γ-Secretase Cleavage and Binding to FE65 Regulate the Nuclear Translocation of the Intracellular C-Terminal Domain (ICD) of the APP Family of Proteins†

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ABSTRACT: Regulated intramembrane proteolysis (RIP) of the amyloid precursor protein (APP) produces amyloid β-protein (Aβ), the probable causative agent of Alzheimer's disease (AD), and is therefore an important target for therapeutic intervention. However, there is a burgeoning consensus that γ-secretase, one of the proteases that generates Aβ, is also critical for the signal transduction of APP and a growing list of other receptors. APP is a member of a gene family that includes two amyloid precursor-like proteins, APLP1 and APLP2. Although APP and the APLPs undergo similar proteolytic processing, there is little information about the role of their γ-secretase-generated intracellular domains (ICDs). Here, we show that APLP1 and 2 undergo presenilin-dependent RIP similar to APP, resulting in the release of a ∼6 kDa ICD for each protein. Each of the ICDs are degraded by an insulin degrading enzyme-like activity, but they can be stabilized by members of the FE65 family and translocate to the nucleus. Given that modulation of APP processing is a therapeutic target and that the APLPs are processed in a manner similar to APP, any strategy aimed at altering APP proteolysis will have to take into account possible effects on signaling by APLP 1 and 2.

Alzheimer's disease (AD)[1] is a progressive neurodegenerative disorder characterized by the presence of intracellular neurofibrillary tangles and extracellular deposits of amyloid in the parenchyma and cerebral vasculature (*1*). Although the precise events that lead to the generation of these pathological hallmarks is not yet understood, strong biochemical and genetic data indicate that the amyloid β-protein (Aβ) plays a critical role in pathogenesis (for review, see ref *2*). Aβ is produced from the amyloid precursor protein (APP) by the action of two proteolytic activities referred to as β- and γ-secretases (*3*−*5*). APP is a member of an evolutionarily conserved protein family (*6*), which includes the mammalian homologues, amyloid precursor like protein 1 (APLP1) (*7*) and amyloid precursor like protein 2 (APLP2) (*8*, *9*). All three mammalian proteins display substantial amino acid and domain homologies, all are predicted to be type I transmembrane proteins, and although APLP1 and 2 lack an Aβ domain, their intracellular C-terminal domains (ICDs) are highly similar to that of APP (*6*).

A NPTY motif in the C-termini of APP, APLP1, and APLP2 interacts with several phosphotyrosine/protein binding (PTB) proteins, including the FE65 protein family (*10*−*13*). FE65 is a nuclear protein which, when overexpressed, prevents activation of the key mitotic S phase gene, thymidylate synthase (TS) (*14*). In this regard, recent experiments have demonstrated that APP functions as an extranuclear anchor to which FE65 is bound (*15*) and that coexpression of APP overcomes the deactivation of the TS gene observed with transfection of FE65 alone (*14*). In addition to the APP−FE65 interaction and its regulation of TS expression, other findings also suggest that APP may play a role in signal transduction. For example, the similarities between the PS-dependent regulated intramembrane proteolysis (RIP) of Notch and APP (*16*, *17*) and the finding that the γ-secretase-generated intracellular domain (ICD) of APP can translocate to the nucleus (*18*) and drive transcription of heterologous reporter genes (*19*) support a role for APP in transcriptional regulation.

Studies of APP and APLP knockout mice suggest a high degree of functional redundancy within the APP family of proteins (*20*). Single disruptions of APP, APLP1, or APLP2 each cause minor abnormalities that are distinct for the different family members (*20*−*22*), but mice with either knock out of APP plus APLP2 or APLP1 plus APLP2 die soon after birth (*20*). Thus, while gene disruption studies

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[1] Abbreviations: Aβ, amyloid β-protein; APP, amyloid precursor protein; APLP1, amyloid precursor-like protein 1; APLP2, amyloid precursor-like protein 2; CHO, Chinese hamster ovarian cells; CTF, C-terminal fragment; ICD, intracellular C-terminal domain; IDE, insulin degrading enzyme; PS, presenilin; RIP, regulated intramembrane proteolysis; TS, thymidylate synthase; 1,10 PTH, 1,10-phenanthroline.

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indicate redundancy between APLP2 and both APP and APLP1, they also demonstrate a key physiological role for APLP2 while pointing to subtly different roles for APP and APLP1. Signal transduction through release of ICDs could provide the mechanism for mediating both the overlapping and distinct functions of the APP family. Here we show that APLP1 and APLP2, like APP, undergo presenilin-dependent RIP resulting in the release of ~6 kDa ICDs. We further demonstrate that the ICDs of APP, APLP1, and APLP2 are extremely labile and are all degraded by insulin degrading enzyme (or a closely similar protease), but they are stabilized in the presence of FE65 and translocate to the nucleus. Thus, while inhibition of the γ-secretase processing of APP is a rational strategy for treating AD, our results caution that inhibition of γ-secretase may have serious repercussions on signal transduction pathways dependent on PS-mediated RIP of APLPs.

EXPERIMENTAL PROCEDURES

Antibodies. The following polyclonal antibodies were described previously (*23*−*25*): C8 antibody to the C-terminus of APP; α-CT1 (which we refer to as 1CT) to the APLP1 C-terminus (Calbiochem, San Diego, CA); α-CT11 (which we refer to as 2CT) to the APLP2 C-terminus (Calbiochem, San Diego, CA); and α-D2−11 (which we refer to as 2NT) raised to full-length APLP2 (Calbiochem, San Diego, CA). The polyclonal 1NT was raised to an unconjugated peptide corresponding to residues 553−592 of APLP1 (Eggert et al., manuscript in preparation). Monoclonal antibody 8E5 directed to $APP_{500-648}$ was a gift of P. Seubert and D. Schenk (Elan PLC). Monoclonal antibody 9E10 to the c-myc epitope was from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids and Transfections. Full-length wild-type human APP_{695}, $APLP1_{650}$, and $APLP2_{751}$ were cloned into vector pcDNA 3.1(+) (Invitrogen Corporation, Carlsbad, CA) containing a hygromycin-resistance gene and used to generate Chinese hamster ovary (CHO) cells stably overexpressing either APP, APLP1, APLP2 or vector alone. The identities of each construct were confirmed by DNA sequencing.

To examine the role of APP and the APLPs in signal transduction, we generated two C-terminal constructs each for APP, APLP1, and APLP2 (see Figure 4a). The first (C60) encodes the C-terminal 59 amino acids of each protein plus an exogenous initiating methionine and corresponds to the expected product following cleavage after residue 40 of the Aβ domain. The second encodes the C-terminal 49 amino acids of APP and APLP2 beginning one amino acid from the epsilon cleavage site of APP and the APLPs (*26*−*28*). Unlike APP and APLP2, APLP1 does not contain an endogenous methionine 49 amino acids from its C-terminus; rather, there is an endogenous methionine 51 residues from the C-terminus. Thus, for APLP1, we generated a construct referred to as C51 that begins 51 amino acids form its C-terminus. To control for the possibility of any increased stability of C51 compared to the C49s of APP and APLP2, we also generated a construct encoding the C-terminal 48 amino acids of APLP1 plus an exogenous initiating methionine (APLP1 C49). All constructs were amplified by PCR, inserted into pcDNA3.1 (+) and confirmed by sequencing.

Plasmids encoding wild-type human FE65, human FE65 L1, and human FE65 L2 were obtained from Drs. D. McLoughlin, S. Guenette, and H. Tanahashi, respectively, and each cloned into pcDNA 3.1 (+) with myc tags added. For transient transfections, 12 μg of each DNA was introduced into COS cells (90% confluent) in 10-cm dishes using Lipofectamine 2000 (Gibco, Invitrogen Corporation, Grand Island, NY), according to the manufacturer's instructions.

Cultured Cells. CHO cells stably overexpressing APP (1B1), APLP1 (3C4) APLP2 (4E10) or vector alone (A5) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO) and 0.4 mg/mL hygromycin (Invitrogen Corporation, Carlsbad, CA).

Whole Cell Lysates of Cells Stably Transfected with APP or APLPs. Cells were grown in 10 cm^2 dishes until nearly confluent, washed with serum-free medium (4 mL × 2), transferred into serum-free medium (4 mL) and incubated for ~16 h. Conditioned media were removed, and the cells were washed twice with ice-cold PBS (10 mL/10 cm^2 dish) and lysed with 500 μL of 1% NP-40 in 50 mM Tris HCl, pH 7.6, containing 150 mM NaCl, 2 mM EDTA (STEN), plus 50 μg/mL leupeptin, 950 μg/mL aprotinin, 20 μg/mL pepstatin A, 120 μg/mL Pefabloc and 2 mM 1,10-phenanthroline (1,10 PTH). Aliquots of lysates (30 μg of total protein per well) were electrophoresed on 10−20% tricine gels. Samples of conditioned media were diluted with 4× Laemmli sample buffer + BME and electrophoresed on 12% tris-glycine gels.

For detection of the γ-secretase generated ICDs, cells were transfected with FE65 and lysed with STEN buffer (100 μL) containing 1% SDS and 2 mM 1,10 PTH. Lysates were each sonicated for 30 s using a Fisher sonic demembranator (model 300) with a semi-micro probe at setting 55. Particulate material was removed by centrifugation at 16000*g* for 10 min and the supernatant diluted 1:10 with STEN buffer containing 2 mM 1,10 PTH. Samples were immunoprecipitated with antibodies specific for the C-terminus of APP, APLP1, or APLP2 (above). The immunoprecitates were electrophoresed on 10−20% tris-tricine gels and Western blotted with the same C-terminal antibodies used for precipitation.

Whole Cell Lysates of Cells Transiently Transfected with APP or APLP C49/51 and C60 Constructs. Approximately 36 h after transient transfection of COS cells, conditioned media were carefully removed and the cells lysed in boiling Laemmli buffer (2×) containing 2 mM 1,10 PTH (150 μL). The lysates were then boiled at 100 °C for 10 min and sonicated for 30 s, and 15 μL of lysate run on 10−20% tricine gels.

Western Blot Analysis. Proteins from cellular lysates electrophoresed on 10−20% tricine gels were transferred onto 0.2 μM nitrocellulose membranes at 400 mA for 2 h; transfer of total protein was assessed by staining with Ponceau S. Filters were then boiled for 10 min in PBS (*29*) and blocked overnight at 4 °C with 5% fat-free milk in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and 0.05% Tween 20 (TBS-T). After the membranes were washed in TBS-T, blots were probed with C8, 1CT, or 2CT as appropriate at 1:1000 dilution in TBST. Bound antibody was visualized using horseradish peroxidase-conjugated anti-

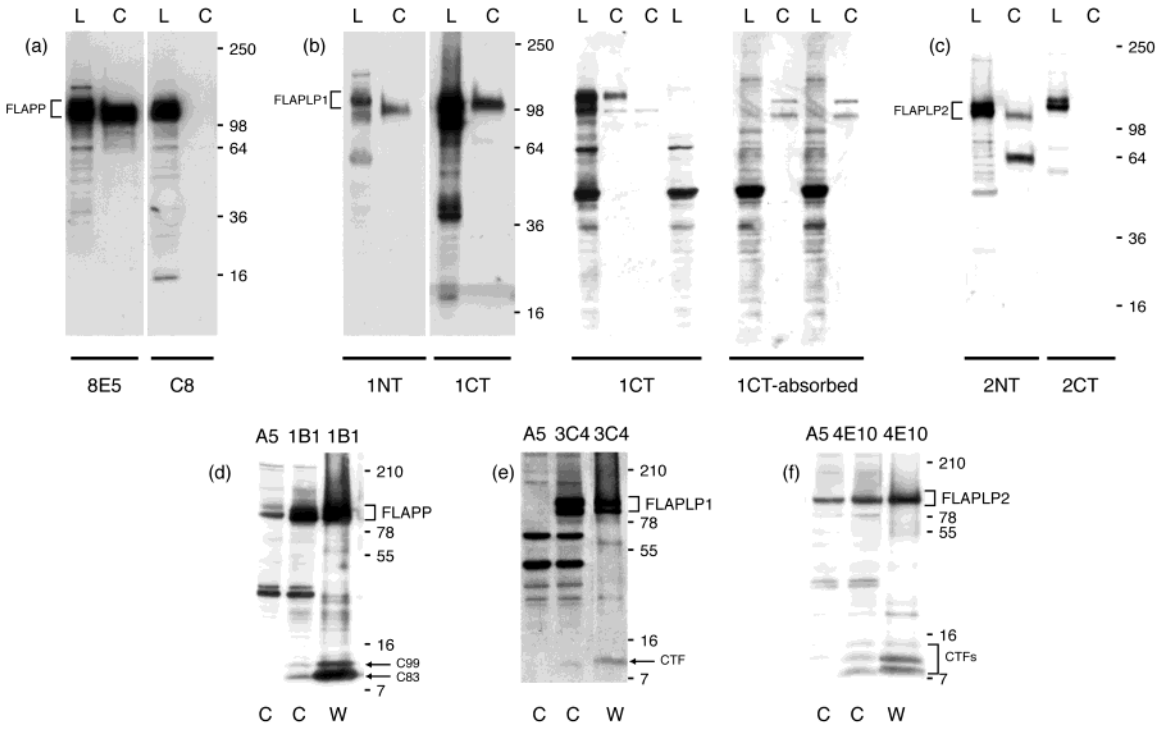

FIGURE 1: APLP1 and 2 are substrates for ectodomain shedding, resulting in secretion of their ectodomains and membrane retention of C-terminal stubs. (a−c): Whole cell lysates (L) and conditioned media (C) from cells overexpressing APP (1B1), APLP1 (3C4), or APLP2 (4E10) were Western blotted with N-terminal (8E5, 1NT, 2NT) and C-terminal (C8, 1CT, 2CT) antibodies. Note that lanes 3 and 4 of (b) panels 2 and 3 contain samples from cells transfected with vector alone (A5). Full length (FL) APP and APLP bands are indicated. (d−f): Crude (C) or sodium carbonate-washed (W) membranes from cells stably transfected with APP (1B1), APLP1 (3C4), APLP2 (4E10), or vector alone (A5) were subjected to Western blotting with C8 (d), 1CT (e), or 2CT (f). Arrows and brackets denote APP (C83 and C99) and APLP C-terminal fragments (CTFs) bands.

rabbit Ig (at 1:20 000) (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and ECL+ detection (Amersham Pharmacia Biotech, Arlington Heights, IL).

Samples from conditioned media were run on 12% tris-glycine gels and transferred onto 0.45 μM nitrocellulose and blocked as above. Filters were probed for APP, APLP1, or APLP2 using the ectodomain directed antibodies 8E5, 1NT, and 2NT, and the C-terminal directed antibodies C8, 1CT, and 2CT.

Immunocytochemistry and Confocal Microscopy. COS cells were plated at 1×10^5 cells/well and 24 h later used for transient transfection with APP C49, C60, APLP1 C49, C60, APLP2 C49, C60 ± FE65. After a further 36 h, cells were fixed and stained for the nuclear marker Histone H1 (monoclonal anti-Histone H1 antibody from Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal antibodies specific for the C-termini of APP, APLP1, or APLP2. For immunofluorescence detection, cells were stained with the secondary antibodies, goat anti-rabbit Cy3 and goat anti-mouse Cy5 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA) and viewed using a Zeiss LSM 510 laser scanning microscope.

Preparation of Crude Membrane Fractions and in Vitro Generation of ICDs. Crude membrane fractions were isolated as described previously (*30*). Briefly, cells were grown to confluence in a 10-cm dish, homogenized in hypotonic buffer (500 μL, 10 mM MOPS, pH 7.0, 10 mM KCl), and the homogenate cleared of nuclei and unbroken cells. A crude membrane fraction was obtained from the post-nuclear supernatant by centrifugation at 16000*g* and 4 °C for 40 min. For in vitro generation of ICDs, membrane pellets were resuspended in 50 μL of 150 mM sodium citrate buffer, pH 6.4 ± 2 mM 1,10 PTH and 250 μg/mL insulin and incubated at 37 °C for 2 h. The reaction was stopped by cooling samples on ice for 10 min, and the membranes pelleted by centrifugation at 100000*g* at 4 °C for 1 h. Forty microliters of the supernatant were removed and diluted with 4× tris-tricine sample buffer, boiled for 10 min and then 20 μL aliquots electrophoresed on 10−20% tris-tricine gels. For analysis of membrane pellets, the remaining supernatant was completely removed and the membrane proteins extracted from the pellet by boiling in 2× tris-tricine sample buffer (20 μL). To remove adventitiously associated proteins, membranes were washed with 100 mM sodium carbonate buffer, pH 11.0, prior to incubation and/or extraction with sample buffer.

RESULTS

APP, APLP1, and APLP2 Are Integral Membrane Proteins that Undergo Ectodomain Shedding. Cell lysates and conditioned media (CM) from CHO cells stably transfected with human APP_{695} (1B1 cells), $APLP1_{650}$ (3C4 cells), $APLP2_{751}$ (4E10 cells) or vector alone (A5 cells) were probed for the presence of APP, APLP1, or APLP2. When lysates and CM from 1B1 cells were Western blotted with the APP N-terminal antibody 8E5, a broad band centered at ~125 kDa was detected in lysates, and a slighter faster migrating species centered at ~118 kDa was detected in the CM (Figure 1a). When the same samples were probed with the APP C-terminal specific antibody C8, a band comigrating with that detected by 8E5 was obvious in lysates but absent in CM, thus confirming that full-length APP undergoes ectodomain

shedding to release a large, C-terminally truncated species into the medium (*31*, *32*). Consistent with this and prior observations (*33*, *34*), the C-terminal stubs (C83 and C99) were detected in both crude and sodium carbonate washed membranes of the 1B1 cells (Figure 1d).

When lysates and CM from 4E10 cells were probed with antibodies to the ectodomain and C-terminal regions of APLP2, a pattern similar to that of APP was observed. The N-terminal antibody 2NT revealed the presence of a tight doublet in cell lysates migrating between 115 and 125 kDa, whereas a faster migrating species running ~110 kDa was detected in CM (Figure 1c). When the same samples were probed with the APLP2 C-terminal specific antibody 2CT, a doublet comigrating with that detected by 2NT was observed in cell lysates but was absent in CM. Thus, as with APP, APLP2 undergoes ectodomain shedding, resulting in the secretion of a large, C-terminally truncated species (*35*). Furthermore, examination of either crude or sodium carbonate washed membranes revealed the presence of both full length APLP2 and its C-terminal stubs (Figure 1f). For the latter, two bands of similar intensity migrating around ~8 and 10 kDa were detected, and a fainter, slower migrating species was also observed at ~12 kDa.

Examination of lysates and CM from 3C4 cells showed a similar but slightly different pattern for APLP1 to those seen in the APP and APLP2 stable transfectants. Western blotting with an N-terminal antibody (1NT) revealed the presence of APLP1 species in both lysates and CM, with the single band in CM migrating slightly faster (~95 kDa) than the smear of bands observed in the cell lysate (95−106 kDa) (Figure 1b). However, when CM was probed with the C-terminal specific antibody 1CT, a band that comigrated with the upper band in the lysate sample (i.e., at ~105 kDa) was observed, but this band migrated slightly slower than the APLP1 species detected in the CM by 1NT (~95 kDa). The authenticity of the C-terminally intact APLP1 species detected in the CM was confirmed by the findings that: (a) this band was not detected in A5 (vector) cells, which do not express APLP1 (Figure 1b, panel 2, lanes 3 and 4); and (b) detection of this band was abolished by preabsorption of the 1CT antiserum with its cognate peptide immunogen (Figure 1b, panel 3, lanes 1 and 2). Thus, these findings suggest that APLP1, like APP and APLP2, undergoes ectodomain shedding (*36*) but that a small amount of full length APLP1 is apparently released into the medium. This phenomenon was not limited to the 3C4 cell line, but was also true of other stable APLP1 clones. Moreover, the cells appeared healthy, as assessed by phase contrast microscopy, reduction of MTT and trypan blue exclusion (not shown). It should also be noted that 3C4 cells were cultured under the same conditions as 1B1 and 4E10 cells, but full-length APP and APLP2 were not detected in the CM of those cells. Consistent with the ectodomain shedding of APLP1, a single C-terminal stub (~9 kDa) was detected in both crude and sodium carbonate washed membranes (Figure 1e). Interestingly, both APLP1 and APLP2 seemed to be less efficiently shed than APP, as judged both by the relative amount of secreted species detected in the CM (Figure 1a−c) and the ratio of C-terminal fragments (CTFs) to full-length protein detected in the membrane fractions (Figure 1d−f; Figure 2a−c).

The APLPs Undergo Presenilin-Dependent Intramembrane Proteolysis. Considerable genetic and biochemical evidence implicates presenilin (PS) as an essential component of the γ-secretase complex responsible for the intramembrane cleavage of APP and a growing list of other type I transmembrane proteins (*16*, *37*−*41*). Indeed, prior work suggested that APLP1 also serves as a γ-secretase substrate (*24*). We therefore assessed the effects of potent γ-secretase inhibitors on the release of the APLP intracellular domains. 1B1, 3C4, and 4E10 cells were treated with two structurally distinct γ-secretase inhibitors (DAPT, (*42*) and compound E (*43*)), and the cells were lysed and Western blotted with appropriate C-terminal antibodies. γ-Secretase inhibitors caused a robust increase in APP, APLP1, and APLP2 CTFs (Figure 2a−c), with the more potent compound E causing a greater increase in each substrate. Importantly, γ-secretase inhibitors did not alter the metabolism of the full-length proteins, thus demonstrating that the APLP CTFs are immediate substrates for γ-secretase cleavage and that this proteolysis of APLP 1 and 2 is preceded by ectodomain shedding. In this regard, APLP1 differs from APP and APLP2, in that APLP1 appears to undergo ectodomain cleavage at only one site rather than two, giving rise to a single CTF (Figure 1e) (*24*, *44*). This may, in part, explain the lower amount of APLP1 ectodomain shedding that we observed (Figure 1b).

Next, we took advantage of CHO cells that stably overexpress APP_{751} plus either wild type human PS-1 and PS-2 (PS-19 cells) or else dominant-negative mutations in both PS-1 (D257A) and PS-2 (D366A) (2A-2 cells). We examined the effect of these loss-of-function PS mutants on both APP and APLP2 processing. As previously reported, the 2A-2 double aspartate mutants show a dramatic accumulation of APP CTFs and concomitant reduction in secreted Aβ (Figure 2d, (*45*)). Similarly, we found that the 2A-2 mutants showed a substantial increase in APLP2 CTFs. Because the C-termini of APP and APLP2 are highly homologous, we sought to confirm the specificity of the 2CT antibody by examining its ability to detect APP CTF in cells that stably overexpress C99 (Figure 2d,e, lanes 3). 2CT did not recognize C99 or the abundant APP CTFs that were readily detected by C8 in the 2A-2 cells. Therefore, loss of functional PS/γ-secretase activity (*45*) results in accumulation of APLP2 CTF.

γ-Secretase cleavage of APP CTFs gives rise to the secreted products, p3 and Aβ, and a product that is released into the cytoplasm, APP ICD (*26*, *28*, *46*). Since the CTFs of the APLPs are likewise γ-secretase substrates (above), we searched for APLP ICDs that are analogous to the APP ICD. To this end, we developed an immunoprecipiation/Western blotting protocol using our 1B1 (APP) cells and then extended this protocol to the study of the 3C4 (APLP1) and 4E10 (APLP2) cells. FE65 is a cytoplasmic protein known to interact with the C-termini of APP and the APLPs (*11*, *12*, *47*), and it has been shown to stabilize recombinant APP ICD (*18*). We searched for ICDs both in the presence and absence of FE65 (Figure 2f−h). For APP-expressing cells, a ~6 kDa band of the size of APP ICD was detected only in the presence of FE65 (Figure 2f). Similarly, in 4E10 cells a faint ~6 kDa band was detected only in cells transfected with FE65 (Figure 2h, lane 2). For APLP1 expressing cells, a ~6 kDa band was detected in cells both

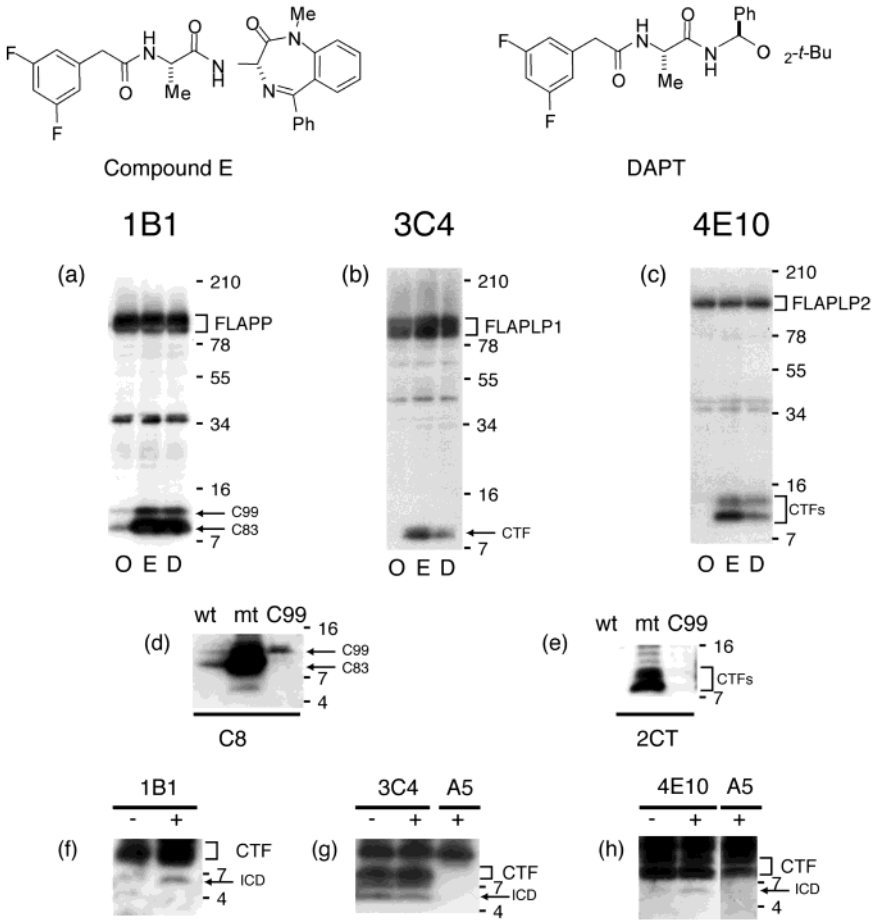

FIGURE 2: γ-Secretase processing of APLP1 and 2 releases their ICDs. (a−c): The structures of compound E and DAPT are shown. Cells expressing APP (1B1), APLP1 (3C4), or APLP2 (4E10) were incubated in the presence of 10^{-8} M compound E (E) or 10^{-6} M DAPT (D) or vehicle (O) for 6 h, and whole cell lysates probed with C8 (a), 1CT (b), or 2CT (c). Arrows and brackets mark CTFs and FL proteins. (d,e): CHO cells that stably overexpress APP_{751} and either wild-type human PS-1 and -2 (wt) or dominant negative mutant D257A PS-1 and D366A PS-2 (mt) were Western blotted with either C8 (d) or 2CT (e) and found to accumulate both APP and APLP2 CTFs. Lysates from cells expressing APP C99 (C99) were used as a negative control for antibody specificity. (f−h): CHO cells stably expressing APP (1B1), APLP1 (3C4), or APLP2 (4E10) or vector alone (A5) were transiently transfected with human FE65 (+) or vector alone (−) and then used for immunoprecipitation/Western blotting with C8 (f), 1CT (g), or 2CT (h). The presence of the C-terminal fragments (CTF) and intracellular C-terminal domains (ICDs) of APP and their APLP equivalents are indicated by brackets and arrows, respectively.

in the absence and presence of FE65, but was slightly more intense in cells transfected with FE65 (Figure 2g, compare lanes 1 and 2). The specificity of the APLP CTFs and ICDs was demonstrated by their absence in the A5 cells, which do not express APLP1 and have low endogenous expression of APLP2 (Figure 2g,h, lanes 3).

The ICDs of APP, APLP1, and APLP2 Can Be Generated in Vitro and Are Substrates of Insulin Degrading Enzyme. To further characterize ICD production, we employed a sensitive in vitro assay recently developed for the detection of the APP ICD (*30*). Crude membrane preparations were isolated from A5, 1B1, 3C4, or 4E10 cells and incubated in the presence or absence of 1,10 PTH and insulin, which potently inhibit insulin-degading enzyme (IDE), a protease known to degrade the APP ICD (*30*). The release of ICDs from the membranes was monitored by Western blotting. A similar pattern was observed for all three proteins of the APP family: minimal ICD was detected in the absence of 1,10 PTH and insulin, whereas robust ICD bands were recovered when these IDE inhibitors were present (Figure 3a−c). The specificity of the ICD detection was confirmed by the finding that little or no ICDs were detected when A5 (vector) cells were used. Moreover, when sodium carbonate-washed membranes were used for ICD generation, the ICDs were readily detected in the absence of the protease inhibitors (Figure 3a−c), suggesting that the activity responsible for degrading ICDs was cytosolic. Our results for APP ICD are in accord with those reported (*30*), but the experiments further demonstrate that APLP ICDs, like APP ICD, are rapidly degraded by a cytosolic protease sensitive to inhibition by 1,10 PTH and insulin, and that the machinery necessary for the generation of ICDs is contained within sodium carbonate-washed membranes.

In another series of experiments, we took advantage of this paradigm to investigate the role of γ-secretase in the generation of ICDs. We assessed the effect of two γ-secretase inhibitors, which we had shown to cause the accumulation of APP, APLP1, and APLP2 CTFs (Figure 2a−c). DAPT and compound E each caused a significant decrease in ICD production, with the more potent compound E being slightly more effective (Figure 3d−f). These results indicate that the proteases which generate and degrade ICDs are the same or highly similar for all three-members of the APP family. In addition to detection of the ICDs released into the cytosolic

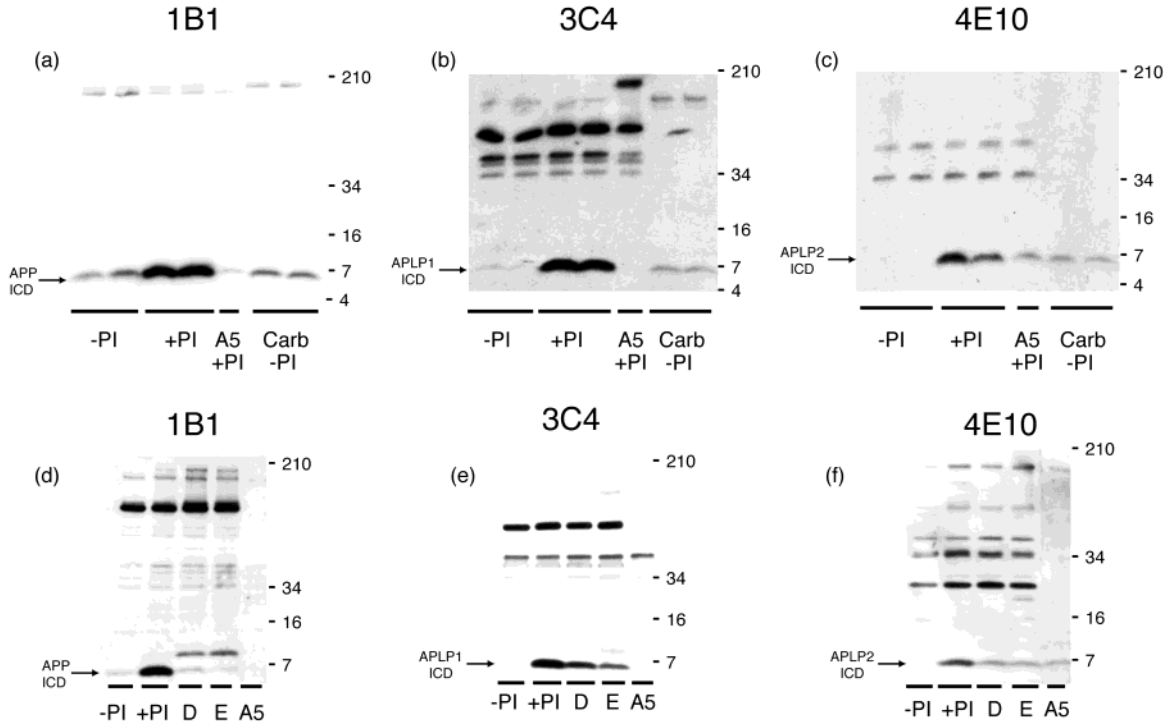

FIGURE 3: In vitro generation of APP and APLP ICDs. (a−c): Crude membranes were prepared as described in the methods and incubated for 2 h at 37 °C in the presence (+PI) or absence (−PI) of the protease inhibitors, 1,10 PTH and insulin; or else were washed with sodium carbonate buffer (carb) and then similarly incubated but in absence of 1,10 PTH and insulin. For cells transfected with vector alone (A5), crude membranes were incubated in the presence of 1,10 PTH and insulin. (d−f): To assess the γ-secretase dependence of ICD generation, crude membranes were incubated in the presence of 1,10 PTH and insulin and 10^{-8} M compound E (E) or 10^{-6} M DAPT (D). +PI, −PI and A5 are as in a−c. ICD bands are marked with an arrow.

phase, we also found small amounts of membrane-associated ICDs (not shown), both in sodium carbonate-washed membranes incubated without protease inhibitors and in unwashed membranes incubated in the presence of 1,10 PTH and insulin. Taken together, our results suggest that IDE (or an IDE-like activity) degrades ICDs both as they emerge from the membrane and within the cytosol.

The ICD of APLP1 Is More Stable than Those of APP or APLP2, But All Are Further Stabilized by FE65 and Translocate to the Nucleus. Consistent with their proposed role in transcriptional regulation, ICDs appear very labile (Figure 3). Consequently, proteins that bind and stabilize ICDs (perhaps by shielding them from degradation) may be critical cofactors for ICD signaling. Because FE65 is known to interact with the C-termini of APP and the APLPs (*11*, *47*) and to stabilize a recombinant C-terminal fragment of APP (*18*), we compared the stability and subcellular location of recombinantly expressed forms of C-terminal APP, APLP1, and APLP2, both in the absence and presence of FE65 (Figures 4 and 5). For these studies, we used two constructs for each protein. The first, referred to as C49, encodes the C-terminal 49 amino acids of APP and APLP2 beginning with the endogenous methionine one amino acid after the epsilon cleavage site of APP (i.e., between Leu645 and Val646 of APP695) (*26*−*28*) and is consistent with ICD fragments detected in cellular extracts and generated in vitro ((*26*) Figure 3). Unlike APP and APLP2, APLP1 does not contain an endogenous methionine 49 amino acids before its C-terminus; rather, there is a methionine 51 amino acids from the C-terminus. Therefore, for APLP1, we generated a construct encoding the C-terminal 51 amino acids of APLP1 (APLP1 C51) and an additional construct encoding the C-terminal 48 amino acids of APLP1 plus an exogenous initiating methionine (APLP1 C49). For each of the three APP family members, we generated longer constructs (C60s) that encode the C-terminal 59 amino acids of each protein, based on the APP product generated by the γ-secretase cleavage after residue 40 of the Aβ domain (plus an exogenous initiating methionine). Although a natural APP C59 species has not yet been detected, APP C60 is known to behave similarly to the APP C49 but to be much more stable (*18*) and therefore of more utility for biochemical analyses.

When COS cells were transfected with either APP C49 or APLP2 C49 virtually no product was detected in the absence of FE65 (Figure 4b,d), whereas in the presence of FE65, a C-terminal protein migrating at ~6 kDa was detected. In agreement with previous work using APP C60 (*18*), transfection of cells with either APP or APLP2 C60 produced robust amounts of a protein that migrated slightly above the C49 band. The C60 proteins (like the C49 proteins) were markedly stabilized when FE65 was coexpressed. In the case of APLP1, its C51 was also stabilized by expression of FE65 (Figure 4c), but APLP1 C51 was readily detected even in the absence of FE65 and appears to be intrinsically more stable than the corresponding C49 proteins of APP and APLP2 (Figure 4, compare panel c to b and d). The APLP1 C60 construct behaved differently from the APP and APLP2 C60s in that it produced two protein bands (Figure 4c); this observation is probably of no physiological significance but rather results from the use of an alternate initiation site. In this regard, it is noteworthy that the lower 1CT-positive band detected in COS cells transfected with APLP1 C60 comigrates with the 1CT-reactive band detected in cells transfected with APLP1 C51 (Figure 4c). Moreover, the RNA

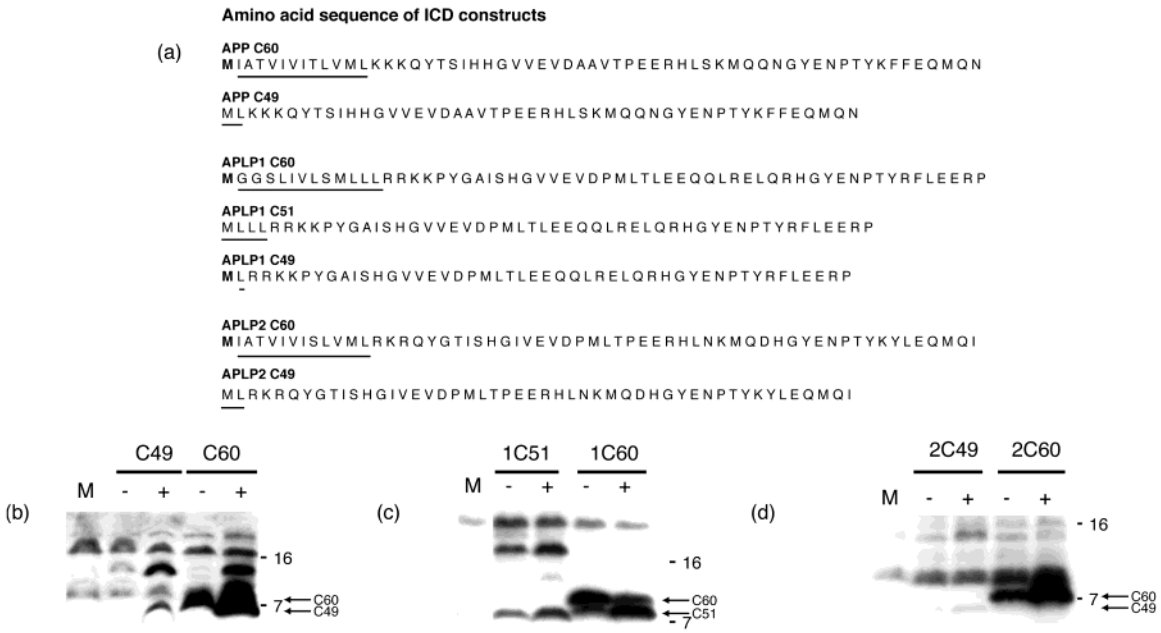

FIGURE 4: Recombinantly produced ICDs are stabilized by FE65. (a): The amino acid sequence of the ICD constructs are shown with exogenous initiating methionine residues in bold and residues from the putative transmembrane domains of the parent proteins underlined. (b−d): COS cells were transfected with vector alone (M), or APP C49 or C60 (b), APLP1 C51 or C60 (c), or APLP2 C49 or C60 (d) in the presence (+) or absence (−) of FE65, and cell lysates were Western blotted with C-terminal antibodies C8, 1CT, or 2CT. Bands of interested are indicated by arrows.

sequence surrounding the ATG that is 51 codons before the 3′ end of the APLP1 C60 construct contains several components compatible for efficient protein translation (*48*), suggesting that the smaller C60 derivative is generated by initiation at codon 51. Also consistent with the latter interpretation is the finding that the lower band present in the APLP1 C60 transfectants behaves very similarly to APLP1 C51, i.e., it is detected in the absence of FE65 and is stabilized similarly by FE65 (Figure 4c, lanes 3 and 4). The increase in the intrinsic stability of APLP1 C51 compared to APP C49 or APLP2 C49 is not attributable to the presence of an additional amino acid in APLP1 C51, since the shorter APLP1 C49 was also intrinsically more stable than the APP or APLP2 C49 proteins (Figure 6).

The increased stability of the APLP1 C49/C51 proteins nonetheless appears at odds with the similar stability of the APLP and APP ICDs generated from their full-length precursors in our in vitro paradigm (Figure 3). To exclude the possibility that these differences arose due to the use of two different cell types (COS cells for the transient transfection experiments and CHO cells for the in vitro ICD generation system), we transfected CHO cells with APP C49, APLP2 C49, or APLP1 C49, with and without FE65. As with the COS cells, in CHO cells, APP C49 and APLP2 C49 were only detectable in the presence of FE65, whereas APLP1 C49 was readily detectable even without FE65 and was only modestly stabilized by FE65 coexpression (data not shown). Thus, when transfected into cells, the APLP1 ICD appears intrinsically more stable than that of either APP or APLP2. Of course, there are other dissimilarities between the transfection experiments and the in vitro system, not least the fact that transfection of cells allows for production and trafficking of proteins through compartments where degradation may not occur and consequently where APLP1 C49/51 may have a longer half-life than the APP and APLP2 C49s. In this regard, our finding that APLP1 ICD is detected in whole cell lysates in the absence of FE65 overexpression (Figure 2f−h), further suggests that APLP1 in whole cells is intrinsically more stable than ICDs of either APP or APLP2.

Confocal microscopy of cells transfected with APP C49, APLP1 C49, or APLP2 C49 revealed the presence of these proteins in both nuclei and vesicular structures, with the APLP1 C49 expressing cells having the highest abundance of nuclear staining (Figure 5a−d). As predicted from our biochemical analysis (Figure 4b−d), the nuclear localization was only seen in cells transfected with FE65 (Figure 5a−d) and was not evident in cells transfected with ICD constructs alone, in which a more diffuse perinuclear staining was evident (Figure 5b). These findings are consistent with evidence that ICDs lack a predicted nuclear localization motif and require other molecules for their transport to the nucleus (*19*). Similarly, when the more stable C60 constructs (exemplified by APLP1 C60, Figure 5e,f) were examined, nuclear localization was only observed in the presence of FE65 (Figure 5f), with perinuclear, cytoplasmic, and vesicular staining obvious in its absence (Figure 5e). Thus, the observed increased stability of ICDs in the presence of FE65 is at least in part due to the altered localization of the ICDs.

ICDs of APP, APLP1, and APLP2 Are also Stabilized by Other FE65 Family Members. FE65 is a member of a family of proteins which include FE65-like protein 1 (L1, (*12*)) and FE65-like protein 2 (L2, (*13*, *47*)). Each of the three FE65 proteins are known to interact with the intracellular domain of APP. It is possible that different FE65 proteins might differentially stabilize the ICDs of the APP protein family. To assess this possibility, we transiently transfected COS cells with C49 and C60 constructs in the absence or presence of the FE65 proteins (Figure 6). The presence of any FE65 protein caused an increase in the amount of the C49 and C60 bands detected. Figure 6 is representative of four experiments, and although there were some variations

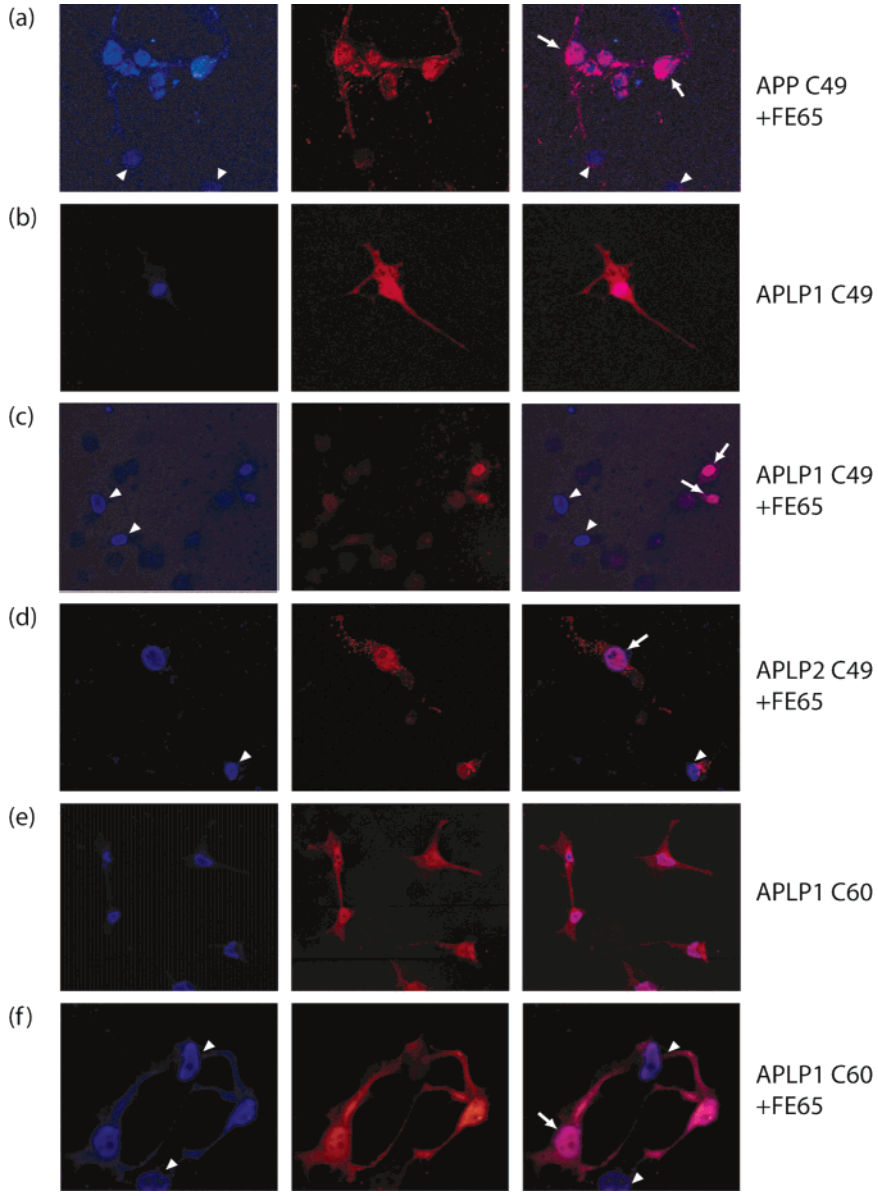

FIGURE 5: Recombinantly produced ICDs translocate to the nucleus in the presence of FE65. COS cells were transfected with C49 or C60 constructs plus or minus FE65 and examined by confocal microscopy. Staining of the nuclear protein histone H1 is shown in blue and staining with C8 (APP), 1CT (APLP1), or 2CT (APLP2) are shown in red. Overlapping staining appears as purple. Note that the images shown are for a single z-plane, but co-localization was always confirmed by examination of multiple z-sections. Co-localization of anti-H1 and C-terminal antibodies is indicated by white arrows, and H1 staining of untransfected cells is indicated by white arrowheads. In (e) all cells shown are transfected. Images were collected at a magnification of 40×.

between experiments, the following findings were observed: (i) FE65 and L1 stabilized APP C49 slightly better than did L2; (ii) FE65 and L1 stabilized APLP1 C49 more effectively than did L2; and (iii) all three FE65 proteins stabilized APLP2 C49 to a similar extent. Interestingly, in addition to the ~6 kDa C49 bands, slightly higher molecular weight bands were also detected for both APLP1 and APP (see also Figure 6a,b). Note in each case, the bands migrated at ~12 kDa, and their amounts reflected those of the respective 6 kDa ICDs, suggesting that these higher species represent dimeric C49 and that binding to FE65 proteins facilitates the dimerization of ICDs.

DISCUSSION

A leading target for the treatment of AD is to reduce production of Aβ (*37*). Aβ is produced by the action of two enzymes, β- and γ-secretases (*3*−*5*). The γ-secretase activity is believed to be a multi-protein complex which includes PS and which has been shown to cleave a number of transmembrane proteins, including Notch, Erb-B4, E-Cadherin, LRP, Nectin 1, Delta and Jagged (*16*, *38*−*41*, *49*, *50*). Here, we show that both APLP 1 and APLP2 undergo PS-dependent RIP in a manner highly similar to APP. While several studies suggest that APP may function in neurite outgrowth and maintenance (*51*−*59*), the true physiological role for APP and its homologues remains obscure. However, by analogy to Notch and the other known γ-secretase substrates, the APP protein family members seem likely to serve as receptors which, upon binding to their cognate ligands, undergo cleavage by a membrane-associated metalloproteinase, thus shedding their ectodomains and retaining C-terminal stubs suitable for intramembrane proteolysis.

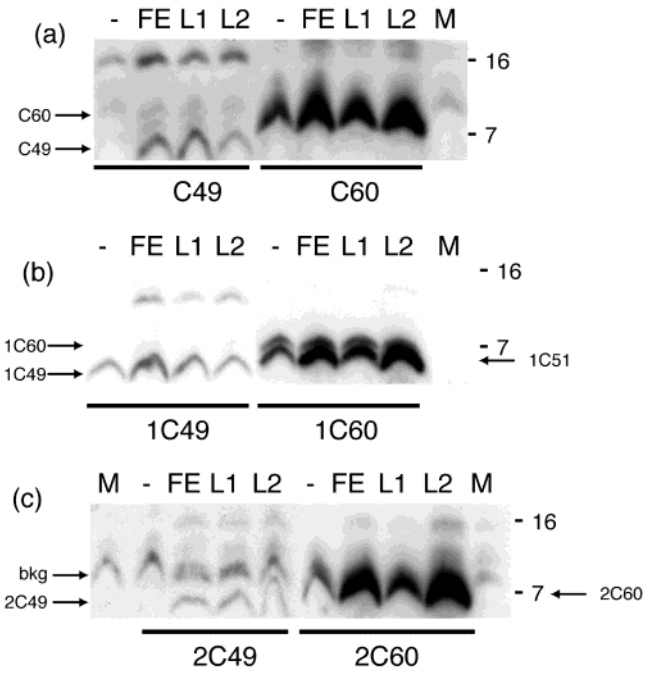

FIGURE 6: FE65-like proteins are capable of stabilizing APP and APLP ICDs. (a−c): COS cells were transiently transfected with APP C49 or C60 (a), APLP1 C49 or C60 (b), or APLP2 C49 or C60 (c) in the presence of FE65 (FE), FE65 L1 (L1), or FE65 L2 (L2). M denotes cells transfected with vector alone. The APLP2 C49 band is indicated with an arrow, as is a nonspecific (bkg) band that comigrates with APLP2 C60, other bands of interest are similarly indicated by arrows. The effects of FE65 proteins on the levels of C60 proteins were somewhat more variable than for C49 proteins but followed a similar pattern.

Once cleaved by γ-secretase, APP and the APLPs release their ICDs, which we have shown are stabilized by the FE65 proteins and can enter the nucleus. This finding is substantiated by recent studies showing that the γ-secretase-generated ICDs of APP and the APLPs can drive transcription of hetrologous reporter genes (*19*, *44*) and the report that a ternary complex containing APP ICD can directly activate transcription of the tetraspanin, Kal1 (*60*).

Only a few studies of APP and APLP functional properties in vivo have appeared. APP−/− mice are viable, fertile, and show locomotor abnormalities, reactive gliosis and relatively subtle cerebral defects in adulthood (*22*, *61*). This rather benign phenotype is assumed to relate to the continued expression of APLP 1 and 2. Indeed, APLP2(−/−)/APP(−/−) mice and APLP2(−/−)/APLP1(−/−) mice had a lethal phenotype (postnatal day 1), whereas APLP1(−/−)/APP(−/−) mice were apparently normal (*20*). These results suggest that APLP2 has the key physiological role among the family members. In accord, mice expressing just a single APLP2 allele [APLP2(±)/APLP1(−/−)/APP(−/−)] showed perinatal lethality. Together, these studies point to a high degree of functional redundancy within the APP family, but with certain specialized functions reserved for each protein.

Our findings that APP and the APLPs are processed similarly to generate labile ICDs, that these ICDs are degraded by the same or highly similar activities (IDE), and that these ICDs bind FE65 proteins and translocate to the nucleus all strongly support the thesis that these proteins share some common signaling functions. But what facilitates each protein's specific function? Clearly differential expression patterns, both spatial and temporal would allow for some specificity, as would interaction with ligands that differ in the their ability to interact with APP, APLP1, and APLP2. Similarly, although ICDs of each protein bind FE65 family members and translocate to the nucleus, their slightly different primary structures may allow interactions with other proteins required for transcription, so that the different ICDs regulate some genes in common as well as some specific genes. Furthermore, our observation that all three FE65 proteins can stabilize one or more of the ICDs suggests that the FE65 proteins play a role in regulating ICD signaling. Thus, differences in the tissue and cell type distributions of the FE65 proteins (*10*, *12*, *13*) and their temporal patterns of expression may mediate differential regulatory effects of the APP and APLP ICDs.

These various possibilities offer both a warning and an opportunity. Clearly, agents that target γ-secretase activity will not only have an effect on APP processing, but also alter the processing and signaling of other γ-secretase substrates, including APLP1 and 2. Long-term inhibition of the common signaling pathways mediated by ICDs would not be desirable. Conversely, strategies aimed at specifically upregulating APLP expression may be beneficial, since not only would this provide a competitive substrate for APP, but the APLP ICDs and the corresponding secreted fragments of the APLPs may actually compensate for the loss of APP ICD and any associated change in secreted APP. Given the many commonalties between APP and the APLPs, including those reported here and previously (*24*, *25*, *36*, *44*), attention should be paid to understanding how modulation of APP processing will impact upon the other members of this protein family.

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