Generation of the \(\beta\)-Amyloid Peptide and the Amyloid Precursor Protein C-terminal Fragment \(\gamma\) Are Potentiated by FE65L1*

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Members of the FE65 family of adaptor proteins, FE65, FE65L1, and FE65L2, bind the C-terminal region of the amyloid precursor protein (APP). Overexpression of FE65 and FE65L1 was previously reported to increase the levels of \(\alpha\)-secretase-derived APP (APP\(\alpha\)). Increased \(\beta\)-amyloid (A\(\beta\)) generation was also observed in cells showing the FE65-dependent increase in APP\(\alpha\). To understand the mechanism for the observed increase in both A\(\beta\) and APP\(\alpha\) given that \(\alpha\)-secretase cleavage of a single APP molecule precludes A\(\beta\) generation, we examined the effects of FE65L1 overexpression on APP C-terminal fragments (APP CTFs). Our data show that FE65L1 potentiates \(\gamma\)-secretase processing of APP CTFs, including the amyloidogenic CTF C99, accounting for the ability of FE65L1 to increase generation of APP C-terminal domain and A\(\beta\)40. The FE65L1 modulation of these processing events requires binding of FE65L1 to APP and APP CTFs and is not because of a direct effect on \(\gamma\)-secretase activity, because Notch intracellular domain generation is not altered by FE65L1. Furthermore, enhanced APP CTF processing can be detected in early endosome vesicles but not in endoplasmic reticulum or Golgi membranes, suggesting that the effects of FE65L1 occur at or near the plasma membrane. Finally, although FE65L1 increases APP C-terminal domain production, it does not mediate the APP-dependent transcriptional activation observed with FE65.

Processing of the amyloid precursor protein (APP)* results in the generation of the amyloidogenic peptide, A\(\beta\), which plays a central role in the pathogenesis of Alzheimer’s disease. Cleavage of C99, the APP C-terminal fragment derived from \(\beta\)-secretase processing of APP, by \(\gamma\)-secretase generates the A\(\beta\) peptide. Furthermore, \(\gamma\)-secretase cleavage of C99 and C83, the \(\alpha\)-secretase derived APP C-terminal fragment (APP CTF), releases the APP C-terminal domain (AICD), a 6-kDa peptide also called CTF\(\gamma\) or AID, that regulates transcription after translocation to the nucleus (1–4).

The majority of proteins reported to bind the 47-amino acid intracellular region of APP (5–7), including the FE65 protein family members FE65, FE65L1, and FE65L2, bind the \(\gamma\)-ENPTY sorting motif of APP via a phosphotyrosine interaction domain (PID/PTB). YENPTY is a clathrin-coated pit internalization domain required for trafficking of APP into the endocytic pathway (8, 9). Previous studies have shown that FE65 protein family members can alter the processing of APP by influencing APP trafficking. Increased maturation of APP and increased \(\alpha\)-secretase-cleaved APP (APP\(\alpha\)) secretion was observed in H4 neuroglioma cells induced for FE65L1 overexpression (10). Furthermore, enhanced secretion of APP\(\alpha\) and A\(\beta\) was reported in Madin-Darby canine kidney APP695 cells stably overexpressing FE65L1 (11). Cell surface APP levels were elevated in these cells, and increased routing of APP into the endocytic pathway from the plasma membrane was suggested to account for the observed increase in A\(\beta\) (11).

The FE65 proteins are adaptor proteins that have three protein-protein interaction domains, a WW domain and two PID/PTB domains, and are thus capable of mediating the formation of protein complexes. The N-terminal PID/PTB domain (PID1) of FE65 mediates the interaction of FE65 with the low density lipoprotein receptor-related protein, LRP (12), the histone acetyltransferase Tip60 (3), and the transcription factor CP2/LSF/LBP-1 (13), whereas the C-terminal PID/PTB domain of the FE65 protein family members binds APP (14–16). In addition, the WW domain binds the mammalian homolog of Drosophila Enabled (17), implicated in cell motility (18).

Biochemical complexes have been demonstrated for FE65-AICD-Tip60 (3) and FE65-APP-Mena (11) but only postulated for APP-FE65-LRP (12). A biologically relevant role for the FE65-AICD-Tip60 complex in transcriptional activation is suggested by its ability to regulate expression of two members of the tetranspanin superfamily (4, 19). In addition, up-regulation of FE65 and Tip60 is observed in mouse brains of APP transgenic mice and results in enhanced expression of the tetranspanin, KAI-1 (4).

Given the importance of the APP CTFs for A\(\beta\) generation and transcriptional activation and the fact that the FE65 proteins bind the NPXY motif present in all APP CTFs, we examined the effect of FE65L1 overexpression on APP CTF processing in H4 neuroglioma cells. Our data show that FE65L1 overexpression results in increased generation of AICD and A\(\beta\) in these cell lines.
cells by increasing γ-secretase processing of both C83 and C99. Furthermore, FE65L1 does not directly alter γ-secretase activity, but binding of FE65L1 to APP and APP CTFs is necessary for FE65L1-dependent γ-secretase processing of APP CTFs. These data suggest that the interaction of FE65L1 with APP CTFs facilitates access of γ-secretase to its substrates. In addition, we provide evidence that these events occur at or near the cell surface.

EXPERIMENTAL PROCEDURES

Reagents—The anti-APP C-terminal polyclonal sera (A5717, Sigma) and the APP C-terminal monoclonal antibodies 136G (a generous gift from Peter Seubert) and C16.1 (a generous gift from Paul Mathews and Ralph Nixon) were used to detect APP and APP CTFs. The 6E10 monoclonal antibody (Signet, Dedham, MA) was used to recognize APPs and C99. The PS1 antisera Ab14 recognizing the N-terminal fragment of PS1 was a gift from Sam Gandy. The anti-FE65 antibody (a gift from Joseph Buxbaum) was previously described (11). The anti-FE65L1 polyclonal sera was raised in rabbit against a gluthathione S-transferase fusion protein coding for amino acids 201–305, which include the WW domain of human FE65L1 (GenBank™ accession number U62325). Antibodies against early endosome marker (EEA1), GM130 (cis-Golgi marker), and syntaxin 6 (trans-Golgi marker) were purchased from Transduction Laboratories. Polyclonal antibodies detecting the endoplasmic reticulum (ER) protein calnexin (Stressgen) and the endosome marker Rab5 (Santa Cruz Biotechnology) were used to identify these membrane fractions. The polyclonal antibody recognizing γ-secretase-cleaved Notch, NICD, was purchased from Cell Signaling Technology, and the anti-Myc antibody, 9E10, was obtained from Santa Cruz Biotechnology, Inc. The monoclonal antibody HA.11 was obtained from CRP Inc. The calpain, proteasome, and lysosomal inhibitors used included calpain inhibitor III, CI-III (Calbiochem) (20), lactacystin (Calbiochem) (21), MG132 (Calbiochem), NH4Cl (Sigma), chloroquine (Sigma), and N-acetyl-leucyl-leucyl-norleucinal, ALLN (Sigma) (21). The γ-secretase inhibitors used were BMS208974, CM-III-115 (22), WPE-III-31C (23), compound E (24), and DAPT (25). Complete protease inhibitor pellets without EDTA (Roche Applied Science) were used for all cell extractions.

Cell Culture, cDNA Constructs, and Transfections—The human H4 neuroglioma cell line, 32-6, is tetracycline-repressible for human HA-tagged FE65L1 and expresses endogenous levels of APP (10, 26). The tetracycline-repressible HA-tagged mutant FE65L1 cell line (C702V) expressing a mutant allele of FE65L1 bearing a C → V substitution at residue 702 of FE65L1 (National Center for Biotechnology accession number XM_051782), a conserved residue located in the APP binding PID2 domain, was established as described (26). The C702V mutation was created in the hFE65L1 plasmid using the primers 5′-GCAC-TTCTGATATCTGAATACAGGGCCGGGTCACGGCT-3′ and 5′-AGCGGTCAGCCGCGGCCTATGTTAGATCACAGAAGTGC-3′ by PCR mutagenesis (QuickChange, Stratagene). Cells induced for 72 h by removal of tetracycline showed similar expression levels for WT HA-FE65L1 and C702V HA-FE65L1. FuGENE 6 (Roche Applied Science) was used for all transient transfections. The mammalian notch N3E construct (a generous gift from R. Kopan) was transiently transfected into H4 neuroglioma cells uninduced and induced for FE65L1 after 24 h post-induction, and cells were harvested at 72 h. Proteasomal, lysosomal, calpain, and γ-secretase inhibitors in Me2SO were added at the indicated concentrations to complete media containing the appropriate nuclear membrane compartmentalization. The immune complexes were separated on a 10–20% Tricine gel. The gels were dried flat, and the radioactive signal was detected using a Cyclone phosphorimaging device (PerkinElmer Life Sciences).

Cell-free AICD Generation—AICD generation was examined in vitro using an active membrane preparation as described by Finnix et al. (30). The cells were incubated with 10,000 × 1 active decay congener stained with 10,000 × 1 nuclear membrane targeting peptide. The immune complexes were separated on a 10–20% Tricine gel. The gels were dried flat, and the radioactive signal was detected using a Cyclone phosphorimaging device (PerkinElmer Life Sciences).

 Luciferase Assays—H4 neuroglioma naive cells were transiently transfected for measurement of APP-dependent transcriptional activation of a GAL4-luciferase construct as previously described (3). Each transfection contained the pG5512-Luc luciferase reporter construct pCMVlacz, p-galactosidase, pCMVHA, pCMVluc, pCMVhA, and pCMVhAβ galactosidase control plasmid pHAT (GAL4) or pMST-APP (APP-GAL4) plasmids in combination with one of the FE65 plasmids, FE65-myc (CMV promoter), rat FE65 (RSP promoter), hFE65L1 (CMV promoter), or mouse FE65L1 (CMV promoter). DNA amounts used for transfection were as previously described (3).
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RESULTS

Increased γ-Secretase Cleavage of the γ-Secretase-derived APP CTF, C83, in H4 Cells Overexpressing FE65L1—We examined APP CTF levels in H4 neuroglioma cells overexpressing FE65L1 to determine whether FE65L1 influenced APP CTF stability. Western blot analysis of total protein lysates obtained from cells uninduced or induced for FE65L1 overexpression using an APP CTF antibody (13G8) showed a single 10-kDa APP CTF fragment corresponding to the α-secretase-derived APP CTF, C83, that was dramatically decreased in lysates obtained from H4 cells induced for FE65L1 overexpression (Fig. 1A).

To determine whether the effect of FE65L1 on C83 steady state levels was due to increased degradation of C83, we tested lysosome and proteasome inhibitors for their ability to block the FE65L1-dependent decrease in C83. Our data show that neither ammonium chloride nor chloroquine had any effect on the observed decrease in C83 upon FE65L1 overexpression (Fig. 1B). This indicates that the lysosomal pathway is not responsible for the observed decrease in C83 levels. MG132 cell treatments resulted in accumulation of APP CTFβ and Aβ40, indicating that the proteasome plays a role in APP CTFβ degradation (30). Similarly, calpain was previously implicated in the degradation of C83 and Aβ40 and in the generation of Aβ, particularly Aβ42 (20, 31). To test the possibility that calpain or proteasomal processing of C83 was increased in H4 cells overexpressing FE65L1, we treated cells with the calpain inhibitor CI-III and the proteasome inhibitors MG132 and lactacystin and examined C83 levels. Neither proteasomal nor calpain inhibitors were able to abolish the FE65L1-dependent decrease in C83 (Fig. 1B). However, a comparison of the APP CTF levels in Me2SO-treated versus drug-treated cells showed that the drug-treated cells had higher APP CTF levels in both uninduced and induced cells than the Me2SO controls. These data suggest that these inhibitors affect activities that alter APP CTF levels but do not prevent the FE65L1-dependent C83 decrease. ALLN, reported to inhibit calpain, the proteasome, and cathepsins (31), was able to partially inhibit the FE65L1-dependent decrease in C83, but did not completely reverse the effects of FE65L1 on APP CTF processing. For ALLN treatments, C83 levels were higher in the uninduced cells treated with ALLN by 1.8-fold, and accumulation of a higher APP CTF fragment was also observed (possibly C89) (Fig. 1B). We can conclude from these data that the observed decrease in C83 in H4 cells overexpressing FE65L1 is not due to increased catabolism of C83 by calpain, the proteasome, or the lysosome.

Both C99 and C83 are substrates for γ-secretase (32–34). To determine whether FE65L1 can modulate γ-secretase cleavage of C83, we treated H4 neuroglioma cells uninduced and induced for FE65L1 overexpression with structurally different γ-secretase inhibitors (22, 24). Our results show that all five γ-secretase inhibitors can restore C83 levels to those of the uninduced controls (Fig. 1C). In fact, γ-secretase inhibitor treatments produce C83 levels that are higher in cells overexpressing FE65L1 than in the control cells, consistent with our previous observation that APP undergoes increased processing by γ-secretase (10). These data indicate that C83 processing by γ-secretase can account for the FE65L1-dependent increase in C83 turnover.

FE65L1 Overexpression Decreases the Half-life of C83 and Facilitates AICD Generation—Cleavage of C83 by γ-secretase results in the generation of p3 and AICD (35). Given that C83 processing by γ-secretase is increased in H4 neuroglioma cells overexpressing FE65L1, we predicted that AICD generation from these cells would be increased. To test whether FE65L1 decreases the half-life of C83 and produces higher levels of AICD, H4 cells uninduced and induced for FE65L1 overexpression were metabolically labeled for 20 min and chased with excess methionine for up to 4 h (Fig. 2A). Our results show that C83 is produced within 30 min of chase in cells uninduced for FE65L1 (Fig. 2A). However, in cells induced for FE65L1 the levels of C83 detectable at the 30 min chase time point is significantly lower than in uninduced cells, and a 6-kDa band with the mobility of AICD is observed (Fig. 2A). These data suggest that C83 is processed by γ-secretase to generate AICD in H4 cells overexpressing FE65L1. AICD detection was ob-
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**Fig. 2.** FE65L1 overexpression decreases the half-life of C83 and results in AICD generation. A. H4 32-6 cells were metabolically labeled with [35S]methionine and [35S]cysteine for 20 min and chased for the times indicated. APP full-length and APP CTFs were immunoprecipitated from 1000 μg of total protein using 1 μl of A8717 antibody, which recognizes the last 20 C-terminal residues of APP, and 20 μl of protein A/G PLUS-agarose followed by electrophoresis in 10–20% Tris-Tricine gels. Detection of labeled APP and APP CTFs was achieved using a Cyclone phosphorimaging system. In the top panel a 1-h exposure of the dried gel shows mature and immature full-length APP, and the bottom panel shows a 3-day exposure of the same gel for detection of APP CTFs and AICD. B. Cells were pretreated with the inhibitors indicated for 2 h before labeling and throughout the pulse/chase experiment, which included a 2-h chase interval. The inhibitors used include the γ-secretase inhibitors, WPE-III-31C and DAPT, the calpain inhibitor, CI-III, the proteasomal inhibitor, MG132, and the lysosomal inhibitor, chloroquine. C. AICD generation was examined in a cell-free assay by incubating membranes (10,000 × g postnuclear pellet) in reaction buffer for 2 h at 37 °C. APP CTFs released in the reaction supernatant were immunoprecipitated with the A8717 antibody, and the membrane pellets were detected by immunoblotting with the 83717 antibody. The membrane blots were reprobed with the anti-PS1 N-terminal antibody, Ab14. These data show a representative example of three independent experiments.

**Fig. 3.** Binding of FE65L1 to APP is required for the APP and APP CTF processing effects that occur upon FE65L1 overexpression. A. Substitution of the conserved cysteine within the FE65L1 PID2 domain to valine (C702V) blocks its binding to APP. Immunoprecipitates were obtained from lysates of uninduced (−) or induced (+) 32-6 and C702V cell lines using an anti-HA antibody. The precipitated proteins were immunoblotted with the APP antibody (369W) and the bottom panel shows a 3-day exposure of the same gel for detection of APP CTFs and AICD. B. Cells were pretreated with the inhibitors indicated for 2 h before labeling and throughout the pulse/chase experiment, which included a 2-h chase interval. The inhibitors used include the γ-secretase inhibitors, WPE-III-31C and DAPT, the calpain inhibitor, CI-III, the proteasomal inhibitor, MG132, and the lysosomal inhibitor, chloroquine. C. AICD generation was examined in a cell-free assay by incubating membranes (10,000 × g postnuclear pellet) in reaction buffer for 2 h at 37 °C. APP CTFs released in the reaction supernatant were immunoprecipitated with the A8717 antibody, and the immune complexes as well as the APP CTFs present in the membrane pellet were detected by immunoblotting with the 83717 antibody. The membrane blots were reprobed with the anti-PS1 N-terminal antibody, Ab14. These data show a representative example of three independent experiments.
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Fig. 4. **FE65L1 overexpression does not alter NICD production.** The N341 notch construct was transiently transfected into 32-6 cells, uninduced (−) and induced (+), for 24 h and CHO cells stably overexpressing PS1 WT or PS1 D385A. Cell lysates were collected at 48 h post-transfection. Total protein (100 μg) was separated on 4–12% Tris-glycine gels and analyzed for NICD by immunoblotting with an NICD-specific antibody. The same blot was reprobed with an anti-Myc antibody, 9E10, recognizing N341 and an anti-β-tubulin antibody to verify protein loads.

specific for the substrates that it binds, APP and APP CTFs, we examined the effects of FE65L1 overexpression on NICD generation in cells expressing the Notch N341 proteins (39). Our data show that FE65L1 overexpression does not alter NICD generation in H4 cells (Fig. 4), whereas an expected increase and decrease in NICD is observed in double stable CHO APP751/PS1WT and CHO APP751/PS1D385A, respectively. These data suggest that FE65L1 does not have a direct effect on γ-secretase activity and are consistent with an effect of FE65L1 on γ-secretase APP CTF processing that is dependent on the interaction of FE65L1 with APP CTFs.

**FE65L1-dependent γ-Secretase Processing of C83 Occurs at the Plasma Membrane or in the Early Endocytic Pathway**—As a first step to determining the subcellular compartment for the γ-secretase-dependent C83 processing, we performed ER/Golgi subcellular fractionations and examined C83 levels in each of these fractions. Although full-length APP was readily detected by Western blot analyses of the ER/Golgi fractions showing both immature and mature APP in the Golgi fractions and immature APP in ER vesicles (Fig. 5A), APP CTFs were not detected (data not shown).

We have previously reported the presence of FE65L1 in a crude light membrane fraction obtained from FE65L1-induced H4 cells (10). To further examine the association of FE65L1 with a subcellular membrane compartment, we performed Western blot analysis of the ER/Golgi fractions with an anti-HA antibody and found that FE65L1 localizes to both ER and Golgi membranes (Fig. 5A). In addition, FE65L1 was detected in association with early endosome vesicles enriched from a light vesicle fraction by immunoprecipitation with EEA1 or Rab5 antibodies (Fig. 5B). These data indicate that FE65L1 localization to membranes is not restricted to a specific membrane compartment and is consistent with the distribution of APP and APP CTFs, its binding partners.

Given that low levels of APP CTFs may not be detectable by Western blot analyses of ER/Golgi fractions, immunoprecipitations were performed on pooled ER fractions (calcinein positive) and Golgi fractions (GM130 positive) using an APP CTF antibody. Using this approach C83 levels were detectable in ER and Golgi pools but were found to be different in cells overexpressing FE65L1 than in uninduced cells (Fig. 6A), suggesting that the FE65L1-dependent γ-secretase cleavage of C83 does not occur in these compartments. The subcellular localization of constitutive α-secretase cleavage of APP has been reported to occur in a post-Golgi compartment in the secretory pathway of H4 neuroglioma cells (36), suggesting that C83 is generated in the late secretory pathway. Localization of C83 in ER/Golgi membranes suggests that a small fraction of C83 may be trafficked back into the ER and Golgi after it is generated.

In contrast to the ER/Golgi fractions where only C83 is detected, several APP CTFs were detected in early endosome vesicles. Their mobility on SDS/PAGE gels between the 7- and 17-kDa protein molecular mass markers suggests that these APP CTFs represent C83, C89, and C99 (Fig. 6B). The APP CTF fragment migrating at 14 kDa showed 6E10 immunoreactivity, confirming that it is in fact C99 (Fig. 6B). Interestingly, although APP levels are unchanged in early endosome vesicles, the levels of all three APP CTFs are reduced upon FE65L1 induction. These data suggest that FE65L1-dependent γ-secretase cleavage of the APP CTFs occurs either at the plasma membrane or in early endosomes (Fig. 6B). The localization of PS1 to the early endosome compartment, determined by detection of the N-terminal PS1 fragment with Ab14, suggests that APP CTFs may be cleaved by γ-secretase at this subcellular site (data not shown).

**Secreted Aβ40 Levels Are Increased in Cells Induced for FE65L1 Expression**—To examine the effect of FE65L1 on Aβ generation we collected conditioned media from cells uninduced and induced for FE65L1 overexpression and measured Aβ by Sandwich enzyme-linked immunosorbent assay. A 26% increase in secreted Aβ1–40 (p < 0.00000001) is observed for cells induced for FE65L1 compared with the uninduced control (Fig. 7). The data in Fig. 7 show a representative experiment that approximates the average increase in Aβ1–40 observed upon FE65L1 overexpression for four independent experiments. The increased Aβ40 secretion from cells overexpressing FE65L1 is significant for all four experiments. When FE65L1 C702V mutant protein was overexpressed, an increase in Aβ1–40 was still observed (6%), but the increase in Aβ1–40 is not statistically significant (Fig. 7). The average increase observed in four independent experiments for FE65L1 C702V is
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**FIG. 6.** The FE65L1-dependent reduction in APP CTFα levels is detected in early endosome vesicles but not in ER or Golgi vesicles. A, APP CTFα levels were analyzed by immunoprecipitation of pooled Golgi-rich (fractions 2–6) and ER-rich (fractions 9–12) containing 1.2 mg of protein isolated from either uninduced (−) or induced 32-6 cells using the A8717 antibody and Western blot analysis using the 13G8 antibody as in Fig. 1C. B, early endosome vesicles from uninduced (−) or induced (+) 32-6 cells were immunopurified with the EEA1 antibody, and full-length APP and APP CTF levels were examined by immunoblotting with 13G8. C99 levels were examined with the 6E10 antibody. The blots were reprobed with anti-Rab5 to verify equal protein loads.

**FIG. 7.** Secreted Aβ1–40 levels are increased by FE65L1 overexpression, and this effect requires binding of FE65L1 to APP. Conditioned medium from H4 cells overexpressing either wild type FE65L1 or the C702V mutant FE65L1 were analyzed for Aβ levels. Compared with uninduced control cells (−), Aβ40 was significantly increased (p < 0.000000601) in H4 cells induced (+) for WT FE65L1 expression. The C702V mutant FE65L1, which does not interact with APP, showed a small increase in Aβ40 that was not significantly different from the uninduced control.

4%, and only one experiment showed a significant difference in the levels of Aβ1–40 (data not shown). The increase in Aβ1–40 observed with the mutant protein most likely reflects residual binding of the mutant protein to APP that is not detected in other assays (see Fig. 3A). No statistically significant differences were observed for secreted Aβ1–42 levels, but an average 17% increase was observed, suggesting that the trend is similar to that of Aβ1–40 (data not shown). Our observations that Aβ40 levels are increased in FE65L1-overexpressing cells, that APP levels in the early endosome vesicles are no different for cells induced for FE65L1 compared with the uninduced control, that C99 was only detected in early endosome vesicles, and that FE65L1 influence on γ-secretase cleavage of APP CTFs is only detected in early endosome vesicles suggest that FE65L1 potentiates APP CTF cleavage at or near the cell surface. This interpretation is consistent with data indicating that secreted Aβ in non-neuronal cells is generated at the cell surface (40) and that Aβ40 generation, unlike Aβ42, occurs predominantly in the endocytic pathway (9, 41).

**FIG. 8.** FE65L1 does not promote AICD-dependent transcriptional activation. A, results of transactivation assays obtained from luciferase activity assays. All cells were transiently transfected with the APP-GAL4, GAL4-luciferase, and pCMV-lacZ constructs previously described (3) and the FE65 plasmids shown. The luciferase activity was normalized for transfection efficiency measured by lacZ activity assays, and the data are reported as -fold increase over GAL4, which corresponds to the ratio of the normalized luciferase activity over the luciferase activity obtained with the control pMST plasmid. B, Western blot analysis of total protein (20 μg) from transiently transfected cells in A using an anti-FE65L1 antibody that recognizes human (h) and rodent (r) FE65 and FE65L1.

Increased routing of cell surface APP into the endocytic pathway, where Aβ is known to be generated, was proposed as a mechanism for increased Aβ secretion in Madin-Darby canine kidney cells (11). However, an increase in Aβ production was reported in HEK293/APP695 cells overexpressing FE65L2 without any observed effects on APPα secretion (42), suggesting that effects on full-length APP trafficking are not required for increased Aβ production. Our data suggest that enhanced γ-secretase cleavage of C99 rather than processing of full-

**DISCUSSION**

Members of the FE65 protein family that bind the NPYXY motif of APP have the capacity to modulate APP processing by influencing trafficking of full-length APP molecules (10, 11). Increased routing of cell surface APP into the endocytic pathway, where Aβ is known to be generated, was proposed as a mechanism for increased Aβ secretion in Madin-Darby canine kidney cells (11). However, an increase in Aβ production was reported in HEK293/APP695 cells overexpressing FE65L2 without any observed effects on APPα secretion (42), suggesting that effects on full-length APP trafficking are not required for increased Aβ production. Our data suggest that enhanced γ-secretase cleavage of C99 rather than processing of full-
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length APP in the endocytic pathway is responsible for the higher Aβ40 levels measured in conditioned media of H4 cells induced for FE65L1 overexpression. This conclusion is based on our observation that FE65L1 overexpression does not produce increased levels of full-length APP in early endosomes, whereas C99 levels, which were detected solely in this compartment, are decreased. Given that cleavage of APP by α-secretase precludes the formation of Aβ, our results showing that FE65L1 modulates both C99 and full-length APP processing provides a means by which increases in Aβ levels can be observed in the presence of elevated levels of APPα.

Although we cannot exclude the possibility that the observed increase in Aβ40 is due to increased production of C99 through effects of FE65L1 on β-site APP cleaving enzyme activity, we believe that the increased Aβ production is due to effects of FE65L1 on C99 trafficking or enhanced access of C99 to γ-secretase for the following reasons. First, C99 levels detected in the early endosomes, like those of C83, are decreased by the presence of excess FE65L1, and we have demonstrated that the decrease in C99 levels is due to increased γ-secretase cleavage of this fragment. Second, all APP CTFs have the FE65L1 binding site, and binding of FE65L1 to APP is required for both increased γ-secretase processing of C83 and Aβ40 generation. Third, we demonstrate that at least one secretase activity involved in Aβ40 generation, γ-secretase, is not directly affected by FE65L1 because NICD production from the γ-secretase substrate Notch, which does not bind FE65L1, is not affected by FE65L1 overexpression. Although the identification of the JLK protease inhibitors, which block Aβ generation but not NICD production, suggested that the enzyme responsible for Aβ generation may be distinct from the enzyme generating NICD (43), subsequent studies using these inhibitors showed that they do not block γ-secretase activity (44). Taken together, these data suggest that it is unlikely that FE65L1 directly alters any of the secretase activities.

The 27% increase in Aβ40 secretion we observed in H4 neuroglioma cells overexpressing FE65L1 is comparable with the 40% increase reported for HEK293APP695 cells overexpressing FE65L2 using a Sandwich enzyme-linked immunosorbent assay (42). These increases in Aβ40 are modest compared with the reported 4.2-fold increase in Aβ observed for Madin-Darby canine kidney APP695 cells overexpressing FE65 detected by autoradiography of secreted metabolically labeled Aβ (11). This discrepancy may in part be due to the different methods used for measurement of Aβ or to cell type-dependent differences in secretase and APP substrate subcellular localization. Our study and those of others (11, 42) with the exception of one published report on FE65 (45) show that all three members of the FE65 protein family increase Aβ production.

In a separate report we demonstrated that FE65L1 overexpression in H4 neuroglioma cells reduces functional endogenous cellular levels of the LRP endocytic receptor protein (46). A reduction in LRP levels has previously been reported to influence Kunitz protease inhibitor–APP processing, the major APP isoforms in H4 neuroglioma cells (39), by increasing APPα and decreasing Aβ secretion in receptor-associated protein-treated cells (47). These data raise the possibility that modulation of APP processing by FE65L1 in H4 neuroglioma cells is mediated through its effects on LRP. However, the data presented here suggest that the FE65L1-dependent decrease in LRP is unlikely to contribute to the effects of FE65L1 on APP processing because the effects of FE65L1 on APPα and Aβ secretion can be blocked by abrogation of the binding of FE65L1 to APP. Furthermore, contrary to the reported effects of LRP reduction on APP processing, FE65L1 increases both APPα and Aβ.

Our data do not conclusively define the subcellular site for the FE65L1-dependent APP CTF processing by γ-secretase. However, given what is known about the subcellular sites of action of the secretases, the simplest explanation accounting for all the effects of FE65L1 on APP and APP CTF processing in H4 neuroglioma cells is that FE65L1 overexpression facilitates the cleavage of APP and APP CTFs (both C83 and C99) by α- and γ-secretase by increasing the residence time of the substrates at or near the cell surface. In support of this notion are studies showing that constitutive α-secretase cleavage and biologically active γ-secretase occur at the cell surface (48–51).

In addition to increased production of Aβ40 upon FE65L1 overexpression, we also observed FE65L1-dependent generation of AICD in pulse/chase experiments and an increase in the ratio of AICD-generated APP CTF substrate in cell-free assays. These data indicate that FE65L1 also potentiates AICD generation in H4 cells. FE65 was previously reported to stabilize AICD in COS cells (1). The contribution of FE65L1 to AICD stabilization could not be determined from our pulse/chase experiments because the substrate for AICD, C83, is generated from full-length APP for several hours during the chase, whereas AICD production from C83 could only be detected for up to 1 h of chase, making it difficult to separate the effect of FE65L1 on generation of AICD from its possible effects on AICD stabilization. All APP CTFs are decreased as a result of FE65L1 overexpression, indicating that they all contribute to increased AICD generation. However, because C83 is the most abundant APP CTF in these cells, it is likely that AICD is predominantly derived from γ-secretase processing of C83, as previously reported for SH-SY5Y cells (32). Although AICD generation is elevated in cells overexpressing FE65L1, unlike FE65, FE65L1 does not mediate transcriptional activation. A similar lack of transcriptional activation was recently reported for FE65L2 even though FE65L2 was detected in nuclear fractions (42). Thus, if the cellular role of the FE65 protein family is AICD-dependent transcriptional regulation, only FE65 appears to take part in transcriptional activation.

A fraction of the FE65 and FE65L1 proteins has previously been shown to be associated with membranes (10, 11). In this study, we show that membrane-associated FE65L1 localizes to all membrane compartments examined. This is likely due to APP and APP CTF localization in membranes throughout the secretory and endocytic pathways.

In conclusion, the results presented here show that FE65L1 overexpression facilitates γ-secretase cleavage of APP CTFs, resulting in increased production of Aβ40 and AICD, that these events occur at or close to the cell surface either at the plasma membrane or early in the endocytic pathway, and that FE65L1, like FE65L2, does not promote AICD-dependent transcriptional activation.

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