Dissection of Amyloid-β Precursor Protein-dependent Transcriptional Transactivation*‡

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Amyloid-β precursor protein (APP) forms a transcriptionally active complex with the adaptor protein Fe65 and the histone acetyltransferase Tip60, but the mechanism of transcriptional activation that is mediated by APP and Fe65 remains unclear. APP is cleaved by γ-secretase similar to Notch, whose intracellular domain activates transcription by interacting with nuclear translocation factors. To test whether the APP intracellular domain (AICD) functions analogously, we investigated how APP and Fe65 transactivate a Gal4 fusion protein of Tip60. Consistent with the Notch paradigm, we observe that γ-cleavage of APP and nuclear translocation of Fe65 are required for transactivation. Surprisingly, however, we find that nuclear translocation of the AICD may be dispensable and that only membrane-tethered AICD (i.e. AICD coupled to a transmembrane region) and not free AICD (i.e. soluble AICD) is a potent transactivator of transcription. Membrane-tethered AICD recruits Fe65 and mediates the activation of bound Fe65 that is then released for nuclear translocation by γ-cleavage together with the AICD. Our data suggest that transcriptional transactivation by APP and Notch may involve distinct mechanisms; whereas the Notch intracellular domain directly functions in the nucleus, the AICD acts indirectly by activating Fe65.

The amyloid-β precursor protein (APP) is a ubiquitous type I membrane protein with a large extracellular domain, a single transmembrane region (TMR), and a short cytoplasmic tail (reviewed in Refs. 1 and 2). Under physiological conditions, APP is processed by proteolytic cleavage in a series of reactions that are carried out by enzymes referred to as secretases. α- and β-secretases initially sever APP just outside of the TMR, releasing virtually all of the extracellular APP domains. Subsequently, γ-secretase cuts in the middle of the TMR, generating small hydrophobic extracellular peptides, including the Aβ peptide that is believed to be the major neurodegenerative agent in Alzheimer’s disease (AD) (reviewed in Ref. 3). γ-Secretase also cleaves APP at a site three residues inside the cytoplasmic membrane boundary (called ε-cleavage (4)). γ- and ε-cleavage produces an intracellular fragment named the APP intracellular domain (AICD) that contains a few amino acids from the TMR followed by the cytoplasmic tail of APP.

In addition to APP, mammals express two closely related proteins called amyloid precursor-like proteins 1 and 2 (APLP1 (5) and APLP2 (6)), both of which are also proteolytically processed similar to APP (7–9). Parts of APP, especially its cytoplasmic sequence, are conserved throughout the human APP gene is well established (reviewed in Ref. 3), the normal function of APP and its homologues remains elusive, and roles in axonal transport, neurite outgrowth, neuronal survival, synapse formation, and gene regulation were suggested (reviewed in Refs. 1 and 2). Mice deficient in either APP (10), APLP1 (11), or APLP2 (12) are viable. In contrast, mice lacking both APP and APLP2 or both APLP1 and APLP2 die shortly after birth without obvious histopathological abnormalities (11), suggesting that the APP family has an important function in mammals.

The sequential proteolytic processing of APP is strikingly similar to that of Notch, a cell surface receptor essential for cell fate determination (reviewed in Ref. 2). Upon ligand binding, Notch undergoes ectodomain shedding followed by intramembrane cleavage by γ-secretase. This results in the release of the Notch intracellular domain (NICD) that enters the nucleus where it directly participates in transcriptional activation. The resemblance of the serial processing of Notch and APP prompted us to test whether the AICD is also involved in regulating gene transcription. Indeed, we found that the cytoplasmic tail of APP forms a tripartite complex with the multi-domain adaptor protein Fe65 and the histone acetyltransferase Tip60 (13). When Tip60 is fused to the DNA-binding domain of yeast transcription factor Gal4, it cannot induce expression of a Gal4-dependent reporter gene, suggesting that Tip60, although a nuclear protein that is part of a DNA-binding protein complex (14), is transcriptionally inactive. Co-expression of either APP or Fe65 with Gal4-Tip60 also fails to activate reporter gene expression. However, co-expression of both APP and Fe65 with Gal4-Tip60 leads to dramatically enhanced expression of the reporter gene, indicating that APP and Fe65 may play a role in activating gene transcription (13).

In suggesting a role for APP in transcription, these results raised several questions. For instance, is intramembrane cleavage of APP required for transactivation? Do the AICD and Fe65 translocate into the nucleus to activate gene expression?
do APP and Fe65 activate transcription? In the present study, we investigated the mechanistic basis of the transcriptional function of APP and Fe65. We found that consistent with the Notch paradigm, γ-secretase of APP and nuclear translocation of Fe65 are required for transcriptional activation. Surprisingly, however, we show that APP acts primarily outside of the nucleus as a membrane-tethered recruiting device for Fe65. The AICD can only potently transactivate Gal4-Tip60 in the presence of Fe65 when the AICD is bound to a membrane, either in the context of APP or of another membrane protein that is a γ-secretase substrate. We postulate that membrane recruitment of Fe65 is essential to activate its transcriptional function by an unknown membrane-associated process and that subsequent γ-secretase of APP is required to release Fe65 for nuclear translocation. Our data thus suggest that although APP and Notch are both cleaved by γ-secretase and can mediate transcriptional activation, they follow distinct mechanistic paradigms.

MATERIALS AND METHODS

Plasmids Used for the Study

Standard Plasmids

pG5E1B-Luc, pcMV-LacZ, and pM-hTip60 have been described previously (13).

APP Plasmids

**Mammalian Expression Plasmids**—pCMV5-APP encodes full-length human APP695 inserted into the blunted EcoRI/XbaI sites of pCMV5. pCMV5-APP-NES encodes APP-NES. APP cDNA without a stop codon was inserted into the blunt Clal site of pCMV5, then oligonucleotides XC154 (AGCTTACGCTAGCCGCACCTGCGGCTTTCAAGGCTA) were annealed and cloned into the HindIII/XbaI sites. The sequence of NES is derived from protein kinase inhibitor (APPct, residues 652–695). pCMV5-APP R672A, pCMV5-APP Q678A, and pCMV5-APP N680A are in the pcI-neo vector (Promega). These six mutants are gifts from Dr. Edward H. Koo. They are based on APP731, but the numbering is based on APP695 for easy comparison with the APP695-based mutants.

**Bacterial Expression Plasmids**—pGEX-4T-1-APPct is SGLRSIPPG-(APP695 652–695) into the NheI site of pGEX-4T-1 (Amersham Biosciences). The linker between GST and APP is GSPEFPGIPPG. pGEX-4T-1-APPct* is generated by cloning the DNA fragment encoding APPct* (with NPTY to NATA mutation) into EcoRI/SalI sites of pGEX-4T-1 (Amersham Biosciences). The linker between GST and APP is GSPEFPGIPP.

**Fe65 Plasmids**

**Mammalian Expression Plasmids**—pCMV5-Fe65 encodes full-length rat Fe65 (711 residues). This plasmid has been described previously (13). pDNA3-N-Myc-Fe65 encodes Myc-Fe65, generated by cloning rat full-length Fe65 cDNA (residues 1–711) into the NheI site of pDNA3-N-Myc-Fe65. pCMV5-APP 1–59 HA-Fe65 encodes HA-Fe65, generated by cloning rat full-length Fe65 cDNA (resides 2–711) into the blunt SalI/XbaI sites of pCMV5-APP. pDNA3-N-Myc-Fe65 encodes NES-Fe65. Oligonucleotides XC148 (CGATGCCCTACAGAAGAAGCTGGAAGAACTGGAACTGGCA) were annealed and inserted into the Clal site of pDNA3-N-Myc-Fe65. The sequence of NES, which is derived from mitogen-activated protein kinase/extracellular signal-regulated kinase inhibitor, is ALAKKLEELDE. The linker between NES and Fe65 is FIDIEFRL. pCMV5-Fe65 encodes full-length Fe65. The linker between NES and Fe65 is FIDIEFRL. pCMVMyc-Fe65 encodes Myc-Fe65.

**Bacterial Expression Plasmids**—pGEX-4T-1-Fe65 encodes FIDIEFRL. pcDNA3-N-Myc-mNES-Fe65 encodes mNES-Fe65, generated by cloning the DNA fragment encoding Fe65 into the EcoRI/XbaI sites of pDNA3-N-Myc-Fe65. The linker between GST and Fe65 is GSPEFPGIPP.
**Bacterial Expression Plasmids**—pGEX-KG-Fe65(1–313) encodes GST-Fe65 \(^{1-313}\) generated by subcloning the DNA fragment encoding residues 1–313 of rat Fe65 into the M1ul site of pGEX-KG. pGEX-KG-Fe65(238–303) encodes GST-Fe65/WW. The DNA fragment encoding residues 238–303 of rat Fe65 was PCR-amplified and inserted into the EcoRI/HindIII sites of pGEX-KG. pGEX-KG-Fe65(238–303/\(mW4\) encodes GST-Fe65/WW*.

The DNA fragment encoding residues 298–303 of rat Fe65 was PCR-amplified and inserted into the EcoRI/HindIII sites of pGEX-KG. pGEX-KG-Fe65(287–533) encodes GST-Fe65/PTB1. The DNA fragment encoding residues 287–533 of rat Fe65 was inserted into the EcoRI site of pGEX-KG. pMAL-C2-Fe65(339–711) encodes MBP-Fe65/PTB2. The DNA fragment encoding residues 339–711 of rat Fe65 was inserted into the blunted EcoRI/HindIII sites of pMAL-C2. pMAL-C2-Fe65(287/303) encodes GST-Fe65/WW*. The DNA fragment encoding residues 238–303 of rat Fe65 was inserted into the BamHI site of pGEX-KG. pGEX-KG-Fe65(287–533) encodes GST-Fe65/PTB1.

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**Gal4-Tip60 Transactivation Assays**

Gal4-Tip60 transactivation assays were performed with COS, HeLa, and HEK293 cells essentially as described (13). The data shown are corrected for transfection efficiency using a co-transfected constitutively expressed \(\beta\)-galactosidase vector and are further normalized to the transactivation observed either with Gal4-Tip60 alone (expressed as Fold over Gal4-Tip60) or with both Gal4-Tip60 and Fe65 (expressed as Fold over Gal4-Tip60/Fe65). The cells were transfected with the \(\gamma\)-secretase inhibitors inhibitor IV and DAPT (Calbiochem) for 20–24 h prior to harvesting. For experiments performed in mouse embryonic fibroblasts, LipofectAMINE Plus (Invitrogen) was used as the transfection reagent to achieve better transfection efficiency.

**Immunocytochemistry**

HeLa cells plated on cover glass in a 12-well plate were transfected with 0.2 \(\mu\)g of plasmid encoding Myc-tagged Fe65 or GFP-APP fusion proteins using FuGENE 6. Two days after transfection, the cells were washed twice with PBS, fixed with 3.7% formaldehyde for 15 min at 4 °C, then blocked, and permeabilized in the blocking solution (PBS containing 3% goat serum and 0.2% Triton X-100) for 30 min at room temperature. The fixed cells were incubated with anti-Myc monoclonal antibody (9E10; Santa Cruz Biotechnology; 1:200 dilution) or anti-GFP polyclonal antibody (T3743; 1:500 dilution) for 1 h. After three washes with PBS and one wash with water, the cells were mounted with Vectashield (Vector Laboratories) and examined with a Bio-Rad confocal microscope.

**Immunoblotting, Co-immunoprecipitation, and Pull-down Experiments**

COS7 cells in 60-mm dishes or 6-well plates were transfected using FuGENE 6 with single or combinations of expression vectors and harvested 48 h after transfection. To examine protein expression levels or the size of APP cleavage products, the cells were directly harvested in SDS-PAGE sample buffer. For immunoprecipitation experiments, the cells were washed once with cold PBS, harvested in 1 ml of lysis buffer (50 \(\mu\)l HEPES-NaOH, pH 7.5, 150 \(\mu\)M NaCl, 10% glycerol, 1% IGE-PAL CA-630, 1.5% MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, 0.1 g/liter phenylmethylsulfonyl fluoride, 10 mg/liter leupeptin, 10 mg/liter aprotonin, and 1 mg/liter pepstatin A). The cell extracts were clarified by centrifugation at 20,800 \(\times\) g for 10 min. The supernatants were incubated with 10 \(\mu\)l of a polyclonal antibody raised against GFP (T3743) or the C terminus of APP (U855) for 2 h at 4 °C. 30 \(\mu\)l of 50% slurry of protein A-Sepharose (Amersham Biosciences) was then added. The beads were incubated with the reactions for 2 h at 4 °C with rotation and then collected by centrifugation. The beads were washed three times with lysis buffer and resuspended in 0.1 ml of SDS-PAGE sample buffer. 20 \(\mu\)l of the protein solutions were resolved on SDS-PAGE and detected by immunoblotting with antibodies to the Myc (9E10; Santa Cruz Biotechnology; 1:1,000 dilution) or HA epitope (H11; BabCo) (1:1,000). For pull-down experiments, 10 \(\mu\)g of GST-Fe65/WW/Tip60, 2 \(\mu\)g of GST-Fe65/WW/TiP60, or 2 \(\mu\)g of GST-Fe65/WW/TiP60 was added to the cell lysates and incubated for 4 h at 4 °C. The rest of the steps are the same as the co-immunoprecipitation experiments.

**Mechanism of Transcriptional Activation by APP**

COS7 cells in 60-mm dishes or 6-well plates were transfected using FuGENE 6 with single or combinations of expression vectors and harvested 48 h after transfection. To examine protein expression levels or the size of APP cleavage products, the cells were directly harvested in SDS-PAGE sample buffer. 20 \(\mu\)l of the protein solutions were resolved on SDS-PAGE and detected by immunoblotting with antibodies to the Myc (9E10; Santa Cruz Biotechnology; 1:200 dilution) or anti-GFP polyclonal antibody (T3743; 1:500 dilution) for 1 h. After three washes with PBS and one wash with water, the cells were mounted with Vectashield (Vector Laboratories) and examined with a Bio-Rad confocal microscope.

**RESULTS**

**\(\gamma\)-Secretase Is Required for APP-dependent Transactivation of Gal4-Tip60**—We originally established two transactivation assays in which the DNA-binding domain of Gal4 is fused either to APP or to Tip60, and transactivation is monitored with a Gal4-dependent reporter plasmid (13). In the present study, we chose to use the Gal4-Tip60 assay because Tip60 normally exists in a nuclear multiprotein complex that possesses DNA binding activity (14), whereas APP and Fe65 have not been shown to bind DNA. Thus in the Gal4-Tip60 assay, the only artificial element is the Gal4 DNA-binding domain that is fused to a protein which is normally bound to DNA. Furthermore, because both APP and Fe65 are unmodified in the Gal4-Tip60 assay, this assay allows us to test whether APP or Fe65 are directly involved in transcription.

We first asked whether \(\gamma\)-cleavage of APP is necessary for APP-dependent transactivation and tested the effects of two \(\gamma\)-secretase inhibitors, inhibitor IV and DAPT (Fig. 1, A and B). Both inhibitors significantly decreased transactivation (>50% inhibition). However, neither inhibitor completely blocked transactivation, probably because the treatment period was short, and not all \(\gamma\)-secretase activity may have been abolished at the dose used. To probe the role of \(\gamma\)-secretase in APP-dependent transactivation with an independent approach, we therefore carried out transactivation assays in embryonic fibroblasts derived from presenilin knockout mice. In vertebrates, the two presenilins, presenilin 1 and 2, are essential for \(\gamma\)-secretase activity (15). Compared with wild type embryonic fibroblasts, APP-dependent transactivation of Gal4-Tip60 was decreased >50% in presenilin 1 knockout cells and completely abolished in presenilin 1/2 double knockout cells (Fig. 1C). These results suggest that \(\gamma\)-secretase activity is essential...
for the APP-dependent transactivation of transcription by Gal4-Tip60.

Nuclear Translocation of Fe65 Is Required for Transactivation Mediated by Gal4-Tip60, Fe65, and APP—Although transactivation of nuclear Gal4-Tip60 requires both APP and Fe65, endogenous APP (16) and Fe65 (17, 18) are largely cytoplasmic, and in transfected cells, co-expressed APP and Fe65 are detectable only in cytoplasmic vesicles (13). In contrast, Fe65 expressed without APP is enriched in the nucleus (19). At least two principal mechanisms could account for these findings. First, it is possible that a low amount of Fe65 and/or of the AICD resides in the nucleus and is sufficient to activate transcription (20). The NICD has been shown to function in this manner (21). Alternatively, it is conceivable that Fe65 and/or APP initiate a signal in the cytoplasm without physically participating in gene transcription. A combination of these two mechanisms could also apply; for example, it is possible that only Fe65 functions directly in the nucleus but has to be activated by APP in the cytoplasm.

To distinguish between these possibilities, we examined the consequence of preventing the nuclear translocation of Fe65 and APP. We fused the NES from mitogen-activated protein kinase (22) to the N terminus of Fe65, using as a negative control a mutant NES (mNES) in which four leucine residues are replaced by alanines. As shown by immunostaining of transfected HeLa cells, addition of the NES translocated Fe65 (expressed without APP, when it is nuclear) from the nucleus to the cytoplasm, whereas Fe65 containing the mutant mNES (mNES-Fe65) continued to be largely nuclear (Fig. 2, A–C).

We then tested whether cytoplasmic Fe65 containing the NES was still capable of transcriptional transactivation. As expected, we observed no activation of Gal4-Tip60-dependent reporter gene transcription with any Fe65 construct in the absence of APP. In the presence of APP, unmodified Fe65 strongly stimulated transcription (Fig. 2D). Strikingly, cytoplasmic NES-Fe65 was unable to activate transcription, whereas the NES-dependent inhibition of transcriptional activation by Fe65 was reversed when the NES was mutated in mNES-Fe65 (Fig. 2D).

Nuclear Translocation of the AICD May Be Dispensable for Gal4-Tip60-mediated Transactivation—In a corresponding experiment, we took a similar “NES tagging” approach to test whether nuclear translocation of the AICD of APP is essential for Gal4-Tip60-mediated transactivation. Unfortunately, the NES and mNES from mitogen-activated protein kinase severely depressed APP expression, no matter whether the sequences were placed right after the TMR or at the C terminus of APP (data not shown). Therefore, we employed the NES from protein kinase inhibitor α, again using a mutant NES as a negative control (23). Fusion of both wild type and mutant NES to the C terminus of APP reduced the level of APP expression to about 30% and may at least partially suppress APP cleavage because lower levels of α/β-cleavage products from the NES-tagged APPs accumulated upon inhibition of γ-secretase (Fig. 3A). Nevertheless, the APP fusion proteins containing either the wild type or the mutant NES were more potent activators of Gal4-Tip60-dependent transcription than unmodified APP (Fig. 3B). As a negative control, we analyzed mutant APP* that contains a point mutation in the cytoplasmic tail of APP, which abolishes Fe65 binding (13), showing that it was unable to transactivate. Similar results with NES-tagged APPs were obtained in HEK293 (Fig. 3B), COS, and HeLa cells (data not shown).

The results with the APP-NES fusion proteins suggest that nuclear translocation of the AICD is not essential for transactivation. It is possible, however, that the NES, when fused to the cytoplasmic domain of APP, is inactive. It is difficult to directly determine whether the NES attached to the C terminus of APP is functional because APP is localized in membranes outside of the nucleus, because the cleaved AICD is unstable, and at best only a small percentage of the AICD enters the nucleus (20). To circumvent these difficulties, we expressed the cytoplasmic tail of APP as a more stable GFP fusion protein (see also Fig. 5 below). When we examined the localizations of GFP-APPct-NES and -mNES and their parent molecules (GFP and GFP-APPct) in transfected HeLa cells, we detected GFP and GFP-APPct throughout both the cytoplasm

![Fig. 2. Nuclear translocation of Fe65 is required for transactivation.](image-url)
and the nucleus (Fig. 3, C1 and C2). The wild type NES selectively kept GFP-APPct out of the nucleus (Fig. 3C3), whereas the mutant NES had no effect on the amount of GFP-APPct in the nucleus (Figs. 3C4). Thus the C-terminal NES fused to the cytoplasmic tail of APP appears to prevent nuclear translocation of the AICD, even though it has no effect on APP-dependent transactivation of Gal4-Tip60.

A 21-residue Fragment of the AICD Is Sufficient for Transactivation—In the next set of experiments, we asked whether the cytoplasmic tail of APP only interacts with Fe65 in transactivating transcription by Gal4-Tip60 or has other potential activities during transactivation. For this purpose, we defined the minimum sequence in APP that is necessary and sufficient for transactivation. We examined three deletion mutants of APP in which the cytoplasmic tail (normally 47 residues) was reduced to 32, 21, or 15 residues (APP-C32, -C21, and -C15, respectively; Fig. 4C). In transfected COS cells, the three deletion mutants were cleaved and, as expected, produced C-terminal fragments that were smaller than wild type C-terminal fragments (Fig. 4A). Co-immunoprecipitation experiments demonstrated that APP-C32 interacted with Fe65 as strongly as wild type APP, and APP-C21 exhibited reduced Fe65 binding, whereas APP-C15 no longer bound to Fe65 (Fig. 4A). In the Gal4-Tip60 transactivation assay, both APP-C32 and APP-C21 transactivated, although less strongly than wild type APP. In contrast, APP-C15 was inactive (Fig. 4B).

Next, we measured the effects of single or double point mutations in the 21-residue region on Fe65 binding and Gal4-Tip60-dependent transactivation (Fig. 4D). Most mutations had no significant effect on either activity. Four mutations (Y682A, E683A, N684A, and P685A) similarly decreased Fe65 binding and Gal4-Tip60 transactivation, and three mutations (G681A and the double mutations Y682A/P685A and P685A/Y687A) abolished both. Thus the point mutations, like the truncation mutants, always affected both Fe65 binding and transactivation similarly, and no sequence in the AICD was required for transactivation but not for Fe65 binding.

Soluble AICD Is Not Sufficient for Strong Transactivation—Is the AICD alone sufficient for transactivation of Gal4-Tip60? We first tested whether the AICD (residues 639–695 of APP, corresponding to the intracellular fragment generated by γ-cleavage at A642 site with an N-terminal methionine) still binds to Fe65. Even when co-expressed with Fe65, the levels of the AICD in transfected cells were very low. Nevertheless, the AICD was still co-immunoprecipitated with Fe65 (Fig. 5A). However, despite being bound to Fe65, the AICD was unable to transactivate Gal4-Tip60 even at high concentrations (Fig. 5B).

The lack of transactivation by the soluble AICD could conceivably be caused by its low expression level. A shorter AICD fragment without the N-terminal residues that are derived from the TMR of APP (i.e. a fragment containing only the cytoplasmic tail of APP) exhibited even lower expression levels than the full-length AICD and thus was no more suitable for transactivation experiments (data not shown). However, when we fused the cytoplasmic tail of APP to GFP, the resulting GFP-APPct protein was expressed at dramatically increased levels (Fig. 5C) and at least partly transported into the nucleus (Fig. 3C). As demonstrated by co-immunoprecipitation experiments, GFP-APPct bound to Fe65 as well as a full-length APP-GFP fusion protein in which GFP was inserted into full-length APP at the same position as in the GFP-APPct protein (Fig. 5, C and E). Conversely, a mutant GFP-APPct protein (GFP-APPct*) that includes the NPTY to NATA mutation (13) was expressed at equally high levels as APP-GFP and GFP-APPct but was unable to bind to Fe65 as expected (Fig. 5C).

We next tested the transactivation activity of GFP-APPct, using GFP-APPct* as a negative control and APP-GFP as a positive control. Strikingly, GFP-APPct was almost completely unable to transactivate Gal4-Tip60 and was only marginally superior to GFP-APPct* (Fig. 5D). Full-length APP-GFP, on
Fig. 4. The 21-residue Fe65-binding sequence in the cytoplasmic tail of APP is sufficient for transactivation. A, co-immunoprecipitation of full-length and truncated APPs with Fe65. APP was immunoprecipitated from COS cells that had been co-transfected with plasmids encoding HA-tagged Fe65 and the APP molecules shown in C. and the immunoprecipitates were analyzed by immunoblotting with an HA antibody to detect Fe65. B, transactivation of Gal4-Tip60 dependent transcription in HeLa and COS cells by wild type APP and the APP mutants shown in C. APP*, APP with the NPTY to NATA mutation that abolished Fe65 binding and transactivation. C, schematic diagrams of wild type and truncated APP molecules used in the experiments of A and B. The intracellular sequences of the APP molecules are shown above the diagrams. Underlined residues, cloning linkers. The open and filled squares below the bottom sequence indicate residues that were examined in the point mutant analysis in D. Open squares, no effect on Fe65 binding and transactivation; filled squares, inhibits binding and transactivation. D, co-immunoprecipitation (top panel) and transactivation experiments (bottom panel) with full-length APP molecules carrying point mutations in the cytoplasmic tail. COS cells were co-transfected with Fe65 and the various APP molecules indicated at the bottom. APP, wild type APP. Other designations reflect the point mutations introduced, with the amino acid numbering based on APP695. Assays were performed in HEK293 cells transfected with 50 ng of plasmids encoding full-length wild type APP and mutant APP* or with 10–1000 ng of plasmid encoding the AICD. C, the cytoplasmic tail of APP (APPct) stabilized by fusion to GFP strongly binds to Fe65. COS cells were co-transfected with Myc-tagged Fe65 and GFP fusion proteins of full-length APP (APP-GFP) or the wild type or mutant cytoplasmic tails of APP (GFP-APPct and GFP-APPct*, respectively). The cell lysates were immunoprecipitated with anti-GFP antibodies and probed with anti-Myc antibodies. D, Gal4-Tip60 transactivation assays with the GFP fusion proteins of APP. Two different cell types were examined to ensure reproducibility. E, schematic diagram of the various proteins used in A–D. IP, immunoprecipitation.

Fig. 5. Full-length APP is a much more potent Gal4-Tip60 transactivator than the isolated cytoplasmic tail of APP. A, the AICD co-immunoprecipitates with Fe65. Lysates of COS cells co-transfected with Myc-tagged Fe65 and APP, APP* (with the NPTY to NATA mutation), or AICD were immunoprecipitated with an antibody to the cytoplasmic tail of APP. B, the AICD is unable to transactivate Gal4-Tip60. Assays were performed in HEK293 cells transfected with 50 ng of plasmids encoding full-length wild type APP and mutant APP* or with 10–1000 ng of plasmid encoding the AICD. C, the cytoplasmic tail of APP (APPct) stabilized by fusion to GFP strongly binds to Fe65. COS cells were co-transfected with Myc-tagged Fe65 and GFP fusion proteins of full-length APP (APP-GFP) or the wild type or mutant cytoplasmic tails of APP (GFP-APPct and GFP-APPct*, respectively). The cell lysates were immunoprecipitated with anti-GFP antibodies and probed with anti-Myc antibodies. D, Gal4-Tip60 transactivation assays with the GFP fusion proteins of APP. Two different cell types were examined to ensure reproducibility. E, schematic diagram of the various proteins used in A–D. IP, immunoprecipitation.

the other hand, potently transactivated Gal4-Tip60, demonstrating that the GFP fusion by itself does not inactivate the AICD (Fig. 5D). These findings were obtained in multiple cell lines over a large concentration range of transfected plasmids, indicating that binding of Fe65 to the AICD alone is not sufficient for robust transactivation.

A Membrane-tethered Fe65-binding Site from APP Is Sufficient for Transactivation—Are the extracellular domains
and/or the TMR of APP involved in transactivation, or do these sequences only serve to tether the AICD to the membrane? To address this question, we fused the signal peptide and TMR of neurexin 1β, a neural cell-adhesion molecule (24), to the full-length, 44-residue and the truncated, 32-residue cytoplasmic tails of APP (Fig. 6). This resulted in fusion proteins (called Nstm-APPct and Nstm-C32) that contain only a short extracellular stub, making them γ-secretase substrate (25), and include no APP sequences except for the cytoplasmic tail with the Fe65-binding site. As a negative control, we introduced into the cytoplasmic tail of Nstm-APPct the NPTY to NATA mutation) was also tested as a negative control (Nstm-APPct*) analogous to the corresponding APP mutant (APP*). The structures of APP, Nstm-APPct, and Nstm-C32 are shown below the bar diagram depicting the transactivation assay results.

**The WW Domain of Fe65 Functions as a Transcriptional Activator**—Because our results point to a central role of nuclear Fe65 in the APP-dependent transactivation of Ga4-Tip60, we tested whether Fe65 possessed a transactivation domain. For this purpose, we fused different fragments from Fe65 to the DNA-binding domain of Ga4 and examined the transcriptional activity of the resulting Ga4 fusion proteins in absence of co-factors (Fig. 7). We found that neither the first 200 residues that include a negatively charged region nor the entire Fe65 molecule could stimulate reporter gene expression. Unexpectedly, however, strong transactivation was achieved as soon as the WW domain was fused to Ga4 together with the N-terminal sequence (Fig. 7). A mutation in the central YYW motif of the WW domain decreased transactivation, suggesting that a folded WW domain is essential for transactivation. As soon as we added either the PTB1 or the PTB2 domain with flanking sequences to the WW domain, transactivation was inhibited >20-fold (Fig. 7). Together these data indicate that Fe65 includes an N-terminal activation sequence that requires the WW domain but is normally inhibited by the C-terminal PTB domains.

The WW and PTB Domains of Fe65 May Interact with Each Other—In a final set of experiments, we investigated whether the PTB domains of Fe65 might inhibit the transactivation activity of the WW domain via a direct interaction. To this end, we produced in transfected COS cells Myc-tagged full-length Fe65 and Fe65 fragments containing either both PTB domains (Fe65/PTB1-2) or all Fe65 domains except for the PTB2 domain (Fe65APTB2). We then measured the binding of these proteins to various Fe65 domains using pull-downs with immobilized GST or MBP fusion proteins (see boxes in Fig. 8 for the domain structures of the proteins used). Upon immunoblotting of bound protein with antibodies to the Myc epitope, we observed interactions between all pairs of Fe65 fragments in which one of the binding partners contains the WW domain, and the other partner contains the PTB2 domain of Fe65 with flanking sequences (Fig. 8A).

These results suggested a possible interaction between the WW and PTB2 domains of Fe65. Therefore we tested whether the isolated WW domain of Fe65 was able to pull down fragments of Fe65 containing either both PTB domains of Fe65 or only one full PTB domain with part of the other domain. As a positive control, we used the cytoplasmic tail of APP, which specifically interacts with the PTB2 domain of Fe65; as negative controls, we employed GST alone and/or the mutant WW domain that is unable to transactivate. As shown in Fig. 8B, the wild type WW domain specifically interacted with all of the Fe65 PTB domain proteins tested, whereas GST and the mutant WW domain did not.

Viewed together, panels A and B of Fig. 8 suggest that the Fe65 WW domain interacts with a sequence of Fe65 at the boundary between the two PTB domains, whereas the cytoplas-
mic tail of APP specifically binds the Fe65 PTB2 domain. Does the cytoplasmic APP tail influence the binding of the Fe65 WW domain to the PTB domains? To address this, we preincubated HA-tagged Fe65 with different concentrations of GST fusion proteins containing either the wild type cytoplasmic tail of APP (GST-APPct) or the mutant tail that cannot bind to Fe65 (GST-APPct*, as a control). As a further control, we employed a point mutant of Fe65 (Fe65C655F) whose C-terminal PTB domains are unable to bind to the cytoplasmic tail of APP but still bind to the WW domain or Fe65. After the preincubations, we bound the Fe65 to the immobilized WW domain of Fe65 (produced as a maltose-binding protein fusion protein). GST-APPct strongly reduced Fe65 binding to the immobilized WW domain, whereas mutant GST-APPct* had no effect (Fig. 8C). Even low concentrations of APPct inhibited binding of wild type Fe65; in contrast, even high concentrations of APPct had no effect on mutant Fe65C655F, which still binds the WW domain but is unable to bind APPct. These findings indicate that the cytoplasmic tail of APP and the Fe65 WW domain cannot bind simultaneously to the PTB domains of Fe65.

DISCUSSION

In a previous study, we demonstrated that the cytoplasmic tail of APP forms a transcriptionally active complex with the adaptor protein Fe65 and the histone acetyltransferase Tip60 (13). This finding suggested mechanistic similarities of APP with Notch whose NICD, like the AICD of APP, is also liberated by intramembranous cleavage. In a well characterized pathway, the released NICD enters the nucleus where it functions as a transcriptional activator (reviewed in Ref. 2). The question thus arises of whether the mechanism of transcriptional activation by APP is similar to that of Notch. Surprisingly, we found that APP activates transcription by an indirect mechanism, suggesting that apart from their common cleavage by /H9253-Cleavage of APP is essential for transactivation (Fig. 1), thereby suggesting that in APP-dependent transactivation, release of the AICD is important for transcriptional activation similar to the Notch pathway. 2) Nuclear translocation of Fe65 is required for transac-
tivation, whereas nuclear translocation of AICD is apparently unnecessary (Figs. 2 and 3). Thus even though the majority of Fe65 is retained in the cytoplasm upon co-transfection with APP, a small amount of Fe65 is normally translocated to the nucleus to activate transcription. 3) The only sequence of the AICD that is essential for transactivation is its Fe65-binding sequence (Fig. 4), indicating that the AICD does not include an independent, secondary transactivation activity. 4) Expression of the AICD alone, without tethering to a membrane, causes little transactivation even at high expression levels (Fig. 5). Again, this is different from the NICD of Notch, which, when expressed as a soluble cytoplasmic fragment, potently activates Notch-responsive promoters even at low levels (21) and induces a constitutive gain-of-function phenotype in transgenic flies and worms (reviewed in Ref. 2). 5) A membrane-anchored Fe65-binding site from the AICD, expressed in the context of a chimeric \( \beta \)-secretase substrate, is a potent transactivator of Gal4-Tip60 (Fig. 6). Thus the Fe65-binding motif of APP, its only sequence that appears to be required for transactivation, is active as long as it is attached to a membrane. 6) The isolated WW domain of Fe65, which is essential for Fe65-dependent transactivation of Gal4-Tip60 in the presence of APP but does not bind to APP, constitutes a strong transactivation domain that is normally inhibited in the context of Fe65 by the C-terminal PTB domains (Fig. 7). 7) The WW and the PTB domains of Fe65 bind to each other in vitro, potentially forcing Fe65 into an inactive conformation, and the cytoplasmic tail of APP competes with the WW domain for binding to the PTB domains (Fig. 8).

Based on these observations, we propose a working model for how APP and Fe65 may function in gene transcription (Fig. 9). According to this model, Fe65 normally adopts a closed conformation via an intramolecular interaction between the WW domain and the PTB2 domain. The intramolecular interaction inhibits the transactivation activity of the WW domain but can be reversed by the concerted action of two mechanisms. First, binding of the cytoplasmic tail of APP directly interferes with the interaction between the WW and the PTB2 domains of Fe65, thus partially opening the closed conformation of Fe65. Second, APP recruits Fe65 to an as yet unknown membrane-associated factor that stabilizes the opened conformation. Proteolytic processing of APP by \( \alpha \) - or \( \beta \)-secretase followed by \( \gamma \)-secretase then releases the AICD and enables activated Fe65 to detach from the membrane and to enter the nucleus where it interacts with Tip60 through the PTB1 domain and other transcription factors through the WW domain. According to the model, the AICD fragment is dispensable at this point and may be degraded by cytosolic proteases immediately after being generated or accompany Fe65 into the nucleus.
membrane to stimulate activation of Fe65 and suggest that this requirement may arise because an unidentified factor activates Fe65 in addition to AICD binding. The major evidence for the latter proposal consists of the observation that overexpression of the soluble AICD alone, in any form, does not cause major Fe65-dependent transactivation of Gal4-Tip60 (Fig. 5), but as soon as the AICD is placed into the context of a membrane protein that is a γ-secretase substrate, it becomes a potent transactivator (Fig. 6). The membrane-associated factor that activates APP-bound Fe65 may be a protein kinase because both APP and Fe65 contain multiple phosphorylation sites, or it may be a special lipid. Indeed, in preliminary experiments, we observed that the PTB2 domain of Fe65 avidly binds to liposomes containing phosphatidylinositol 4,5-biphosphate (data not shown), a phospholipid enriched in actin-rich structures such as membrane ruffles in the plasma membrane (reviewed in Ref. 26). Interestingly, APP and Fe65 are known to concentrate in actin-rich lamellipodia (27). According to our model, γ-cleavage of APP is required to release activated Fe65 from the membrane, which then translocates into the nucleus where its WW domain interacts with other transcription factors to activate gene expression.

Our model proposes a transactivation mechanism for APP that is very different from that of Notch. We suggest that in APP, the AICD only acts to bind Fe65 to the membrane and that the only need for γ-secretase activity is to release Fe65 for nuclear entry after it has been activated by a membrane-dependent process. However, alternative interpretations of our results are possible, although not as well supported by the data. For example, Fe65 could be a transcriptional inhibitor that needs to be drained from the nucleus by a membrane-tethered Fe65-binding site in APP. The potent transcriptional activation mediated by the WW domain of Fe65 (Fig. 7) and the inhibition of transcriptional activation of Fe65 by the NES (Fig. 2) argue against this hypothesis. Another alternative model is that the AICD functions analogous to the NICD and directly activates transcription in the nucleus. Against this model is the observation that the addition of an NES to the cytoplasmic tail of APP did not inactivate it. More significantly, overexpression of the AICD in various forms, even as a GFP fusion protein that we demonstrate enters the nucleus (Fig. 3), caused at best marginal transactivation (Fig. 5), much less than that observed for full-length APP (Fig. 4) or for an Fe65-binding site fused to a cleavable membrane anchor (Fig. 6). Consistent with its smaller size, the function of the AICD thus appears to be more limited than that of the NICD. The ternary complex of Tip60, Fe65, and AICD has been found at the promoter region of the KAI gene (28), a putative target of APP-mediated transcription. Although our model proposes no nuclear function for the AICD (Fig. 9), our model does not necessitate a dissociation of the AICD from Fe65 before Fe65 enters the nucleus and is thus consistent with this result. Moreover, our data do not exclude a second, independent function of the AICD in the nucleus that is unrelated to Tip60.

It should also be noted that our understanding of the transcriptional activation pathway by APP is severely hampered by the lack of information on APP mutants (we do not know which parts of APP are actually essential for function) and on target genes, information that has been available for Notch and greatly helped in understanding the Notch pathway. Genetic studies are required to complement the biochemical and cell biological assays reported here. In the absence of genetic information, the interpretation of biochemical and cell biological data is limited, similar to the limitations of genetic data in the absence of biochemical and cell biological data. Because of these limitations, at present we can only propose a model that is based on the best available evidence obtained with the best available approaches. Clearly this model is only a working model that has to be tested and adapted. Although our data strongly argue against a precise functional correspondence between Notch and APP and suggest that their respective transcriptional pathways differ, the model we propose should not be considered a conclusion but a hypothesis that may guide future experiments.

An important question regarding the function of APP and Fe65 in gene transcription is how it is regulated. Our model includes at least two potential regulatory steps, the release of activated Fe65 by intramembranous cleavage of APP and the conversion of Fe65 into a transcriptionally active form. γ-Cleavage is probably controlled by the initial extracellular α- and β-cleavage, because γ-secretase seems to cleave by default any type I transmembrane protein with a short extracellular sequence (25). The extracellular cleavage of Notch is tightly controlled by ligand binding (21, 29), but the regulation of the extracellular α/β-cleavage of APP remains unclear. The second potential regulatory step, Fe65 activation, depends on the nature of the modification that activates Fe65. For example, if the modification is phosphorylation, the activation of the kinase would control the generation of the nuclear signal. Conversely, if the modification is phospholipid binding, the activity of lipid kinase or phosphatase would mandate whether a nuclear signal is initiated. If Fe65 activation requires several modifications, then the combined regulation of APP processing and of multiple Fe65 modifications would indeed subject the transcriptional signal mediated by Fe65 and APP to a tight and complex regulation.

Is the function of APP in gene transcription important for understanding AD? Based on two lines of evidence, we think that the nuclear signaling of APP is probably not a major effect on involved in the pathogenesis of AD. First, mutations in the presenilin and APP genes that cause AD generally do not have a major effect on the generation of AICD and thus should not have a significant impact on transactivation activity. Second, besides APP, APLP1 and APLP2 also undergo presenilin-dependent intramembrane cleavage, resulting in the release of intracellular fragments (7, 8). Both also transactivate Gal4-Tip60 (9). However, neither APLP1 nor APLP2 has been associated with AD, suggesting that the transactivation activity of APP family proteins and AD are unrelated. Nevertheless, the transcriptional function of APP may be indirectly important for the pathogenesis of AD. The rate of Aβ production determines the progression of AD (3). Aβ production is mediated by APP cleavage that in turn is probably regulated to control the transcriptional activity of Fe65. Thus Aβ production is embedded in a regulatory cascade whose end point, among others, probably includes a transcriptional read-out.

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