-3′) and inserted into the pGBKT7 vector (Clontech) so that an in-frame fusion protein GAL4-DNA-binding domain (DBD)-AICD can be expressed and used as a bait in the two-hybrid screening. The sequence of the candidate plasmid was verified by DNA sequencing (Core Facility, College of Medicine, National Taiwan University). This plasmid (pGBKT7-AICD) was transformed into yeast strain AH109 and the transformants were selected using dropout medium lacking tryptophan (SD/-Trp). AH109 harboring pGBKT7-AICD was then transformed with a human brain cDNA library (in yeast expression vector pACT2) (Clontech) and the cotransformants were selected on dropout medium lacking tryptophan and leucine (SD/-Trp/-Leu). The selected cotransformants were replica-plated onto dropout medium lacking tryptophan, leucine, adenine (SD/-Trp/-Leu/-Ade) as well as medium lacking tryptophan, leucine, adenine, and histidine, but supplemented with 5 mM 3-AT (SD/-Trp/-Leu/-Ade/-His/3-AT) and allowed to grow until colonies appeared. AH109 cotransformed with pGBKT7/pCL1 and pGBKT7-53/pGADT7-T (all control plasmids were from Clontech) was used as positive controls, whereas the ones cotransformed with pGADT7/pGBKT7 and pGBKT7-Lam/pGADT7-T were used as negative controls. Plasmids isolated from the positive colonies were transformed into competent *Escherichia coli* strain DH5α and plated onto ampicillin-containing LB agar plates. The identities of the positive library plasmids were verified by DNA sequencing (Core Facility, College of Medicine, National Taiwan University), and the sequencing results were compared with the GenBank non-redundant database using the BLAST programs from NCBI.

Yeast protein extraction and Western blotting. AH109 harboring both pGBKT7-AICD and the AD/library plasmid of interest was grown in 50 mL of SD/-Trp/-Leu medium overnight at 30 °C. The cells were harvested by centrifugation (3000*g* for 5 min at 4 °C). Protein extraction was conducted using the Y-PER yeast protein extraction reagent (Pierce, Rockford, IL, USA) according to manufacturer's suggestion. For Western blotting analysis, proteins on the polyacrylamide gel were transferred to a PVDF membrane (Pall, East Hills, NY, USA) and probed with either the anti-APP C-terminal polyclonal antibody (AB5352, Chemicon, Temecula, CA, USA) or the anti-HA monoclonal antibody (sc-7392, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Anti-HA antibody was used to detect GAL4-AD fusion proteins since the pACT2 vector was specially designed to express HA epitope-tagged fusion protein. For GAL4-AD-FLOT1$_{137–427}$ encoded by pACT2-FLOT1$_{137–427}$, anti-flotillin-1 monoclonal antibody (clone18, BD Biosciences Pharmingen, San Diego, CA, USA) was used. The membrane was then incubated with anti-rabbit or anti-mouse IgG HRP-conjugated antibody (Amersham Biosciences). Proteins of interest (i.e., GST-AICD and flotillin-1) were visualized with ECL™ Western blotting detection reagent (Amersham Bioscience) by enhanced chemiluminescence method.

Yeast two-hybrid assay. All three further-truncated flotillin-1 constructs were derived from pACT2-FLOT1$_{137–427}$, which was identified by yeast two-hybrid screening as described in the previous section. pACT2-FLOT1$_{189–427}$, pACT2-FLOT1$_{282–427}$, and pACT2-FLOT1$_{388–427}$ were generated by *Bam*HI, *Xma*I, and *Nco*I digestion/re-ligation of pACT2-FLOT1$_{137–427}$, respectively. The sequences of the candidate plasmids were verified by DNA sequencing (Core Facility, College of Medicine, National Taiwan University). The obtained further-truncated flotillin-1 construct was then cotransformed with pGBKT7-AICD into yeast strain AH109. The cell growth of the cotransformants was checked on dropout medium (SD/-Trp/-Leu/-Ade and SD/-Trp/-Leu/-Ade/-His/3-AT) as described previously. Again, AH109 cotransformed with pGBKT7/pCL1 and pGBKT7-53/pGADT7-T was used as positive controls, whereas the ones cotransformed with pGADT7/pGBKT7 and pGBKT7-Lam/pGADT7-T were used as negative controls.

GST-AICD expression and purification. Human AICD cDNA was subcloned from pGBKT7-AICD (see previous section) into the pGEX4T-1 vector (Amersham Biosciences, Piscataway, NJ, USA) so that an in-frame fusion protein GST-AICD can be expressed. The sequence of the candidate plasmid was verified by DNA sequencing (Core Facility, College of Medicine, National Taiwan University). This plasmid (pGEX4T-1-AICD) was transformed into *Escherichia coli* strain BL21 and the overexpression of GST-AICD was induced by adding 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) into a liquid culture at an OD_{600} of 0.4. The culture was let grow for an additional 2 h. An empty plasmid pGEX4T-1 was used to overexpress GST under similar culturing conditions. The *E. coli* cells were harvested by centrifugation (6000*g* for 20 min at 4 °C) and lysed by sonication in the presence of 0.3 mg/mL of lysozyme and 1× protease inhibitor mixture (lysis buffer: 10 mM Tris–HCl, pH 7.4, 100 mM NaCl). Both GST-AICD and GST were partially purified by affinity chromatography (GSTPrep FF 16/10, Amersham Biosciences, elution buffer: 50 mM Tris–HCl, pH 8.0, and 10 mM reduced glutathione).

GST pull-down assays and Western blotting. Partially purified GST-AICD and GST were immobilized on glutathione Sepharose 4B beads (Amersham Biosciences) following manufacturer's suggestion. SH-SY5Y cells were grown in a 1:1 mixture of Ham's F12 nutrients and minimal essential medium (MEM, GibcoBRL/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C, 95% air and 5% CO_2, and replaced with fresh media every two to three days until cells became confluent. Cells were washed with phosphate-buffered saline (PBS, 2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4), trypsinized with 0.05% trypsin/0.53 mM EDTA (GibcoBRL), and lysed by brief sonication in 100 μL lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM DTT, 1 mM PMSF, and 1× protease inhibitor mixture). The cell lysate was obtained by

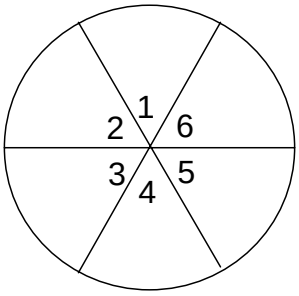
centrifugation at 15,000*g* for 30 min at 4 °C and incubated with above GST-AICD- or GST-immobilized glutathione Sepharose 4B beads for 2 h at 4 °C with gentle agitation. The beads were washed with PBS. Proteins bound to the beads were eluted by boiling in 1× Laemmli sample buffer for 5 min and were separated by 12% SDS–PAGE. For Western blotting analysis, proteins on the polyacrylamide gel were transferred to a PVDF membrane and probed with either the anti-APP C-terminal polyclonal antibody (Calbiochem, San Diego, CA, USA) or the anti-flotillin-1 monoclonal antibody (clone18, BD Biosciences Pharmingen). The membrane was then incubated with anti-rabbit or anti-mouse IgG HRP-conjugated antibody (Amersham Biosciences). Proteins of interest (i.e., GST-AICD and flotillin-1) were visualized with ECL™ Western blotting detection reagent (Amersham Bioscience) by enhanced chemiluminescence method.

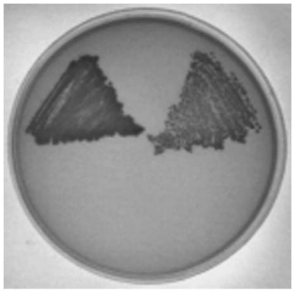
Immunoprecipitation and Western blotting. Approximately 0.5 μL of rabbit anti-APP C-terminal antibody (Calbiochem) or mouse anti-flotillin-1 monoclonal antibody (BD Biosciences Pharmingen) was incubated with 50 μL of 50% protein A Sepharose CL-4B beads slurry (Amersham Biosciences) for 1 h at room temperature. The cross-linking reaction was initiated by the addition of 6.5 μL of 5 mM disuccinimidyl suberate (DSS) and the reaction mixture was incubated for 1 h at room temperature with gentle agitation. Fifty microliters of TBS (25 mM Tris–HCl, pH 7.2, 150 mM NaCl) was added to stop the reaction and wash off excess DSS. Unbound antibodies were removed with 50 μL of 0.1 M glycine (pH 2.8). An appropriate amount of SH-SY5Y crude cell lysate prepared as described in the previous section was incubated with the above antibody-conjugated protein A Sepharose CL-4B beads for 1 h at 4 °C with gentle agitation. The beads were washed with PBS. Proteins bound to the beads were eluted by boiling in 1× Tris–Tricine sample buffer (0.05 M Tris–HCl, pH 6.8, 12% glycerol, 4% SDS, 0.1 M DTT, and 0.01% Coomassie R250) for 5 min and were separated by 16% Tris–Tricine SDS–PAGE. For Western blotting analysis, proteins transferred to a PVDF membrane were probed with either the anti-APP C-terminal polyclonal antibody (Calbiochem) or the anti-flotillin-1 monoclonal antibody (clone18, BD Biosciences Pharmingen) and were visualized with ECL™ Western blotting detection reagent (Amersham Bioscience) by enhanced chemiluminescence method.

Results and discussion

Identification of potential AICD-interacting proteins

To further elucidate the roles the intracellular domain of amyloid precursor protein (AICD) might play in the pathogenic pathway of Alzheimer's disease, a yeast two-hybrid system was used to screen for proteins that may directly interact with AICD. Approximately 3.5×10^6 independent clones from a human brain cDNA library (in pACT2) were screened with a bait plasmid, pGBKT7-AICD, which could express an in-frame fusion protein GAL4-DNA-binding domain (DBD)-AICD. About 400 potential positive colonies out of approximately 1.5×10^8 cotransformants were first identified on dropout medium lacking tryptophan, leucine, and adenine (SD/-Trp/-Leu/-Ade). These colonies were further checked by growth selection on medium lacking tryptophan, leucine, adenine, and histidine, but supplemented with 5 mM of 3-AT (SD/-Trp/-Leu/-Ade/-His/3-AT), and 53 His^+ colonies were obtained. AD/library plasmids isolated from these His^+ yeast colonies were characterized by restriction enzyme digestion and sequence analysis, and were reintroduced into pGBKT7-AICD- or pGBKT7-containing AH109 cells to confirm the interactions between bait and prey proteins (Fig. 1). Sequences of the insert of the plasmids from the true positive clones





	GAL4-DBD fusions	GAL4-AD fusions
1	pGBKT7	pGADT7
2	pGBKT7-53	pGADT7-T
3	pGBKT7-Lam	pGADT7-T
4	pGBKT7-AICD	pGADT7
5	pGBKT7	$pGADT7\text{-}FLOT_{137\text{-}427}$
6	pGBKT7-AICD	$pGADT7\text{-}FLOT_{137\text{-}427}$

Fig. 1. Yeast two-hybrid analysis of the interaction of AICD with flotillin-1. Yeast AH109 cells were cotransformed with indicated plasmids (left). The protein–protein interactions were checked by the growing conditions of the cotansformants on selective medium (SD/-Trp/-Leu/-Ade/-His/3-AT) (right). pGBKT7-53 and pGADT7-T encode fusions between the GAL4-DBD and murine p53, and GAL4-AD and SV40 large T-antigen, respectively. pGBKT7-Lam encodes a fusion of GAL4-DBD and human lamin C. The construction of the plasmids used in the assays was described in detail under Materials and methods. AH109 cotransformed with pGBKT7-53/pGADT7-T was used as a positive control since p53 is known to interact with SV40 large T-antigen, whereas the ones cotransformed with pGADT7/pGBKT7 (both are empty vectors) and pGBKT7-Lam/pGADT7-T were used as negative controls.

were compared with the GenBank non-redundant database using the BLASTN program from National Center for Biotechnology Information (NCBI). The partial sequences of 24 such plasmids were found to be identical to portions of the open reading frame of nine different known proteins (data not shown). Among those, three plasmids contained fragments encoding the C-terminal 291 amino acids of human flotillin-1 (FLOT1, GenBank Accession No. NP_005794), which was in the same reading frame as that of GAL4-AD, therefore should be able to express an in-frame GAL4-AD-FLOT1$_{137–427}$ fusion protein. A Western blotting analysis was performed to verify that both GAL4-DBD-AICD and GAL4-AD-FLOT1$_{137–427}$ were expressed in the pGBKT7-AICD/pACT2-FLOT1$_{137–427}$ cotransformed AH109 (Fig. 2). Fig. 2 shows that the bait and prey fusion proteins were expressed at the expected sizes (~27.6 and ~50.7 kD, respectively) in the cotransformants but not in the control (i.e., untransformed AH109). Yeast two-hybrid results from Fig. 1 suggest that flotillin-1 is a potential AICD-interacting protein. Since neither AH109 harboring both pGBKT7-AICD and pGADT7 nor that cotransformed with pGBKT7 and pACT2-FLOT1$_{137–427}$ was able to grow on selective medium, the interaction occurred only in the presence of both AICD and flotillin-1$_{137–427}$ in the form of fusion proteins.

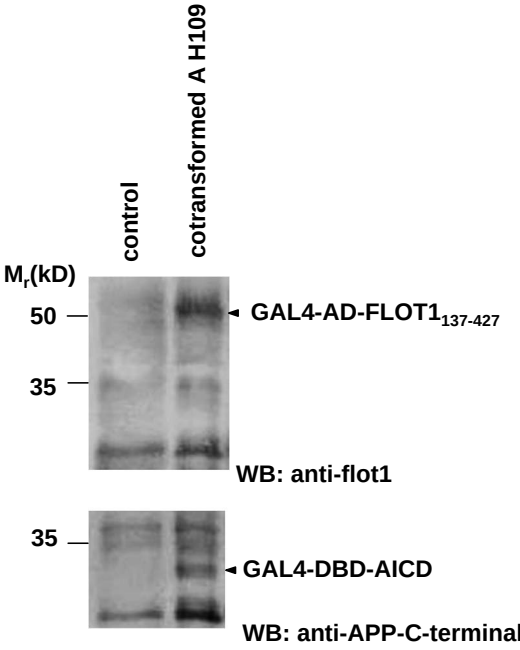


Fig. 2. Expression of the GAL4-DBD-AICD and GAL4-AD-FLOT1$_{137–427}$ fusion proteins in yeast strain AH109 cotransformed with both pGBKT7-AICD and pACT2-FLOT1$_{137–427}$. Protein extracts from the cotransformants were prepared as previously described and separated by 12% SDS–PAGE. The predicted sizes of GAL4-DBD-AICD and GAL4-AD-FLOT1$_{137–427}$ are ~27.6 and ~50.7 kD, respectively. For Western blotting analysis, proteins on the polyacrylamide gel were transferred to a PVDF membrane and probed with either the anti-APP C-terminal polyclonal antibody (Calbiochem) (lower panel) or the anti-flotillin-1 monoclonal antibody (clone18, BD Biosciences Pharmingen) (upper panel). Cell extracts from untransformed AH109 were used as a control.

AICD interacts with flotillin-1

To confirm the interaction between AICD and flotillin-1 as revealed by the yeast two-hybrid result, a glutathione *S*-transferase (GST) pull-down assay was conducted (Fig. 3). Partially purified GST-AICD or GST expressed in *E. coli* was immobilized to glutathione Sepharose 4B beads and then incubated with crude lysate from SH-SY5Y cells. Proteins bound to the glutathione Sepharose beads were separated by SDS–PAGE and analyzed by Western blotting using either the anti-APP C-terminal rabbit polyclonal antibody (Calbiochem) or the anti-flotillin-1 mouse monoclonal antibody (BD Biosciences Pharmingen). As shown in Fig. 3, both GST-AICD and flotillin-1 were detected at the predicted sizes of ~33 and ~48 kD, respectively (shown by arrowheads). Our results indicate that GST-AICD, but not GST alone, is able to bind to the full-length flotillin-1 (Fig. 3A).

The interaction was further verified by coimmunoprecipitation assays of lysate from SH-SY5Y cells expressing these proteins at endogenous levels. Cell lysate was immunoprecipitated with either anti-APP C-terminal antibody or anti-flotillin-1 antibody and was analyzed by Western blotting. Results shown in Fig. 4 reveal that flotillin-1 could be coprecipitated with APP and/or APP C-terminal fragment(s) and vice versa. AICD, however, was not detected in this study, probably due to its relatively low level in vivo [2]. Although it is not yet clear whether other parts of APP interact with flotillin-1 or not, the experi-

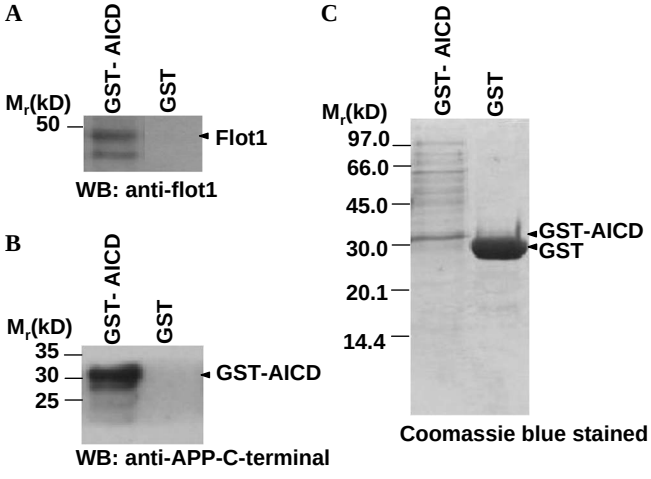


Fig. 3. GST pull-down assays. GST-AICD or GST immobilized on glutathione Sepharose 4B beads (Amersham Biosciences) was incubated with crude lysate from SH-SY5Y cells. Proteins bound to the glutathione Sepharose beads or the proteins interacting with the beads were separated by a 12% polyacrylamide gel and analyzed by Western blotting using either the anti-flotillin-1 mouse monoclonal antibody (Clone18, BD Biosciences Pharmingen) (A) or the anti-APP C-terminal rabbit polyclonal antibody (Calbiochem) (B). GST-AICD and flotillin-1 were detected at the predicted sizes of ~33 and ~48 kD, respectively (shown by arrowheads). (C) The Coomassie Blue-stained SDS–polyacrylamide gel depicting the amounts of the protein samples (GST-AICD or GST) used in the experiments.

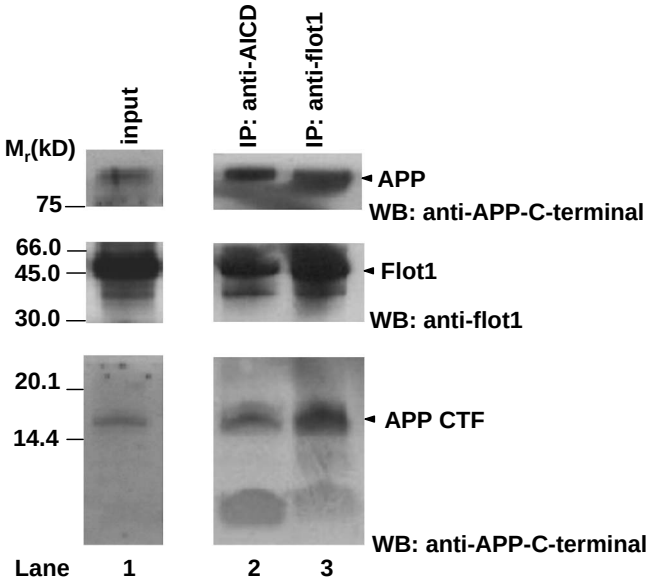


Fig. 4. Coimmunoprecipitation of APP C-terminal fragment(s) (APP CTF) and flotillin-1. Cell lysate from SH-SY5Y cells was immunoprecipitated with either anti-APP C-terminal antibody (lane 2, IP: anti-AICD) or anti-flotillin-1 antibody (lane 3, IP: anti-flot1). Proteins bound to the antibody-conjugated protein A Sepharose CL-4B beads (Amersham Biosciences) were separated by 16% Tris–Tricine SDS–PAGE and analyzed by Western blotting using either the anti-flotillin-1 mouse monoclonal antibody (Clone18, BD Biosciences Pharmingen) (middle panel) or the anti-APP C-terminal rabbit polyclonal antibody (Calbiochem) (upper and lower panels). Aliquots of cell lysate were also loaded directly onto the gels and analyzed by Western blotting (lane 1, input).

ments described above suggest that AICD could at least form a physical complex with flotillin-1 in intact cells and in vitro, and it is very likely that this interaction is direct.

Truncated flotillin-1 fusion protein lacking residues 1–282 does not interact with GAL4-DBD-AICD

As mentioned previously, it has been suggested that AICD may play a signaling role. The PTB domain located in the C-terminal half of Fe65 is responsible for its interaction with AICD, presumably via interaction with the PTB domain of AICD [2–4]. Other proteins not containing PTB domain(s) have also been identified to be interacting with AICD [32–34]. Flotillin-1 is not similar to any of these previously identified AICD-interacting proteins in terms of primary structure. A conserved domain search (CD-search) against the conserved domain database of NCBI using human flotillin-1 as the query sequence [35,36] did not reveal any structural resemblance to these proteins either. Two putative conserved domains were identified from CD-search (Fig. 5A). It is shown in Fig. 5 that the N-terminal fragment of FLOT1$_{137–427}$ corresponds to the C-terminus of the SPFH domain of Band_7 family (Pfam01145, which ends at approximately the 185th amino acid residue of the full-length flotillin-1) and contains portions of the second hydrophobic domain of flotillin-1, whereas the rest of the protein (FLOT1$_{137–427}$) corresponds to the flotillin

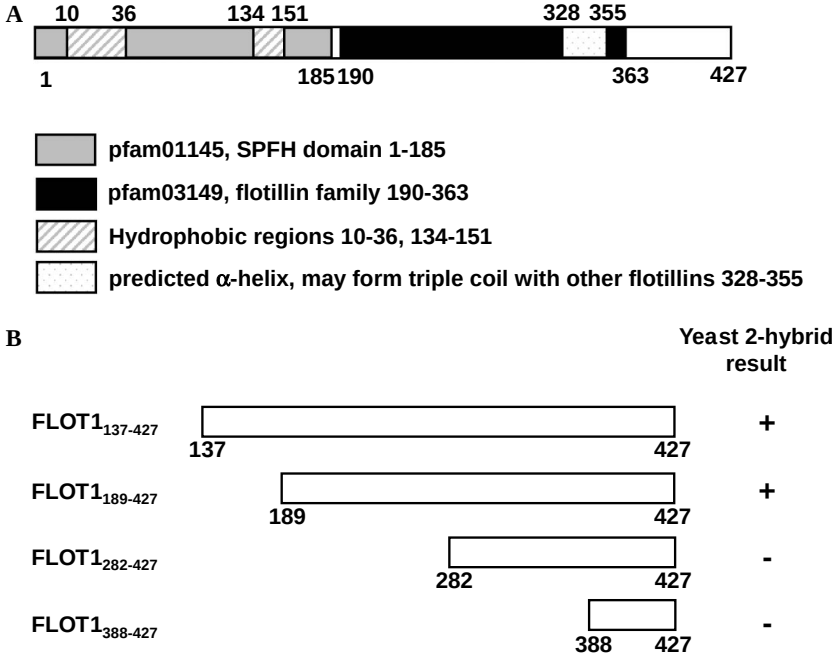


Fig. 5. Schematic representations of the proposed structural elements of human flotillin-1 (FLOT1, GenBank Accession No. NP_005794) and the various truncated forms of flotillin-1 used in the yeast two-hybrid assays. (A) Two putative conserved domains, pfam01145 and pfam03149, were identified from CD-search as described in the text. In addition, two hydrophobic domains (positions 10–36 and positions 134–151) and an α helix that may be involved in oligomerization or interaction with other proteins were also predicted from the primary structure of flotillin-1 [27]. (B) Yeast two-hybrid assays for the interaction between the further-truncated flotillin-1 and AICD. Plasmid expressing GAL4-AD-FLOT1$_{137–427}$ fusion protein (i.e., pACT2-FLOT1$_{137–427}$) was obtained from the yeast two-hybrid screening. All three further-truncated flotillin-1 constructs were derived from pACT2-FLOT1$_{137–427}$ and were cotransformed with pGBKT7-AICD into yeast strain AH109. The cell growth of the cotransformants was checked on dropout medium (SD/-Trp/-Leu/-Ade/-His/3-AT) as described previously. A plus sign (+) indicates growth and a minus sign (−) indicates no growth on selective medium.

family (Pfam03149) (Fig. 5A). To identify regions of flotillin-1 important for its interaction with AICD, three further-truncated flotillin-1 constructs (pACT2-FLOT1$_{189–427}$, pACT2-FLOT1$_{282–427}$, and pACT2-FLOT1$_{388–427}$, Fig. 5B) were generated from pACT2-FLOT1$_{137–427}$ and cotransformed into AH109 cells with pGBKT7-AICD for yeast two-hybrid assays. Growth selection results, as shown in Fig. 5B, indicated that neither GAL4-AD-FLOT1$_{282–427}$ nor GAL4-AD-FLOT1$_{388–427}$ was capable of interacting with GAL4-DBD-AICD, whereas GAL4-AD-FLOT1$_{189–427}$ remained to be able to interact with GAL4-DBD-AICD, suggesting that the fragment containing residues 189–282 of flotillin-1 was required for the interaction. Previous studies have suggested that the FLOT1$_{131–192}$ fragment is important for the oligomerization of other SPFH family proteins, e.g., stomatin [37], and is probably important for the interaction between flotillin-1 and neuroglobin [38]. On the other hand, oligomerization of flotillin-2, another flotillin family member whose sequence shares 40% identity and 62% similarity with flotillin-1, has been shown to be mediated by its C-terminal region from amino acids 208 to 355, corresponding to the amino acid residues 253–400 of flotillin-1 [39]. The size of this region may be narrowed down even further since it has been predicted from the primary structure of flotillin-1 that an α helix positioned within this region (residues 328–355, Fig. 5A) may be involved in oligomerization or interaction with other proteins [27]. It seems that FLOT1$_{189–282}$ does not overlap with either proposed oligomerization domain. More experiments are being conducted to see whether the interaction between flotillin-1 and AICD (or APP and other proteolytic products of APP) interferes with the oligomerization of flotillins, which has been suggested to be involved in its raft association, probably in a manner similar to that of caveolins [39–42].

The accumulation of flotillin-1 in human brain has been suggested to be associated with the progression of AD pathology [9–14]. Although there is growing evidence indicating that partitioning of APP in lipid rafts may regulate amyloidogenic processing and physiological function(s) of APP, the issue of whether or not flotillin-1 interacts with APP and therefore modulates the aforementioned partitioning process as caveolin-1 or -3 does had not yet been addressed. This is, to our knowledge, the first report that demonstrates the direct interaction between AICD and flotillin-1. Functional studies are in progress to further clarify how the interaction between AICD (or APP and other proteolytic products of APP) affects the localization and processing of APP.

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References

[1] D.J. Selkoe, Biochemistry of altered brain proteins in Alzheimer's disease, Annu. Rev. Neurosci. 12 (1989) 463–490.
[2] P. Cupers, I. Orlans, K. Craessaerts, W. Annaert, B. De Strooper, The amyloid precursor protein (APP)-cytoplasmic fragment generated by γ-secretase is rapidly degraded but distributes partially in a nuclear fraction of neurones in culture, J. Neurochem. 78 (2001) 1168–1178.
[3] W.T. Kimberly, J.B. Zhang, S.Y. Guenette, D.J. Selkoe, The intracellular domain of the β-amyloid precursor protein is stabilized by Fe65 and translocates to the nucleus in a notch-like manner, J. Biol. Chem. 276 (2001) 40288–40292.
[4] X. Cao, T.C. Sudhof, A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60, Science 293 (2001) 115–120.
[5] J.P. Borg, J. Ooi, E. Levy, B. Margolis, The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein, Mol. Cell. Biol. 16 (1996) 6229–6241.
[6] F. Fiore, N. Zambrano, G. Minopoli, V. Donini, A. Duilio, T. Russo, The regions of the Fe65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein, J. Biol. Chem. 270 (1995) 30853–30866.
[7] G. Minopoli, P. De Candia, A. Bonetti, R. Faraonio, N. Zambrano, T. Russo, The β-amyloid precursor protein functions as a cytosolic anchoring site that prevents Fe65 nuclear translocation, J. Biol. Chem. 276 (2001) 6545–6550.
[8] M.A. Leissring, M.P. Murphy, T.R. Mead, Y. Akbari, M.C. Sugarman, M. Jannatipour, B. Anliker, U. Muller, P. Saftig, B. De Strooper, M.S. Wolfe, T.E. Golde, F.M. LaFerla, A physiologic signaling role for the γ-secretase-derived intracellular fragment of APP, Proc. Natl. Acad. Sci. USA 99 (2002) 4697–4702.
[9] R.G.W. Anderson, The caveolae membrane system, Annu. Rev. Biochem. 67 (1998) 199–225.
[10] R.G.W. Anderson, K. Jacobson, A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains, Science 296 (2002) 1821–1825.
[11] D.A. Brown, E. London, Functions of lipid rafts in biological membranes, Annu. Rev. Cell Dev. Biol. 14 (1998) 111–136.
[12] M. Edidin, The state of lipid rafts: from model membranes to cells, Annu. Rev. Biophys. Biomol. Struct. 32 (2003) 257–283.
[13] K. Simons, D. Toomre, Lipid rafts and signal transduction, Nat. Rev. Mol. Cell Biol. 1 (2000) 31–39.
[14] K. Simons, R. Ehehalt, Cholesterol, lipid rafts, and disease, J. Clin. Invest. 110 (2002) 597–603.
[15] R. Ehehalt, P. Keller, C. Haass, C. Thiele, K. Simons, Amyloidogenic processing of the Alzheimer β-amyloid precursor protein depends on lipid rafts, J. Cell Biol. 160 (2003) 113–123.
[16] N. Girardot, B. Allinquant, D. Langui, A. Laquerriere, B. Dubois, J.J. Hauw, C. Duyckaerts, Accumulation of flotillin-1 in tangle-bearing neurons of Alzheimer's disease, Neuropathol. Appl. Neurobiol. 29 (2003) 451–461.
[17] B. Allinquant, K.L. Moya, C. Bouillot, A. Prochiantz, Amyloid precursor protein in cortical neurons: coexistence of two pools differentially distributed in axons and dendrites and association with cytoskeleton, J. Neurosci. 14 (1994) 6842–6854.
[18] C. Bouillot, A. Prochiantz, G. Rougon, B. Allinquant, Axonal amyloid precursor protein expressed by neurons in vitro is present in a membrane fraction with caveolae-like properties, J. Biol. Chem. 271 (1996) 7640–7644.
[19] S.J. Lee, U. Liyanage, P.E. Bickel, W. Xia, P.T. Lansbury Jr., K.S. Kosik, A detergent-insoluble membrane compartment contains Aβ in vivo, Nat. Med. 4 (1998) 730–734.

[20] M. Morisima-Kawashima, Y. Ihara, The presence of amyloid β-protein in the detergent-insoluble membrane compartment of human neuroblastoma cells, Biochemistry 37 (1998) 15247–15253.
[21] A. Rouvinski, I. Gahali-Sass, I. Stav, E. Metzer, H. Atlan, A. Taraboulos, Both raft- and non-raft proteins associate with CHAPS-insoluble complexes: some APP in large complexes, Biochem. Biophys. Res. Commun. 308 (2003) 750–758.
[22] M. Burns, K. Duff, Cholesterol in Alzheimer's disease and tauopathy, Ann. N.Y. Acad. Sci. 977 (2002) 367–375.
[23] D.R. Riddell, G. Christie, I. Hussain, C. Dingwall, Compartmentalization of β-secretase (Asp2) into low-buoyant density, noncaveolar lipid rafts, Curr. Biol. 11 (2001) 1288–1293.
[24] E.T. Parkin, I. Hussain, E.H. Karran, A.J. Turner, N.M. Hooper, Characterization of detergent-insoluble complexes containing the familial Alzheimer's disease-associated presenilins, J. Neurochem. 72 (1999) 1534–1543.
[25] K.S. Vetrivel, H. Cheng, W. Lin, T. Sakurai, T. Li, N. Nukina, P.C. Wong, H. Xu, G. Thinakaran, Association of γ-secretase with lipid rafts in post-Golgi and endosome membranes, J. Biol. Chem. 279 (2004) 44945–44954.
[26] J. Abad-Rodriguez, M.D. Ledesma, K. Craessaerts, S. Perga, M. Medina, A. Delacourte, C. Dingwall, B. De Strooper, C.G. Dotti, Neuronal membrane cholesterol loss enhances amyloid peptide generation, J. Cell Biol. 167 (2004) 953–960.
[27] P.E. Bickel, P.E. Scherer, J.E. Schnitzer, P. Oh, M.P. Lisanti, H.F. Lodish, Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins, J. Biol. Chem. 272 (1997) 13793–13802.
[28] D. Volonte, F. Galbiati, S. Li, K. Nishiyama, T. Okamoto, M.P. Lisanti, Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe, J. Biol. Chem. 274 (1999) 12702–12709.
[29] H. Kokubo, C.A. Lemere, H. Yamaguchi, Localization of flotillins in human brain and their accumulation with the progression of Alzheimer's disease pathology, Neurosci. Lett. 290 (2000) 93–96.
[30] T. Ikezu, B.D. Trapp, K.S. Song, A. Schlegel, M.P. Lisanti, T. Okamoto, Caveolae, plasma membrane microdomains for alpha-secretase-mediated processing of the amyloid precursor protein, J. Biol. Chem. 273 (1998) 10485–10495.
[31] K. Nishiyama, B.D. Trapp, T. Ikezu, R.M. Ransohoff, T. Tomita, T. Iwatsubo, I. Kanazawa, K.K. Hsiao, M.P. Lisanti, T. Okamoto, Caveolin-3 upregulation activates β-secretase-mediated cleavage of the amyloid precursor protein in Alzheimer's disease, J. Neurosci. 19 (1999) 6538–6548.
[32] I. Nishimoto, T. Okamoto, Y. Matsuura, S. Takahashi, T. Okamoto, Y. Murayama, E. Ogata, Alzheimer amyloid protein precursor complexes with brain GTP-binding protein G_o, Nature 362 (1993) 75–79.
[33] P. Zheng, J. Eastman, S. Vande Pol, S.W. Pimplikar, PAT1, a microtubule-interacting protein, recognizes the basolateral sorting signal of amyloid precursor protein, Proc. Natl. Acad. Sci. USA 95 (1998) 14745–14750.
[34] N. Chow, J.R. Korenberg, X.-N. Chen, R.L. Neve, APP-BP1, a novel protein that binds to the carboxyl-terminal region of the amyloid precursor protein, J. Biol. Chem. 271 (1996) 11339–11346.
[35] A. Marchler-Bauer, S.H. Bryant, CD-search: protein domain annotations on the fly, Nucleic Acids Res. 32 (2004) W327–W331.
[36] A. Marchler-Bauer, J.B. Anderson, P.F. Cherukuri, C. DeWeese-Scott, L.Y. Geer, M. Gwadz, S. He, D.I. Hurwitz, J.D. Jackson, Z. Ke, C.J. Lanczycki, C.A. Liebert, C. Liu, F. Lu, G.H. Marchler, M. Mullokandov, B.A. Shoemaker, V. Simonyan, J.S. Song, P.A. Thiessen, R.A. Yamashita, J.J. Yin, D. Zhang, S.H. Bryant, CDD: a conserved domain database for protein classification, Nucleic Acids Res. 33 (2005) D192–D196.
[37] L. Snyers, E. Umlauf, R. Prohaska, Oligomeric nature of the integral membrane protein stomatin, J. Biol. Chem. 273 (1998) 17221–17226.
[38] K. Wakasugi, T. Nakano, C. Kitatsuji, I. Morishima, Human neuroglobin interacts with flotillin-1, a lipid raft microdomain-associated protein, Biochem. Biophys. Res. Commun. 318 (2004) 453–460.
[39] C. Neumann-Giesen, B. Falkenbach, P. Beicht, S. Claasen, G. Lüers, C.A.O. Stuermer, V. Herzog, R. Tikkanen, Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression, Biochem. J. 378 (2004) 509–518.
[40] M. Sargiacomo, P.E. Scherer, Z. Tang, E. Kubler, K.S. Song, M.C. Sanders, M.P. Lisanti, Oligomeric structure of caveolin: implications for caveolae membrane organization, Proc. Natl. Acad. Sci. USA 92 (1995) 9407–9411.
[41] A. Schlegel, M.P. Lisanti, A molecular dissection of caveolin-1 membrane attachment and oligomerization. Two separate regions of the caveolin-1 C-terminal domain mediate membrane binding and oligomer/oligomer interactions in vivo, J. Biol. Chem. 275 (2000) 21605–21617.
[42] K. Simons, W.L.C. Vaz, Model systems, lipid rafts, and cell membranes, Annu. Rev. Biophys. Biomol. Struct. 33 (2004) 269–295.