The β-amyloid precursor protein (APP)-binding protein Fe65 is involved in APP nuclear signaling and several steps in APP proteolytic processing. In this study, we show that Fe65 stimulates γ-secretase-mediated liberation of the APP intracellular domain (AICD). The mechanism of Fe65-mediated stimulation of AICD formation appears to be through enhanced production of the carboxyl-terminal fragment substrates of γ-secretase and direct stimulation of processing by γ-secretase. The stimulatory capacity of Fe65 is isoform-dependent, as the non-neuronal and α2 isoforms promote APP processing more effectively than the exon 9 inclusive neuronal form of Fe65. Intriguingly, Fe65 stimulation of AICD production appears to be inversely related to pathogenic β-amyloid production as the Fe65 isoforms profoundly stimulate AICD production and simultaneously decrease Aβ42 production. Despite the capacity of Fe65 to stimulate γ-secretase-mediated APP proteolysis, it does not rescue the loss of proteolytic function associated with the presenilin-1 familial Alzheimer disease mutations. These data suggest that Fe65 regulation of APP proteolysis may be integrally associated with its nuclear signaling function, as all antecedent proteolytic steps prior to release of Fe65 from the membrane are fostered by the APP-Fe65 interaction.

The β-amyloid precursor protein (APP)-binding protein Fe65 associates with the carboxyl-terminal region of APP, a protein integrally involved in Alzheimer disease (AD) pathogenesis (1). Several groups have demonstrated that Fe65 plays a key nuclear signaling function, in association with the histone acetyltransferase Tip60, following γ-secretase-mediated cleavage of APP (2–4). Although the exact mechanism of APP/Fe65 nuclear signaling is not resolved (5, 6), it remains clear that Fe65 is essential in APP-mediated transcripational regulation. Transcription and mutagenic studies have identified the tyrosine residues in the 685YENPTY697 motif (numbered relative to APP695) within the intracellular domain of APP as the Fe65-binding site (7, 8). Three core domains within Fe65, two PTB domains and one WW domain, mediate the physical associations between APP and other Fe65 interacting factors (1). The carboxyl-terminal PTB domain (or PTB2) is the Fe65 domain that associates with the APP YENPTY motif (1, 7–9) in a phosphotyrosine-independent manner.

Previous work has demonstrated that Fe65 is involved in the regulation of APP processing. The intracellular interaction between APP and the carboxyl-terminal portion of the low density lipoprotein-related protein 1 (LRP) is mediated by Fe65 via its two PTB domains (10). The LRP interaction with APP, mediated by Fe65, may coordinate numerous aspects of APP proteolytic processing, including APP carboxyl-terminal fragment generation and β-amyloid secretion (11). APP is processed in a sequential fashion, in which either α- or β-secretase proteolytic cleavage is a prerequisite for γ-secretase-mediated proteolysis. Consequently, Fe65 regulation of APP proteolytic processing could play a fundamental role in numerous aspects of APP function. Previous work implicating Fe65 in modulation of β-amyloid secretion (10–12) suggests that Fe65 may play a role in proteolytic events associated with AD pathogenesis.

Consistent with the connection between Fe65 and AD etiology, epidemiological studies have suggested that a specific isoform of Fe65 may be protective against late onset sporadic AD. A careful examination of Fe65 isoforms expressed in AD patients and unaffected controls isolated an allele of the Fe65 gene in which a CTA element in intron 13 was deleted adjacent to the splice donor site (13). The deletion results in differential splicing of the nascent Fe65 transcript, leading to an alternative coding sequence at the carboxyl-terminal portion of the Fe65 protein (14). The alternative allele is referred to as the a2 allele and is reported to confer resistance to the late onset form of AD (13–15). Splicing events also generate a second form of variability in Fe65 transcripts. In neuronal cell types, the six nucleotide exon 9 is incorporated into Fe65 transcripts, resulting in the insertion of Arg-Glu into the first PTB domain (16). The role of the a2 and neuronal (exon 9 inclusive) Fe65 isoforms in regulating APP proteolytic processing is explored in this work, which demonstrates that all Fe65 isoforms promote γ-secretase-mediated AICD release, but with the neuronal form of Fe65 being the least efficacious.
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EXPERIMENTAL PROCEDURES

Cell Culture and Transfection Conditions—COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone) and 1 × penicillin/streptomycin (Invitrogen) at 37 °C in 10% CO2. Stock plates of COS7 cells were grown to 50–80% confluence prior to splitting cells for transient transfection experiments. All transactivation assays were done using cells seeded onto 24-well plates. The plates were grown to ~90% confluence prior to transfection. All transfections employed Lipofectamine 2000 (Invitrogen), using 1.5 μl of Lipofectamine 2000 per well and transfection medium supplemented with 10% fetal bovine serum lacking antibiotics. NIH 3T3 cells, HEK293 cells, and JEG3 cells were grown in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium and were transfected using the same methods as employed for COS7 cells. The transfection mix was incubated on the cells for 2–4 h prior to replacement of the transfection medium with fresh growth medium described above.

In all transactivation assays, 1.5–2 μg of DNA was used per well. APPG16 concentrations are defined in each experiment in which titration assays are done. If it is not otherwise specified, then 250 ng/well of APPG16 DNA was used. In all transactivation assays in which Fe65 values are not stated, 1000 ng per well of Fe65 DNA were employed for an APP:Fe65 vector ratio of 1:4. In all experiments with Fe65, the vector preparations used for the transfection were measured and double checked by comparative gel electrophoresis. To ensure that none of the observed effects were because of undetectable variations in vector preparation quality, all experiments were replicated with different preparations of Fe65 vectors. In all experiments, the promoter concentration was held constant by the promoter saturation across conditions. The pFRluc (Invitrogen) promoter present in each condition. In experiments unaccompanied by transactivation assays, the EF2-galactosidase values driven by the identical portion of the EF1α promoter cloned in-frame with Gal4VP16.

The APPG16 M686YENPTYN587 point mutants were generated by the method employed previously (17). Briefly, mutagenic primers were designed with changes in the coding sequence to alter either Tyr686 or Tyr587 to alanine and insert a silent restriction site using the standard QuikChange site-directed mutagenesis approach (Stratagene). The resulting APPG16 Y682A and Y687A vectors were mapped and sequenced to verify the presence of the mutation. The Myc epitope-tagged Fe65 vectors were generously provided by Dr. Qubai Hu and Dr. George Martin. The generation of the pcDNA3.1-Fe65myc vectors has already been described (17), and all of the subclones used the same strategy. The Myc epitope-tagged Fe65 Y682A and Y687A vectors were cloned into pcDNA3.1/Myc-His (+)B at the BamHI and NotI sites (Invitrogen). Untagged versions of these vectors were also generated by leaving the stop codon intact following the subcloning of the cDNA. The amino-terminally truncated Fe65 M260F65327ML vector was also provided by the laboratory of Dr. T. Sudhof and has been described previously (2). The BACE expression vector was obtained from Curagen Corp., in which the human BACE cDNA was PCR-amplified and subcloned into pcDNA3.1. The human presenilin 1 wild-type and FAD mutant expression vectors were described previously (17).

Cleavage Assay, Protein Preparation, and Inhibitor—Luciferase and β-galactosidase assays were performed by standard methods as reported previously (16). The APPG16 protein is cleaved by γ-secretase, liberating the APP AICD-Gal4VP16 moiety to transactivate the pFRluc Gal4-luciferase construct as described previously (17). The cells were lysed on ice 24–48 h post-transfection, and luciferase assays were performed and point to point normalized to β-galactosidase values driven by the EF2-βGal vector. For the assays in which parallel protein concentration levels were assessed, see below for details on protein preparation. All measurements were performed on an EG&G Berthold LB 96V luminometer. The γ-secretase inhibitor DAPT (Sigma) was used at 10 μM, unless otherwise stated, and the cells were treated with inhibitor ~24 h post-transfection.

Western Blots, Aβ ELISA, and Antibodies—Western blots examining protein levels in parallel with transactivation assays used 30 μl per well (90 μl total) of lysates, placed into 180 μl of 1 × RIPA with 1 × proteosome inhibitor mixture (Sigma) or a separate well from the same transfection experiment lysed in front of the coding portion of the cDNA representing the 5’ end of C99, C83, or C50. The 3’ primer used for all of the APP constructs was identical to that used to make APPG16, in which a 5’ XbaI site resides in front of the complementary sequence at the 3’ end of APP695 with the stop codon deleted. Following PCR amplification of C99, C83, and C50, the PCR product was isolated, digested with HindIII and XbaI, and subcloned into the HindIII and XbaI sites of the EF1α-APPG16 vector from which the HindIII-XbaI APP coding cassette had been removed. This resulted in C99, C83, and C50 vectors driven by the identical portion of the EF1α promoter cloned in-frame with Gal4VP16.
1× RIPA supplemented with 1× proteosome inhibitor mixture. The lysates were rocked at 4 °C for 10 min and spun down and prepared for loading. The samples were run on NOVEX 4–12% NuPAGE gradient gels and probed using the rabbit anti-Gal4 DNA binding domain antibody (Calbiochem), the rabbit APP carboxyl-terminal antibody (Cell Signaling), mouse c-Myc antibody (BD Biosciences), and the FE518 Fe65 antibody (14). Western blots employing 6-well plates were transfected and assayed 48 h following transfection. The cells were lysed in 1× RIPA with 1× proteosome inhibitor mixture (Sigma), and prepared as described above. The Aβ40 and Aβ42 sandwich ELISA measurements were performed using the media drawn off of the transiently transfected cells, 48 h post-transfection. The ELISA procedure was performed as reported previously (17).

RESULTS

Fe65 Overexpression Stimulates Liberation of the APP Intracellular Domain—The pivotal role of Fe65 in mediating the interaction between APP and LRP (10, 11) led us to examine its role in the modulation of γ-secretase-mediated proteolysis of APP. It is difficult to quantify the total extent of cleavage of APP by direct biochemical means, because the amino-terminal product of the cleavage is a heterogeneous mix of multiple Aβ-related peptides, and the labile carboxyl-terminal product (AICD) does not accumulate to appreciable levels. Consequently, for many experiments, we employed a system in which cleavage of a carboxyl-terminally modified APP drives transcription of a luciferase reporter plasmid, as we have described previously (17). Preliminary experiments indicated that Fe65 promoted γ-secretase-mediated proteolysis of APP. Consequently, this brought up the question of whether different splice variant products of Fe65 would differ in ability to promote proteolytic liberation of AICD. To assess the role of the neuronal specific exon 9/E9 Arg-Glu insertion into PTB1 domain, we employed Myc-tagged versions of the neuronal and non-neuronal forms of human Fe65 (described under “Experimental Procedures”). Because a second splice variation occurs that alters the carboxyl terminus of Fe65, via incorporation of an alternative exon 14, a Myc-tagged version of the non-neuronal expression vector incorporating the a2 allele was also implemented in this work. Throughout these experiments, a three-way comparison is made between human Fe65 neuronal (E9), Fe65 non-neuronal, and Fe65 non-neuronal a2 to compare the relative efficacy of the PTB1 insertion (neuronal versus non-neuronal) and the a2 allele (non-neuronal versus non-neuronal a2). These vectors were overexpressed in COS7 cells in concert with a titrated range of APPGV16 concentrations which, once cleaved, activates the Gal4-luciferase reporter. Luciferase expression is normalized to constitutively expressed EF2-βGal. This methodology has been described previously (17).

Consistent with previous experiments, we observed that increasing levels of APPGV16 resulted in a plateau in the transactivation of the Gal4-luciferase reporter. All three isoforms of Fe65 substantially increased the proteolytic liberation of AICD-GV16 from the membrane at statistically significant levels (Fig. 1A). However, the three Fe65 isoforms demonstrated significantly different stimulatory capacity, as the neuronal form was considerably less efficacious in promoting cleavage than either the non-neuronal or a2 isoform (Fig. 1A, upper two curves represent the non-neuronal and a2 isoforms). Although in some experiments the a2 allele promoted slightly greater AICD-GV16 liberation than the non-neuronal, this difference was not consistently observed across experiments.

To verify that Fe65 stimulation of APP proteolytic liberation was not because of variation in expression following transfection, a portion of the lysates generated for the transactivation assay was employed for immunoblot analysis. Consistently, APPGV16 expression increased across the vector titration. To obviate small differences that might appear within individual blots, the lysates were run and probed with two separate antibodies to interrogate APPGV16 and Fe65 expression levels (Fig. 1B). We did not observe any difference in APPGV16 expression levels within the different Fe65 isoform transfection conditions. Conversely, we were surprised to see that increasing levels of APPGV16 resulted in a consistent decrement in Fe65 protein.
levels for all isoforms (Fig. 1B, bottom two blots). Although no strict quantitation was performed, it appears that APPGV16 may result in a greater decrement of the Fe65 neuronal form across the titration.

Fe65-mediated Stimulation Is γ-Secretase-mediated—The assay system employed in this work detects release of the AICD-GV16 moiety from the membrane. In previous work, we have shown this to be γ-secretase-dependent. However, the intracellular domain of APP contains a known caspase cleavage site (19–22). To ensure that Fe65 was stimulating proteolytic release of AICD-GV16 through the γ-secretase processing pathway, COS7 cells were transiently transfected with APPGV16 and the Fe65 isoforms in the presence or absence of the γ-secretase inhibitor DAPT. Consistently, all Fe65 isoforms promote statistically significant increases in AICD-GV16 production, and the non-neuronal and a2 isoforms are more efficacious than the neuronal form (Fig. 2). The blockade of transactivation observed in the presence of DAPT confirms that Fe65 stimulation of AICD-GV16 is dependent on γ-secretase, and, consequently, is not likely to be mediated by caspase cleavage. This result was confirmed by comparing effects of transfected Fe65 isoforms on cleavage of AICD-GV16 in wild-type primary mouse embryo fibroblasts and mouse embryo fibroblasts derived from PS1^−/−/PS2^−/− mice. Fe65 isoforms promoted APPGV16-mediated transactivation exclusively in the wild-type cells (data not shown).

Fe65 Stimulation of APP Proteolysis Requires Direct Protein Interaction—Given the demonstrated role of Fe65 in transcriptional regulation (2, 3, 6), we sought to determine whether Fe65 stimulation of γ-secretase-mediated proteolysis was because of direct physical association or through an indirect mechanism. Fe65 association with APP is mediated by the interaction of the phosphotyrosine binding domain (PTB2) with the tyrosine residues within the YENPTY motif (7, 9, 23). Consequently, APP-Gal4VP16 mutants were made that contain alanine substitutions at either of the two tyrosine residues within the YENPTY motif (7, 9, 23). Specifically, APP-Gal4VP16 mutants were made that contain alanine substitutions at either of the two tyrosine residues within the YENPTY motif. The resulting mutations APPGV16 Y682A and Y687A failed to demonstrate statistically significant levels of difference even at the p < 0.01. With APPGV16 Y682A and Y687A the fold stimulation observed with any of the Fe65 isoforms never reached 2-fold.

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FIGURE 2. Fe65 promotes AICD-GV16 release in a γ-secretase-dependent fashion. COS7 cells were transfected with APPGV16 alone or with each of the three Fe65 isoforms. Twenty-four h post-transfection one set of cells per transfection condition were treated with 10 μM DAPT (γ-secretase inhibitor) for 24 h. The Gal4-luciferase data are plotted normalized to constitutive β-galactosidase expression. Unpaired Student’s t test analysis of untreated versus DAPT-treated cells demonstrated a statistically significant difference at p < 0.0005 or less for all conditions. Comparing untreated samples demonstrated statistically significant differences between APPGV16 and Fe65 neuronal (p < 0.0001). The difference between Fe65 neuronal and Fe65 non-neuronal was also statistically significant (p < 0.01). However, there was no significant difference between Fe65 non-neuronal and the a2 isoform. The γ-secretase dependence of Fe65 stimulation is observed in the consistent levels of repression with DAPT treatment. In all conditions, DAPT repressed between 80 and 90% of the Gal4-luciferase signal; the level of repression was slightly higher with co-expression of the Fe65 isoforms (APPGV16 alone, 80%; Fe65 co-expression, 88–89%).

FIGURE 3. Fe65 stimulation of APPGV16 proteolysis is dependent upon direct association. Site-directed mutagenesis of APPGV16 at the tyrosine residues within the YENPTY motif resulted in two alternative APPGV16 constructs, APPGV16 Y682A and APPGV16 Y687A. Wild-type APPGV16, APPGV16 Y682A, and APPGV16 Y687A were transiently transfected into COS7 cells in the presence or absence of the three Fe65 isoforms. All transfection conditions were assayed 48 h post-transfection. Gal4-luciferase values were normalized to constitutive β-galactosidase (βGal) expression. Fold stimulation with all three Fe65 isoforms was consistent with previous observations for wild-type APPGV16, ranging from 4- to 10-fold for the three isoforms. Student’s t test analysis demonstrated that APPGV16 and Fe65 isoforms maintained statistically significant differences at p < 0.0002 or less. However, the APPGV16 Y682A and Y687A mutants failed to demonstrate statistically significant levels of difference even at the p < 0.01. With APPGV16 Y682A and Y687A the fold stimulation observed with any of the Fe65 isoforms never reached 2-fold.
**Fe65 Stimulation of APP Proteolysis Is Dependent on PTB1—**

Based upon the above observation that direct physical interaction between APP and Fe65 is critical for Fe65 enhancement of \( \gamma \)-secretase-mediated proteolysis, we sought to identify the critical Fe65 domains. Fe65 has three functional domains, an amino-terminal WW domain (8) and two carboxyl-terminal PTB domains (9). Consequently, it is possible that Fe65 stimulation of proteolysis occurs by assembling a heterotrimeric or heterotetrameric complex with APP. The Fe65 deletion mutants have been described previously (2). The domain structure of Fe65 and the amino acid positions flanking the three functional domains are diagrammed in Fig. 4A. The region deleted within each of the PTB mutants is diagrammed above the protein, as shown in Fig. 4A, and the position of the WW domain mutation is indicated with an asterisk.

Mapping the critical domains of Fe65 to stimulate APP proteolysis was accomplished by co-transfecting APPGV16 with the Fe65 variants in COS7 cells. The results were normalized as fold stimulation. Consistent with previous observations, full-length Fe65 stimulated an approximate 8-fold increase in proteolysis. Deletion of any of the three core domains of Fe65 decreased its capacity to stimulate APP proteolysis at statistically significant levels (Fig. 4B). However, deletion of the PTB1 domain resulted in the greatest decrease. Indeed, this mutant appears to inhibit, not stimulate, cleavage of APPGV16. The difference between APPGV16 alone and the PTB1 deletion is statistically significant. Consequently, it appears that not only is PTB1 critical to Fe65 function, but lack of it results in a dominant-negative form of the protein with respect to enhancing APP proteolysis. This effect probably reflects interference with the activity of Fe65 natively expressed in COS7 cells. As the two PTB domains of Fe65 are involved in the previously observed linker functions of Fe65 (2, 10), Fe65 stimulation of APP proteolysis may require the assembly of multiprotein complexes. Intriguingly, the deletion of the amino-terminal portion of Fe65 (128–711 variant) resulted in a dramatic and statistically significant increase in the proteolytic release of AICD-GV16 (Fig. 4B). Further truncation of the amino terminus of Fe65 to the beginning of the WW domain reduced the stimulatory effect to a level comparable with full-length Fe65. This prompted us to speculate that a region of Fe65 that can stimulate proteolytic events is normally masked by the amino-terminal portion of the protein, an event that potentially might be regulated in vivo. Similar effects were observed by Sudhof and co-workers (2) for signaling by APP-Gal4. Additional truncation of Fe65 past the WW domain (residues 287–711) resulted in a significant reduction in Fe65 stimulatory efficacy. However, it is interesting to note that truncation of Fe65 down to just the PTB1 domain (residues 287–531) reduces but does not entirely eliminate its ability to stimulate processing of APPGV16.

To ensure that the observed effects were not because of simple differences in level of protein expression, fractions of the lysates used in the transactivation assay were subjected to immunoblot analysis with antibodies against APP and Fe65 (Fig. 4B). The equivalency of plasmid concentrations in Fe65 variants in COS7 cells to assess which domains are requisite for proteolytic stimulation. The three key functional domains of Fe65 are diagrammed (A) with numeric representation of the beginning and end point for each domain. The asterisk above the WW domain refers to site of mutagenesis within the WW domain. The \( \Delta \)PTB1 and \( \Delta \)PTB2 representations diagram the region of each domain deleted in those mutants. B, Gal4-luciferase data were normalized to fold stimulation using the average value of APPGV16 as the referent for each individual value within all conditions. Co-expression with all forms of Fe65 resulted in statistically significant differences from APPGV16 alone (\( p < 0.0001 \)), using Student’s \( t \) tests to perform the analysis. However, overexpression of \( \Delta \)PTB1 repressed AICD-GV16 production at significant levels. Mutations in either the WW domain or PTB2 resulted in significant differences from full-length Fe65 (\( p < 0.003 \) and \( p < 0.0008 \), respectively). Truncation of the amino-terminal region of Fe65 (residues 128–711) resulted in increased stimulation that was statistically significant compared with Fe65 full length (FL) (\( p < 0.0001 \)). Further truncation to the beginning of the WW domain eliminated most of the observed augmentation of stimulation with residues 128–711. Truncations eliminating the WW domain (residues 287–711) or the WW and the carboxyl-terminal region of Fe65 (residues 287–531) reduced the stimulatory capacity of Fe65 at significant levels (\( p < 0.0001 \)). C, Western blots were performed upon the lysates used for the transactivation assay. The top blot is probed with the APP carboxy-terminal antibody. The bottom blot is probed with the FE518 antibody to demonstrate Fe65 protein levels. ARC, acid-rich cluster; PTB, phosphotyrosine binding domain. The M lane refers to control cells that were mock-transfected with the pcDNA3 empty expression vector.

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**FIGURE 4. Mapping Fe65 domains requisite for stimulation of AICD-GV16 liberation.** Fe65 deletion mutants were co-transfected with APPGV16 in COS7 cells to assess which domains are requisite for proteolytic stimulation. The three key functional domains of Fe65 are diagrammed (A) with numeric representation of the beginning and end point for each domain. The asterisk above the WW domain refers to site of mutagenesis within the WW domain. The \( \Delta \)PTB1 and \( \Delta \)PTB2 representations diagram the region of each domain deleted in those mutants. B, Gal4-luciferase data were normalized to fold stimulation using the average value of APPGV16 as the referent for each individual value within all conditions. Co-expression with all forms of Fe65 resulted in statistically significant differences from APPGV16 alone (\( p < 0.0001 \)), using Student’s \( t \) tests to perform the analysis. However, overexpression of \( \Delta \)PTB1 repressed AICD-GV16 production at significant levels. Mutations in either the WW domain or PTB2 resulted in significant differences from full-length Fe65 (\( p < 0.003 \) and \( p < 0.0008 \), respectively). Truncation of the amino-terminal region of Fe65 (residues 128–711) resulted in increased stimulation that was statistically significant compared with Fe65 full length (FL) (\( p < 0.0001 \)). Further truncation to the beginning of the WW domain eliminated most of the observed augmentation of stimulation with residues 128–711. Truncations eliminating the WW domain (residues 287–711) or the WW and the carboxyl-terminal region of Fe65 (residues 287–531) reduced the stimulatory capacity of Fe65 at significant levels (\( p < 0.0001 \)). C, Western blots were performed upon the lysates used for the transactivation assay. The top blot is probed with the APP carboxy-terminal antibody. The bottom blot is probed with the FE518 antibody to demonstrate Fe65 protein levels. ARC, acid-rich cluster; PTB, phosphotyrosine binding domain. The M lane refers to control cells that were mock-transfected with the pcDNA3 empty expression vector.
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apparently reduced expression of the PTB2 mutant in the experiment shown reflects poor efficiency of expression of the plasmid vector or differences in stability or post-translational processing of expressed protein. It should be noted that at longer exposures many of the Fe65 variants did have multiple bands not encountered with the full-length Fe65. Consequently, it is plausible that cleavage products associated with the variants may account for some of the observed differences. However, this is unlikely as the primary protein product of the Fe65 expression vectors was vastly more abundant than the cleavage products observed.

Equivalency of Full-length Fe65 and Fe65 Derived from an Alternative Start Site—The Fe65 knock-out mouse generated by Martin and co-workers (24) was determined to be a hypomorphic mutant as a shorter Fe65 protein product was still expressed. The targeting strategy employed a deletion of exon 2 of the murine Fe65 gene that contains the start codon. Another start site that is in-frame and outside of the exon 2 targeted region occurred at position Met260. Consequently, we sought to test the functional efficacy of the protein resulting from the alternative start site relative to the full-length Fe65 protein. The truncation product associated with the alternative start site maps to the beginning portion of the WW domain. However, it is unclear whether this partial truncation is sufficient to destroy the interaction capacity of the WW domain as Met260 residues after the amino-terminal tryptophan, but two tryptophan residues remain within the WW domain carboxyl terminus to the alternative start site. Interestingly, we observe no difference between effects of full-length Fe65 or M260Fe65M327L on APPG16 proteolysis (Fig. 5). This result is consistent with our antecedent domain mapping studies, as there was little difference between full-length Fe65 and the truncated 242–711 Fe65 (the truncation mutation that preserved the WW domain).

Isoform Difference Is Not Dependent on Protein Concentration—APPG16 may result in a decrement in expression of all three Fe65 isoforms, in which the neuronal form of Fe65 is impacted most profoundly (Fig. 1). To assess whether this observed decrement in protein levels is responsible for the differential efficacy of the Fe65 isoforms in stimulating APP proteolysis, APPG16 expression vector levels were held constant, and the expression vector for each isoform of Fe65 was titrated from 0 to a 10-fold excess relative to APPG16. Fe65 protein concentrations, as assessed by immunoblot analysis, increased almost linearly with increasing quantity of transfected plasmid. However, the stimulation of APPG16-dependent luciferase transcription (measured from the same experiment as the immunoblot analysis) plateaued near a 1:3 ratio of expression vectors (100 ng/well of APPG16 DNA was used) (Fig. 6). The linear increase in protein levels of neuronal, non-neuronal, and a2 Fe65 variants across the titration appeared equivalent despite the difference in transactivation observed. This indicates that the differences in the ability of the Fe65 isoform to promote proteolytic cleavage of APP are not because of protein concentration effects.

Fe65 Stimulation of APP Proteolysis Is Consistent across Heterologous Cell Types—To assess whether Fe65 stimulation of APP proteolysis is a general phenomenon, titration experiments were performed in NIH 3T3, HEK293, and JEG3 cells. The titration experiments were performed in which either Fe65 was incrementally introduced into cells expressing a fixed amount of APPG16, or APPG16 was titrated into cells expressing a fixed amount of each of the Fe65 isoforms. These experiments demonstrated that titrating in Fe65 consistently increased APPG16 proteolysis in each cell type (supplemental Fig. S1, A, C, and E). In these experiments 100 ng of the APPG16 expression vector was employed, corresponding to early plateau values of reporter activity in previous APPG16 titration experiments (Fig. 1). Consistent with the observation in COS7 cells, the Fe65 neuronal form increased the level of transactivation the least, ~2–3-fold in all three cell types. Concordantly, the non-neuronal and the a2 isoform increased AICD release to a much greater extent, up to 8-fold in NIH 3T3 cells. The overall extent of stimulation did vary between cell types, with NIH 3T3 cells and HEK293 cells showing the highest levels of stimulation. Interestingly, the a2 isoform resulted in marginally higher levels of AICD release than the non-neuronal isoform in all three cell types. In the experiments employing APPG16 titration with fixed levels of Fe65 vector employed in each transfection, the same pattern emerged with an attenuated difference between the non-neuronal and a2 isoforms (supplemental Fig. S1, B, D, and F). Again, there was a greater difference between isoforms observed in the NIH 3T3 and HEK293 cells than in JEG3 cells, suggesting some potential cell type specificity to the potency of the Fe65 isoforms. Yet, in

FIGURE 5. Comparison of full-length p97Fe65 and M260Fe65M327L. APPG16 was transfected into COS7 cells alone or co-transfected with either full-length human neuronal Fe65 (designated p97Fe65) or M260Fe65M327L. The M260Fe65M327L is a truncation mutation mapping to an alternative start site resident within Fe65. The additional M327L mutation was added as overexpression of the truncated version of Fe65 resulted in protein species initiating at the Met260 site. To avoid the generation of biologically unobserved Fe65 protein species, Met260 was mutated to examine exclusively the Fe65 variant starting at Met260. The alternative start site abolishes the first portion of the WW domain but preserves the Trp residue mutated in the previous experiment (Fig. 4). Both forms of Fe65 elicited statistically significant differences in proteolysis compared with APPG16 alone at p < 0.0002 or less by Student’s t test. However, the two co-expressed forms of Fe65 demonstrated no statistical difference in stimulating AICD production. Induced Gal4-luciferase values were normalized to constitutive β-galactosidase expression.
both sets of experiments it was observed that Fe65 promotes the proteolytic release of the AICD fragment, suggesting that the role of Fe65 in stimulating APP proteolysis is a cell type independent phenomenon.

**Fe65 Stimulates Precursor Formation and γ-Secretase-mediated APP Proteolysis**—Previous work has suggested that Fe65 may positively regulate both APPα secretion as well as Aβ production (12, 25). Furthermore, LRP association with APP, mediated by Fe65, regulates proteolytic processing and AICD generation (10, 11). This suggests that Fe65 could stimulate γ-secretase-mediated liberation of AICD-GV16 by promoting processing of APP by α- and/or β-secretase, thereby increasing the concentration of substrate for γ-secretase-mediated cleavage. Additionally, it has been shown that Fe65 can both stabilize and target the γ-secretase cleaved AICD to the nucleus (26, 27). Consequently, we sought to explore whether Fe65 stimulation occurs by increasing γ-secretase substrate production, directly stimulating γ-secretase, or through stabilization of the released AICD-GV16 moiety. To accomplish this, we generated expression vectors coding for α- or β-secretase APPGV16 cleavage products, C99-GV16 and C83-GV16. The C50-GV16 γ-secretase cleavage product was also generated to test the Fe65 stabilization effects. These constructs were transfected into COS7 cells either alone or in the presence of the Fe65 isoforms.

The results indicate that Fe65 may play a role at each level of regulation of AICD generation. The most robust stimulation of AICD-GV16 production was observed with the full-length APPGV16 expression vector. Here, the neuronal form produced an approximate 4-fold increase in proteolysis, whereas the non-neuronal and a2 forms stimulated an approximate 7–8-fold increase (Fig. 7A). The augmented stimulation achieved by the non-neuronal and a2 forms of Fe65 was significantly higher than the neuronal form. Fe65-mediated stimulation of AICD-GV16 mobilization was also observed with the immediate substrates for γ-secretase, as the three Fe65 isoforms produced a 3–4-fold increase in proteolysis with both C99-GV16 and C83-GV16. However, the Fe65 isoform dependence in proteolytic stimulation is dramatically attenuated with C99-GV16 and C83-GV16 (Fig. 7A). Although the action of the three isoforms is statistically significantly different with the APP holoprotein, there is no statistical difference between any of the Fe65 isoforms with C99-GV16 or C83-GV16. This further supports the notion that the observed differences in Fe65 stimulation with the holoprotein are not because of small differences in Fe65 protein abundance, as there were identical amounts of Fe65 expression vector co-transfected with the holo-APPGV16, C99-GV16, and C83-GV16. Additionally, there was a small increase in C50-GV16 transactivation with all three Fe65 isoforms, consistent with its previously reported role in AICD stabilization. However, this is only slightly above the nonspecific stimulation observed with the GV16 expression vector. Consequently, it appears that Fe65 may play a role in regulating both production of the immediate substrates of γ-secretase, by promoting α-secretase or β-secretase-mediated cleavage of APP, and by directly stimulating γ-secretase-mediated cleavage of those substrates.

To verify that the results observed do not reflect differences in efficiency of expression of different constructs, protein levels were assessed by Western blotting. The Gal4 antibody was employed to visualize the APPGV16 species, and the Myc antibody was used to elucidate Fe65 expression levels represented within the transactivation experiment (Fig. 7B). Immunoblots with anti-Gal4 demonstrated equivalent levels of APPGV16, C99-GV16, and C83-GV16 across conditions. The observed levels of C50-GV16 were less consistent, likely because of the instability of the γ-cleaved C50 product. Fe65 expression levels also appeared roughly equivalent within the APPGV16 and C99-GV16 conditions, with perhaps slightly less of the Fe65 neuronal form. Interestingly, overexpression of C83-GV16 appeared to diminish the level of expression of Fe65. However, the remaining level of expression of Fe65 is apparently still sufficient to maximally promote C83-GV16 cleavage, as revealed by titrating the amount of Fe65 plasmid transfected (Fig. 6). It is unclear whether the apparent decrement of Fe65 expression resulting from expression of C50-GV16 and GV16 is meaningful. This difference may simply reflect variability in the immunoblot procedure, as the C50-GV16 and GV16 samples were run on a discrete gel from the APPGV16, C99-GV16 and C83-GV16 samples. Intriguingly, in the holo-APPGV16 and C99-GV16 transfected cells, a smaller Fe65 protein fragment
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Fe65 Stimulates Release of the APP Intracellular Domain Independent of Amyloid Production—Because Fe65 appears to promote processing of APP at the level of cleavage by α- and β-secretase and promotes the cleavage of the products of those reactions by γ-secretase, we sought to explore the role of Fe65 in the regulation of Aβ40 and Aβ42 production. COS7 cells were transfected with APPGv16 in the presence or absence of the Fe65 isoforms. Because of the lack of C99-GV16 build up with DAPT (Fig. 8), we assumed that endogenous β-secretase activity is low in COS7 cells. Consequently, BACE was co-transfected to facilitate an examination of the role of Fe65 in amyloid generation. As observed in the previous experiments, Fe65 markedly promoted AICD-GV16-mediated transactivation in an isoform-dependent manner (Fig. 9B). However, in contrast, using the media from the same cells analyzed in the transactivation assay, Fe65 induced only a small statistically insignificant
increase in Aβ40 levels but a consistent decrease in Aβ42 levels (Fig. 9A, right axes). The decrement in Aβ42 levels is statistically significant with the non-neuronal and a2 isoforms of Fe65. The lack of statistical significance with the neuronal isoform of Fe65 results from increased variance within the test set, although the mean Aβ42 level was still lower with Fe65.

Immunoblot analysis with anti-Gal4 revealed that the level of expression of APPGV16 was consistent across the transfection conditions (Fig. 9C). Consequently, the Fe65-mediated reduction in Aβ42 production is not because of variation in APPGV16 expression. Expression levels of Fe65 also were similar across conditions. In accordance with previous observations, there is marginally less of the neuronal form of Fe65 observed at the protein level.

**DISCUSSION**

Fe65 is implicated in regulating numerous aspects of APP proteolytic processing and function, from Aβ secretion to nuclear signaling. In this study, we extend the previously ascribed roles for Fe65 in proteolytic regulation of APP to include the promotion of liberation of the AICD. A primary focus of this work is an exploration of the differences in efficacy of different Fe65 isoforms that result from allelic variation of the gene and from alternative splicing. In neuronal systems alternative splicing incorporates the six-nucleotide exon 9 encoding Arg-Glu into the nascent transcript (16). The Arg-Glu insertion occurs within the middle region of the PTB1 domain of Fe65. The functional significance of the difference between the neuronal E9 inclusive and the non-neuronal forms of Fe65 has remained enigmatic. Brains of AD patients examined post-mortem revealed that the neuronal form of Fe65 was down-regulated in degenerated regions of the brain, whereas both neuronal and non-neuronal isoforms were up-regulated in unaffected areas of the brain (28). This suggests that Fe65 may be protective against the onset of AD pathogenesis.

An allelic variant of Fe65, known as the a2 isoform, was identified as a polymorphism that conferred resistance to late onset dementia of Alzheimer-type (13). This conclusion has been disputed by some groups (29–31) and confirmed by another working with a more age-advanced population (15). The polymorphism falls within the splice donor site of intron 13 and leads to splicing to an alternative splice acceptor site, resulting in a complete change in the coding sequence for the penultimate carboxyl-terminal portion of the Fe65 protein (14).

This study compared the function of the neuronal, non-neuronal, and the non-neuronal a2 forms of Fe65. The consistent and compelling observation is that the non-neuronal and non-neuronal a2 isoforms have substantially greater ability to pro-
expression vector to drive with each of the Fe65 isoforms. All cells were co-transfected with APPGV16 alone or co-transfected which time the cells the media were drawn off each sample, and 100 ng/ml Aβ40 and 0.15 ng/ml Aβ42 were added to two wells within a plate pre-prepared with the 6E10 amyloid cap-standards run in association with the assay. A detection antibody. One well was used for the A

FIGURE 9. Fe65 differentially stimulates AICD and Aβ production. COS7 cells were transiently transfected with APPGV16 alone or co-transfected with each of the Fe65 isoforms. All cells were co-transfected with the BACE expression vector to drive β-amyloid production. Eighteen h following transfection, the media were changed, and 300 µl of fresh media was added to each well. The cultures were maintained for an additional 36 h, following which time the cells the media were drawn off each sample, and 100 µl were added to two wells within a plate pre-prepared with the 6E10 amyloid capture antibody. One well was used for the Aβ40 and the other for the Aβ42 amyloid assay. The remainder of the cells were then washed, lysed, and analyzed by both the transactivation assay and Western blot. A, β-amyloid sandwich ELISA was performed as described under "Experimental Procedures." Detectable levels of Aβ40 and Aβ42 were found in all samples, plotted relative to the Aβ40 values (left axis) or Aβ42 values (right axis). The values were normalized to the linear regression generated from the A

FIGURE 10. Fe65 stimulates AICD-GV16 production in wild-type and FAD mutant PS1. APPGV16 was transiently transfected into COS7 cells in the presence or absence of neuronal Fe65. CMV-PS1 expression vectors containing the wild-type or FAD mutant (M146L and C410Y) human cDNA sequences were co-transfected. Two concentrations of APPGV16 were used within each PS1 set. The low and high concentrations of APPGV16 were 50 and 250 ng of expression vector per well, respectively. The ratio of APPGV16:Fe65 was maintained at a 1:4 ratio across conditions. Levels of AICD-GV16 production were elevated in wild-type relative to FAD mutant PS1 within all conditions examined. PS1 wild-type (wt) was significantly different from M146L and C410Y in low and high concentrations of APPGV16, and in the presence and absence of Fe65 (p < 0.005; condition to condition analysis with Student’s t test). Fe65 induced increases in AICD-GV16 production with both PS1 wild-type and FAD mutant transfected cells. However, the fold stimulation with Fe65 was indistinguishable between wild-type and FAD mutant conditions (low APPGV16 concentration, 4.1-fold (wild type), 3.6-fold (M146L), 3.6-fold (C410Y); high APPGV16 concentration, 3.0-fold (wild type), 3.4-fold (M146L), and 3.0-fold (C410Y)).

In Aβ42 levels between APPGV16 alone and co-expression of Fe65 non-neuronal (p < 0.006) and a2 (p < 0.03) were statistically significant. B, transactiva-

mote APP processing than the neuronal form, whereas the activity of the non-neuronal and non-neuronal a2 isoforms is similar. The differences observed experimentally do not result from differences in the nature or quality of plasmid DNA employed, because the plasmids do not differ significantly except for the coding region inserted (14), and the results were obtained with numerous different plasmid preparations, each of which was carefully characterized with regard to purity and concentration. The majority of the experiments discussed here were performed in COS7 cells; however, similar results were obtained with NIH 3T3, HEK293, and JEG3 cells (supplemental Fig. S1). The use of neuronal cell types was explored, however, as previously noted endogenous expression of Fe65 is greatly elevated in neuronal systems (28); consequently, endogenous Fe65 masked the effects of transfected Fe65.
Previous work with the APPGV16 reporter system has demonstrated that APPGV16-mediated transcriptional activation is dependent upon α-, β-, and γ-secretase proteolytic processing of APP (17). Consistent with this conclusion, this study demonstrates that Fe65-mediated stimulation of this reporter system requires γ-secretase action, as DAPT blocks stimulation of transactivation by Fe65 (Fig. 2). Consequently, it is unlikely that the Fe65 induction of reporter activity is because of non-specific transcriptional effects. Furthermore, as DAPT blocks the Fe65 augmentation of APPGV16 reporter activity, it is unlikely that other proteolytic events associated with the APP carboxyl terminus, such as caspase cleavage (20, 32), are responsible for the Fe65-mediated effects observed.

The stimulation of AICD liberation by Fe65 apparently reflects both increased rate of γ-secretase-mediated cleavage of its immediate substrates (C83 and C99) as well as increased rate of production of those substrates via cleavage of APP by α- and β-secretases. The latter effect apparently predominates, because Fe65 promotes liberation of AICD from APPGV16 more strongly than from C83-GV16 or C99-GV16 (Fig. 7). The ability of Fe65 to promote formation of such γ-secretase substrates was also demonstrated directly, as coordinate expression of APPGV16 and the three Fe65 isoforms in the presence of the γ-secretase inhibitor DAPT shows elevated levels of C83-GV16 accumulation with the non-neuronal and α2 isoforms (Fig. 8A). We observed that all three Fe65 isoforms promoted liberation of AICD from both C83-GV16 and C99-GV16. These observations are consistent with previous studies showing that other members of the Fe65 gene family directly stimulate γ-secretase activity (33). Therefore, Fe65 may promote a complex series of proteolytic events in which both steps prior to Fe65 liberation from the membrane are fostered by its association with APP.

Various studies have concluded that Fe65 either inhibits or promotes secretion of APPα-secretase (10, 12, 18). The results of our analysis of AICD production indicate that Fe65 promotes cleavage of APP by α-secretase, a conclusion that implies that Fe65 should increase rather than decrease production of APPα-secretase. We did not attempt to assess the effects of Fe65 overexpression on secretion of APPα-secretase. However, we did observe that Fe65 overexpression increased the quantity of a band consistent in size with APPα-secretase in cellular fractions (Fig. 8B), suggesting that Fe65 may stimulate α-secretase-mediated cleavage of APP within the Golgi or within endocytic compartments, both sites in which APP processing has been described (34–38). Indeed, variation among cell types in the predominant locus of APPα-secretase (plasma membrane, endosome, or trans-Golgi) might account for the disagreement in published studies concerning whether Fe65 promotes or inhibits secretion of APPα-secretase.

Mutations of either tyrosine residue within the YENPTY motif of the Fe65-binding site of APP abolished stimulation of APP proteolysis (Fig. 3) by all three Fe65 isoforms. Although mutagenic scanning has identified the amino-terminal tyrosine as critical to the association of Fe65 with APP (7), the NPTY forms the core of the PTB domain recognition sequence (1, 8, 39). The YENPTY motif is highly conserved, and hence perturbations would be expected to disrupt associations between binding partners. Mutation of the critical APP-binding site abrogates the Fe65-mediated stimulation, suggesting that direct association is important. Although the association of Fe65 with APP is apparently important for Fe65-mediated effects on APP processing, the affinity of that interaction does not appear to be a major determinant of the effectiveness of the interaction, because the Fe65 α2 isoform is known to associate more weakly with APP than the non-α2 alleles of Fe65 (14); yet the α2 allele stimulates APP proteolysis with equivalent or greater efficacy to the non-neuronal and neuronal isoforms.

Our domain mapping studies demonstrate that all three core domains of Fe65 play a function in stimulating APP proteolysis (Fig. 4). Deletion of PTB1 has the most profound effect upon APP processing, as deletion of PTB1 converts Fe65 from an activator to an inhibitor of APP processing, possibly by acting as a dominant-negative inhibitor of the action of endogenous Fe65. The PTB1 domain of Fe65 mediates association with several binding partners, including LRP (10), Tip60 (2), Tau (40), and CP2/LSF/LBP1 (41), suggesting that the association of one or more of these proteins with Fe65 is necessary for stimulation of APP processing. Interestingly, the neuronal form of Fe65 inserts the residues Arg-Glu into PTB1. This suggests that the differing effects of non-neuronal and neuronal Fe65 on APP processing reflect differing association with one of the aforementioned binding partners. LRP is likely to be the differentially associating binding partner, as association of LRP with the APP-Fe65 complex is known to promote APP processing (10, 11).

Deletion of the PTB2 domain of Fe65, which mediates association of Fe65 with APP (1, 7, 9), results in only a partial loss of Fe65 function. This surprising result is consistent with other studies demonstrating that both Fe65 and Fe65 lacking PTB2 promote APPα-secretase secretion (10). This suggests the possibility that PTB1 may contribute weakly to the association of Fe65 with the YENPTY motif of APP.

Selective mutations to the WW domain that blocks its functionality (2), or deletion of the amino-terminal portion of Fe65 containing the WW domain, also attenuate the Fe65 stimulation of APP proteolysis. Surprisingly, we repeatedly observed small residual levels of stimulation with the Fe65 truncation mutants in which only the PTB1 domain remained. Although the mechanism of this effect requires further investigation, the effect underscores the critical role of PTB1 in Fe65 function.

Truncation of Fe65 amino-terminal to the recently identified acidic residue cluster (ARC) domain (18) enhances Fe65 stimulation of APP proteolysis (Fig. 4). Intriguingly, further truncation of the amino-terminal region past the ARC domain but prior to the WW domain, reduces Fe65 stimulatory capacity to levels similar to the full-length protein. This suggests that the most amino-terminal portion of Fe65 masks the augmentative role of the ARCl domain. Consistent with this notion, it was recently noted that alternative start sites for Fe65 drive the formation of an amino-terminally truncated version of Fe65 in the partial Fe65 knock-out mouse (24). This start site maps to residue 260, leaving the majority of the WW domain intact, including two tryptophan residues. Overexpression of Fe65 from the alternative start site demonstrates indistinguishable levels of APPGV16 proteolysis from the full-length Fe65 protein (Fig. 5), consistent with the deletion studies.
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There have been numerous reports of Fe65 modulation of β-amyloid secretion. However, both positive (12) and negative effects (42) have been reported. In this study, we find that all three isoforms decreased secretion of pathogenic Aβ42 in concert with increased production of AICD-GV16 (Fig. 9, A and B). It is curious to note that none of the Fe65 isoforms significantly affect Aβ40 levels. However, this result is reminiscent of the findings of our previous work, in which we showed that mutation of the APP S3 cleavage site resulted in decreased liberation of AICD-GV16 accompanied by increased production of Aβ42, whereas Aβ40 levels were unaffected (17). As Fe65 increases AICD production, we speculated that overexpression of Fe65 might partially rescue the loss of function observed with PS1 mutants. Although overexpression of Fe65 in this study did increase AICD-GV16 liberation via FAD mutant PS1, the Fe65-mediated fold stimulation of APPG16 processing was the same with both wild-type and FAD mutant PS1 (Fig. 10). Consequently, Fe65 is unlikely to be a useful means to restore the loss of function associated with the PS1 FAD mutants.

The significance of our finding that Fe65 isoforms differ in their ability to promote APP cleavage is due in part to the characterized role of Fe65 as a nuclear signaling moiety. It was originally suggested that transcriptional regulation was mediated by nuclear accumulation of a tripartite complex of AICD-Fe65-Tip60 (2, 3). Recent work has drawn into doubt the necessity of either the AICD or Tip60 for functional nuclear signaling, suggesting that Fe65 alone is sufficient for transcriptional initiation (6). The observation that hold up, prior to γ-secretase-mediated cleavage, functions to sequester Fe65 from the nucleus is widely recognized (2, 6, 43). Consequently, the liberation of Fe65 from the cytosolic face of the membrane may be necessary for Fe65 nuclear signaling, whether it functions in a complex with AICD or independently. Although there is little doubt that Fe65 plays a transcriptional regulatory role, as deletion of Fe65 in mice negatively impacts both neurodevelopment and memory formation (24, 44), which genes are actively regulated remains a hotly contended issue (3, 45–49).

The role of Fe65 in stimulating APP processing at multiple cleavage steps suggests a tight coupling between the nuclear signaling function of Fe65 and its role in proteolytic regulation of APP. The biological significance of the finding that the Fe65 isoform specific to neuronal systems promotes APP processing less effectively than other Fe65 isoforms is unclear. Possibly the lower activity of the neuronal form counter-balances the much greater level of Fe65 protein expressed in neurons (16). Our results indicate that Fe65 stimulates cleavage of APP by α-secretase and decreases pathogenic amyloid production. Because stimulation of α-secretase-mediated cleavage of APP and Aβ42 suppression are both speculative venues for AD therapeutics, the mechanism involved in Fe65 regulation of APP proteolysis may provide insights into novel therapeutic approaches to AD.

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