The adaptor protein FE65 interacts with the β-amyloid precursor protein (APP) via its C-terminal phosphotyrosine binding (PTB) domain and affects APP processing and Aβ production. Our previous data demonstrate that the apoE receptor ApoEr2 co-precipitated with APP and suggest that there are extracellular and intracellular interactions between these two transmembrane proteins. We hypothesized that FE65 acts as an intracellular link between ApoEr2 and APP. Co-immunoprecipitation experiments in COS7 cells demonstrated an interaction between ApoEr2 and FE65 that depended on the N-terminal PTB domain of FE65. Full-length FE65 increased co-immunoprecipitation of ApoEr2 and APP. Full-length FE65 also increased surface expression of ApoEr2, as determined by surface protein biotinylation and live cell surface staining. Constructs containing both the C- and N-terminal PTB domains of FE65 increased secreted APP, secreted ApoEr2, APP C-terminal fragment, and ApoEr2 C-terminal fragment, but constructs containing only single PTB domains did not affect APP or ApoEr2 processing. In addition, full-length FE65 decreased Aβ to a significantly greater extent than individual FE65 domains. These data suggest that FE65 can bind APP and ApoEr2 at the same time and affect the processing of each.

Alzheimer disease is characterized by the presence of β-amyloid plaques, composed predominantly of the Aβ peptide, a 40- or 42-amino acid cleavage product of the β-amyloid precursor protein (APP).1 APP, a transmembrane protein, undergoes extracellular cleavage by α- or β-secretase, resulting in the formation of a large N-terminal extracellular fragment (APPx) and smaller, membrane-bound C-terminal fragments (CTF). If the initial cleavage event occurs via β-secretase, then cleavage of the CTF by γ-secretase results in the formation of Aβ (1). The cytoplasmic domain of APP contains a -GYENPTY- sequence that serves as a binding motif for adaptor proteins that possess a phosphotyrosine binding (PTB) domain, such as members of the FE65, X11, JIP, and Dab protein families. Such PTB domain interactions have been shown to play critical roles in tyrosine kinase-mediated signal transduction, protein trafficking and localization, phagocytosis, cell fate determination, and neuronal development (2). Additionally, several studies have shown that interactions between these cytoplasmic proteins and APP lead to altered processing of APP. FE65 adaptor protein family members, including FE65, FE65-like (FE65L or FE65L1), and FE65L2 (3, 4), are expressed at high levels in neurons (5). FE65 family members possess three protein binding domains as follows: a WW domain and two PTB domains, which display distinct binding specificities. The WW domain binds Mena (mammalian enabled), which binds actin and thus links FE65 and APP to cytoskeletal dynamics and cellular motility and morphology (6, 7). The interaction between FE65 and APP is mediated via the second phosphotyrosine binding (PTB2) domain of FE65 (8) and modulates APP processing and trafficking in several cell lines (9, 10). FE65 increases Aβ secretion in Madin-Darby canine kidney cells stably transfected with APP695 (11). In HEK293 cells, FE65 stabilizes immature APP and inhibits APPs formation and Aβ secretion (12). In H4 cells, FE65L1 increased production of secreted APP Aβ (4). In addition, mice transgenic for FE65 and APP displayed lower Aβ accumulation in the cerebral cortex and a lower level of APP-CTFs (10).

The N-terminal PTB1 domain of FE65 proteins binds an apoE receptor, the low density lipoprotein receptor-related protein (LRP1) (13). LRP1 is a large type I transmembrane protein whose small cytoplasmic tail contains two NPXY motifs (14). FE65L1 affects LRP metabolism, resulting in decreased LRP steady state levels, secreted LRP, and LRP endocytic receptor function (3). LRP and APP interact in their extracellular domains (15), and LRP can affect the processing of APP, reducing surface APP and APPsα and increasing Aβ production (16, 17) through extracellular and intracellular interactions between LRP and APP (18). FE65 acts as a cytoplasmic linker between LRP and APP (18, 19).

Our previous studies have shown that another apoE receptor, ApoEr2, also interacts with APP and modulates APP processing (1). Thus we tested whether ApoEr2 could bind to FE65 forming a similar intracellular connection with APP. We found that FE65 interacts with the intracellular domain of ApoEr2 through the PTB1 domain, affecting its trafficking and processing. Data suggest that there exists a tripartite complex of APP, FE65, and ApoEr2, affecting processing of the membrane-bound molecules.
ApoEr2 and FE65

MATERIALS AND METHODS

Vector Construction—We generated several constructs of the cytoplasmic domain of ApoEr2 containing exon 18 and 19 (construct 1), exon 19 and 20 (construct 2), exon 18 and exon 20 (construct 3), or exons 18–20 (construct 4). We produced an NPXY mutant of full-length ApoEr2 using site-directed mutagenesis (Stratagene), substituting alanine for asparagine. Full-length FE65 with C-terminal Myc tag and several deletion constructs of FE65 with C-terminal Myc tags were the kind gifts of Dr. Thomas Sudhof (University of Texas, Southwestern). APP770 was expressed from a PsecTag2/HygroB vector. Recombinant DNA sequences were confirmed by sequencing, and expression of correctly sized proteins was confirmed by Western blot (data not shown).

Antibodies—We used anti-HA (Abcam), anti-c-Myc (Abcam), and anti-FE65 (Abcam: Ab17469; anti-pan-FE65 (20)). Polyclonal FE65 antibody (5916) was the gift of Dr. Edward Koo (University of California). Rabbit polyclonal antibody 5810 against FE65 antibody (5916) was the gift of Dr. Edward Koo (University of California). Rabbit polyclonal antibody 5810 against FE65 antibody (5916) was the gift of Dr. Edward Koo (University of California).

Western blot (data not shown).

Recombinant DNA sequences were confirmed by sequencing, and expression of correctly sized proteins was confirmed by Western blot (data not shown).

Co-immunoprecipitations—Transfected COS7 cells were washed with PBS and lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Nonidet P-40, and phosphatase and protease inhibitors. For immunoprecipitations, lysates were incubated for 2 h at 4 °C with the anti-HA antibody or an anti-6E10 antibody and protein G-Sepharose beads (Amer sham Biosciences). The precipitates were washed three times with lysis buffer and resuspended in SDS sample buffer. The samples were separated by SDS-PAGE on 4–15% polyacrylamide gels, transferred electrophotographically to nitrocellulose membranes, and blocked with 5% nonfat dry milk. The blots were incubated with antibodies at room temperature for 1 h. Horse radish peroxidase-conjugated secondary antibodies were visual ized by ECL detection system and exposed to film.

Biotin-labeled Cell Surface Proteins—ApoEr2-transfected COS7 cells were washed twice with PBS, and surface proteins were labeled with sulfo-NHS-SS-biotin (Pierce) under gentle shaking at 4 °C for 30 min, according to the manufacturer’s protocol. Quenching solution was added to cells, which were washed twice with Tris-buffered saline. Cells were lysed in 500 μl of lysis buffer, collected with a cell scraper, disrupted by sonication on ice, incubated for 30 min on ice, and clarified by centrifugation (10,000 × g, 2 min). To isolate biotin-labeled proteins, lysate was added to immobilized NeutrAvidinTM gel and incubated 1 h at room temperature. Gels were washed five times with wash buffer and incubated 1 h with SDS-PAGE sample buffer, including 50 mM dithiothreitol. Equal levels of total biotinylated proteins were analyzed by immunoblotting.

Immunocytochemistry—Primary neuronal cells were fixed with 4% paraformaldehyde. After fixation, cells were washed with PBS and incubated with 5810 and anti-FE65 antibody overnight at 4 °C. After primary incubation, cells were washed three times in PBS and then incubated with Alexa Fluor 555 (red color) goat anti-rabbit antibody (Molecular Probes) and Alexa Fluor 488 (green color) goat anti-mouse antibody for 1 h at room temperature. Stained cells were viewed with a confocal laser scanning microscope.

Live Cell Surface Staining—Human embryonic kidney cells were washed with PBS and incubated with primary antibody diluted in ECS (containing 145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 5 mM HEPES, 5 mM glucose, 15 mM sucrose, 0.25 mg/liter phenol red, and 10 μM d-serine (all from Sigma), adjusted to pH 7.4 with NaOH) for 8 min at room temperature. Cells were washed three times in ECS and then incubated with 5810 antibody (recognizing ApoEr2) for 8 min, washed with ECS, and incubated with Alexa Fluor 555 goat anti-rabbit antibody for 8 min at room temperature. Final washes were carried out with ECS. Stained cells were imaged on an Axioskop FS microscope (Zeiss) equipped with a ×63, 0.9 N.A., Achroplan, water-immersion objective or with a Nikon E600 microscope equipped with a ×60, 1.0 N.A. objective.

Mouse Brains—Brain lysates were prepared from Swiss Webster mice in IP buffer (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1% Nonidet P-40, and phosphatase and protease inhibitors). In addition, brains were isolated from 3-month-old male...
FE65 knock-out mice and controls (C57BL/6 × 129SvEv Brdley) (20).

Statistical Analyses—Experiments were repeated a minimum of four times unless otherwise noted. All data were analyzed using analysis of variance with Graphpad Prism 4 software, using Tukey’s multiple comparison test for post hoc analyses with significance determined as p < 0.05. Descriptive statistics were calculated with StatView 4.1 and displayed as an expressed mean ± S.E.

RESULTS

FE65 Interacts with APP in COS7 Cells—FE65 interacts with APP and alters APP processing in several cell types. To confirm that APP interacts with FE65 in our system, we transfected COS7 cells with APP and FE65, immunoprecipitated APP with 6E10 antibody, and probed for FE65 with anti-c-Myc antibody. Full-length FE65 co-precipitated with APP (Fig. 1A, upper panel) in cells expressing both APP and FE65. Western blot analysis of COS7 cell extracts confirmed that levels of total APP were consistent across transfections (Fig. 1A, lower panel). We tested whether FE65 affected APP processing in these cells by measuring secreted APP and APP CTF. We transfected COS7 cells with full-length APP; secreted APP was measured in conditioned media, and APP CTF was measured in cell lysates. We found that full-length FE65 strongly increased secreted APP and APP CTF in COS7 cells (Fig. 1B). Because FE65 affects APP processing, we examined whether it affected Aβ production. We found that full-length FE65 significantly decreased secreted Aβ42 levels by 55% in COS7 cells (p < 0.05) (data not shown). Thus, we confirmed that FE65 interacted with APP in COS7 cells and altered its processing.

We also asked whether FE65 affected APP processing in CHO cells and whether this processing depended on the apoE receptor LR. We transfected CHO cells containing or lacking LR with full-length APP; secreted APP was measured in conditioned media, and APP CTF was measured in cell lysates. In contrast to the findings in COS7 cells, we found that full-length FE65 strongly decreased secreted APP and APP CTF in CHO cells and altered its processing.
ApoEr2 and FE65

FIGURE 4. Co-localization of ApoEr2 and FE65. After 10 days in culture, primary cortical neurons were fixed and immunostained for 5810 and α-FE65. Antibodies were detected with Alexa Fluor 555 anti-rabbit antibody (in red) (left panel) and Alexa Fluor 488 anti-mouse antibody (in green) (center panel), observed with a confocal laser-scanning microscope (×40). Co-localization of ApoEr2 and FE65 appears yellow as vesicular staining in the right panel. Higher magnifications (×100) of neuronal processes are shown in the lower panels.

A. C-terminal of apoEr2

| TM | Exon | 18 | 19 | 20 |
|----|------|----|----|----|
|    |      | HA |    |    |
| 1  |      | HA |    |    |
| 2  |      |    | HA |    |
| 3  |      |    |    | HA |
| 4  |      |    |    |    |

B. ER2

| ER2 | 1 | 2 | 3 | 4 |
|-----|---|---|---|---|
|     |   |   |   |   |

C. ER2

| ER2 | 95 | 95 |
|-----|----|----|
|     |    |    |

D. ER2

| ER2 | 95 |
|-----|----|
|     |    |

FIGURE 5. FE65 interacts with the NPXY motif of ApoEr2. A, constructs of the ApoER2 cytoplasmic domain with HA tags were generated, including exons 18 and 19 (construct 1), exons 19 and 20 (construct 2), exons 18 and 20 (construct 3), and exons 18–20 (construct 4). B, proteins of expected sizes were expressed to similar levels from each ApoER2 construct, as determined by Western blotting. C, COS7 cells were transfected with different ApoER2 C-terminal constructs (indicated along the top of the film) and FE65. Cell lysates (200 µg) were immunoprecipitated (IP) with anti-HA antibody and probed with anti-Myc antibody. Only ApoER2 constructs containing exon 18 immunoprecipitated with FE65. D, COS7 cells were transfected with FE65 and wild-type ApoER2 (lanes 1 and 3) or an ApoER2 NPXY to APXY mutant (lanes 2 and 4). Cell lysates (200 µg) were immunoprecipitated with anti-HA antibody and probed with anti-Myc antibody. The ApoER2 APXY mutant showed decreased co-immunoprecipitation with FE65. ApoER2 and FE65 immunoblots (middle and lower panels) of cell lysates showed that ApoER2 was expressed at similar levels in all transfected cells.
ApoEr2 and FE65

**FIGURE 6. PTB1 domain of FE65 interacts with ApoEr2.** A, constructs of FE65 with C-terminal Myc tags containing PTB1 and PTB2 (construct 1), WW and PTB1 (construct 2), only PTB2 (construct 3), and full-length FE65 (construct 4). B, lysates from FE65-transfected cells (20 μg) were probed with anti-Myc to demonstrate protein expression (construct 1, 65 kDa; construct 2, 85 kDa; construct 3, 85 kDa; and construct 4, 95 kDa). C, COS7 cells were transfected with ApoEr2 and the FE65 constructs indicated along the top of the film. Cell lysates (200 μg) were immunoprecipitated with anti-HA antibody and probed with anti-Myc antibody. FE65 constructs containing the PTB1 domain (constructs 1, 2, and 4) immunoprecipitated (IP) with ApoEr2. An ApoEr2 immunoblot (lower panel) showed that ApoEr2 was expressed to similar levels in all transfected cells.

**FE65 Co-localizes with ApoEr2 in Primary Neuronal Cells**—To develop an assay for protein clustering, we performed immunocytochemistry on primary neuronal cells. Cells were fixed and immunostained with 5810 (recognizing ApoEr2) and anti-FE65 antibodies. ApoEr2 immunoreactivity was strong in all neurons and was punctuate throughout processes (Fig. 4, left panel). Similarly, FE65 was present in cell bodies and expressed in a punctate pattern throughout neuronal processes, but only some of neurons were FE65 positive (Fig. 4, middle panel). ApoEr2 co-localized with FE65 within the neuronal processes and cell bodies (Fig. 4, right panel). Higher magnifications of co-localizations are shown in Fig. 4, lower panels.

**ApoEr2 Exon 18 (Containing the NPXY Motif) Interacts with FE65**—To determine which domain of ApoEr2 interacts with FE65, we co-transfected COS7 cells with full-length FE65 and ApoEr2 constructs of different C termini with HA tags (Fig. 5A, constructs 1–4). Proteins of expected sizes were expressed to similar levels from each ApoEr2 construct, as determined by Western blots (Fig. 5B). We immunoprecipitated ApoEr2 C termini with the anti-HA antibody and probed for FE65 with anti-c-Myc antibody. FE65 co-precipitated with ApoEr2 constructs possessing exon 18 (constructs 1, 3, and 4) but not the one lacking exon 18 (construct 2) (Fig. 5C). The specificity of the co-precipitation of FE65 with only some ApoEr2 constructs again suggests that the interaction is not due to overexpression of proteins in the same cells. An FE65 immunoblot (Fig. 5C, lower panel) showed that FE65 was expressed to similar levels in all transfected cells.

To test whether the NPXY motif of ApoEr2 was important in the binding of ApoEr2 to FE65, we mutated the NPXY sequence to APXY in a full-length ApoEr2. We transfected COS7 cells with FE65 and wild-type or mutant ApoEr2. ApoEr2 was immunoprecipitated with the anti-HA antibody, and precipitates were probed for FE65 with anti-c-Myc antibody. Mutant ApoEr2 showed significantly less co-precipitation with FE65, to 65% of the level of wild-type ApoEr2 (p < 0.05) (Fig. 5D). The levels of ApoEr2 and FE65 did not vary across conditions (Fig. 5D, middle and lower panels). There was still some interaction observed between ApoEr2 (APXY) and FE65, suggesting that alteration of the asparagine residue was not sufficient to prevent FE65 binding, and the larger context of the cytoplasmic domain of ApoEr2 contains other recognition sequences for FE65. However, the reduced interaction does suggest that the NPXY motif of ApoEr2 is important in the interaction with FE65.

**ApoEr2 Interacts with the N-terminal PTB1 Domain of FE65**—After examining which domain of ApoEr2 interacted with FE65, we examined which domain of FE65 interacted with ApoEr2. In addition to full-length FE65, we examined deletion constructs of the PTB1 and PTB2 domains, the WW and PTB1 domains, and only the PTB2 domain (Fig. 6A). We co-transfected COS7 cells with full-length ApoEr2 and these FE65 deletion mutants. Expected protein sizes were expressed from each construct, as determined by Western blots with anti-c-Myc antibody (Fig. 6B). We found that all three constructs containing the FE65 PTB1 domain co-precipitated with ApoEr2, but the construct with only the PTB2 domain did not, even after overexposure of the blots. Western blot analysis of COS7 cell extracts confirmed that levels of total ApoEr2 were consistent across transfections (Fig. 6C, lower panel). Thus, the interaction of FE65 with ApoEr2 depends on PTB1.

**FE65 Increases ApoEr2 Processing and Levels of Cell Surface ApoEr2**—We tested whether the interaction of FE65 with ApoEr2 affected processing of ApoEr2. COS7 cells were transfected with full-length ApoEr2 alone or ApoEr2 and each of the four FE65 constructs (Fig. 7A). Western blots demonstrated that each of the four FE65 constructs were expressed in transfected cells (Fig. 7B). Secreted ApoEr2 was measured in condi-
**ApoEr2 and FE65**

**A. COS7**

| FE65 | 1 | 2 | 3 | 4 |
|------|---|---|---|---|
|      |   |   |   |   |
| 110  |   |   |   |   |
| 25   |   |   |   |   |

| sApoEr2 | Total ER2 | ER2 CTF |
|---------|-----------|---------|
|         |           |         |
| 15      |           |         |
| 95      |           |         |
| 60      |           |         |

α-myc

**B. COS7**

| FE65 | 1 | 2 | 3 | 4 |
|------|---|---|---|---|
|      |   |   |   |   |
| 110  |   |   |   |   |
| 25   |   |   |   |   |

| sApoEr2 | Total ER2 | ER2 CTF |
|---------|-----------|---------|
|         |           |         |
| 15      |           |         |
| 95      |           |         |
| 60      |           |         |

**C. CHO**

| FE65 | 1 | 2 | 3 | 4 |
|------|---|---|---|---|
|      |   |   |   |   |
| 110  |   |   |   |   |
| 25   |   |   |   |   |

| sApoEr2 | Total ER2 | ER2 CTF |
|---------|-----------|---------|
|         |           |         |
| 15      |           |         |
| 95      |           |         |
| 60      |           |         |

**D. Cell surface labeling**

| PTB2 | FL |
|------|----|
| 110  | 110|

| Surface ER2 | Total ER2 |
|-------------|-----------|
|             |           |
|             |           |

**E. Cell surface staining**

| DiC | ApoEr2 |
|-----|--------|
|     | PTB2   |
|     | FL     |

**FLFE65**

**FIGURE 7.** **FE65 affects ApoEr2 processing.** A, COS7 cells were transfected with ApoEr2 and vector (lane 1) or FE65 constructs (lanes 2–5). Secreted ApoEr2 was measured in conditioned media (15 μl) with antibody 5810, and ApoEr2 CTF were detected in cell lysates (20 μg) with anti-HA. Full-length FE65 (lane 4) and the construct containing PTB1 and PTB2 domains (lane 1) increased secreted ApoEr2 and ApoEr2 CTF. B, proteins of expected sizes were expressed to similar levels from each FE65 construct, as determined by Western blots. C, CHO cells were transfected with ApoEr2 and vector (1st and 3rd lanes) or ApoEr2 and FE65 (2nd and 4th lanes). Secreted ApoEr2 was measured in conditioned media (15 μl) with 5810 antibody, and ApoEr2 CTF was measured in cell lysates (20 μg) with α-HA. FE65 decreased secreted ApoEr2 and ApoEr2 CTF. Immunoblot of cell lysates showed similar levels of ApoEr2 (middle panel). D, COS7 cells were transfected with ApoEr2 and FE65 PTB2 or ApoEr2 and full-length FE65 (FL). Cell surface proteins were biotin-labeled, isolated with avidin beads, and immunoblotted with 5810 for ApoEr2. Full-length FE65 increased surface levels of ApoEr2 (upper blot). Cell lysates showed similar levels of total ApoEr2 (lower blot). E, HEK cells were transfected with ApoEr2 and FE65 PTB2 (upper panel) or ApoEr2 and full-length FE65 (lower panel). Cells were incubated with antibody 5810 and Alexa Fluor 555 anti-rabbit antibody (red color, right panel). Cells were imaged with differential interference contrast (DIC, left panels). Stained HEK cells were imaged at ×63. Full-length FE65 increased surface levels of ApoEr2 (lower panel).
FE65 knock-out brains did not have the 95-kDa FE65 band by immunoblots (Fig. 8A). We immunoprecipitated ApoEr2 from brain lysates, and we found that an FE65 immunoreactive band was only present in wild-type and not FE65 knock-out brain lysates (Fig. 8B, left panel). Similarly, immunoprecipitation of FE65 resulted in co-precipitation of ApoEr2 only in the wild-type brain lysates (Fig. 8B, right panel).

Total levels of ApoEr2 were unchanged in the FE65 knock-out brains (Fig. 8B), as were levels of the control protein β-actin (Fig. 8C). Levels of ApoEr2 CTF, however, were markedly decreased in the FE65 knock-out brains. Thus, similar to findings in transfected cells, where increased FE65 led to increased ApoEr2 CTF, decreased FE65 in vivo led to decreased ApoEr2 CTF (Fig. 8C).

Full-length FE65 Increased Co-immunoprecipitation of APP with ApoEr2—Because FE65 PTB1 interacts with ApoEr2 and FE65 PTB2 interacts with APP, we hypothesized that FE65 may form an intracellular link between ApoEr2 and APP. To test for the ApoEr2-FE65-APP complex, we transfected COS7 cells with ApoEr2, APP, and full-length FE65 or ApoEr2, APP, and only the PTB2 domain of FE65. ApoEr2 was immunoprecipitated with anti-HA antibody, and APP was measured with 6E10. Full-length FE65 significantly increased co-precipitation between APP and ApoEr2 compared with the PTB2 alone (Fig. 9A, upper panel). No differences in APP precipitation were observed when PTB2 was expressed compared with cells not transfected with FE65 (data not shown). Levels of total APP and ApoEr2 in cell extracts were consistent across transfections (Fig. 9A, lower panels), and both FE65 constructs were strongly expressed as determined by Western blot (Fig. 9B). Quantification of Western blots demonstrated that full-length FE65 increased co-immunoprecipitation between APP and ApoEr2 by 130% (p < 0.01).

To test whether the APP-FE65-ApoEr2 complex was affected by the FE65-interacting protein LRP, we transfected CHO LRP+/+ or CHO LRP−/− cells with ApoEr2, APP, and full-length FE65 or ApoEr2, APP, and vector. APP was immunoprecipitated with 369 antibody, and ApoEr2 was measured with 5810. ApoEr2 was detected in precipitates from wild-type lysates but not FE65 knock-out brains (Fig. 9C, 5th to 8th lanes). In contrast, FE65 decreased co-precipitation of APP and ApoEr2 in CHO LRP+/+ cells (Fig. 9C, 1st to 4th lanes). Quantification of triplicate experiments demonstrated that levels of co-precipitation between APP and ApoEr2 were significantly increased by 154% in CHO LRP−/− cells but significantly decreased by 166% in CHO LRP+/+ cells (p < 0.05). Levels of total APP and ApoEr2 in cell extracts were consistent across transfections (Fig. 9C, lower panels). Unexpectedly, the LRP+ cells showed higher levels of co-precipitation of APP and ApoEr2 (Fig. 9C, compare 1st and 3rd lanes with 5th and 7th lanes). The data with FE65 co-expression suggest that LRP could compete with ApoEr2 for binding to FE65.

To test whether FE65 affected APP processing in the presence of ApoEr2 in CHO cells, we transfected CHO LRP+/+ cells or CHO LRP−/− cells with ApoEr2, APP, and FE65 or with ApoEr2, APP, and vector. In CHO LRP+/+ cells, FE65 slightly decreased levels of APP CTF and secreted APP (Fig. 9D) but increased levels of APP CTF and secreted APP in CHO LRP−/− cells (Fig. 9E). These data suggest further that LRP alters the effects of FE65 on APP processing.

To test whether the observed effects of FE65 on APP processing (Fig. 1) depended on the kind of protein complexes that we hypothesize here, we transfected COS7 cells with ApoEr2
and APP alone or with each of the FE65 constructs from Fig. 4. Full-length FE65 (construct 4) and the construct containing both PTB1 and PTB2 domains (construct 1) increased levels of APP CTF and secreted APP, but the constructs containing the PTB1 domain (construct 2) or the PTB2 domain (construct 3) did not affect either APP proteolytic product (Fig. 10A).

To more directly test the effect of ApoEr2 on APP processing, we transfected COS7 cells with APP, FE65, and ApoEr2 or APP, FE65, and vector. ApoEr2 increased levels of APP CTF and secreted APP in COS7 cells (Fig. 10B). These data suggest that ApoEr2 alone can affect APP processing in COS7 cells, but ApoEr2-FE65 complexes could also greatly affect APP processing.

We also tested whether the FE65 constructs had differential effects on production of another APP proteolytic product, Aβ42. As expected, full-length FE65 and the construct containing both PTB1 and PTB2 domains of FE65 significantly decreased secreted Aβ42 levels compared with COS7 cells transfected with APP and ApoEr2 alone (by 75 and 88%, respectively, \( p < 0.01 \)) (Fig. 10C). Surprisingly, the other FE65 constructs (constructs 2 and 3) also caused reductions in secreted Aβ42 levels, although these changes were significantly smaller than the reduction seen with full-length FE65. These data suggest that the effects of FE65 on APP processing require interactions with apoE receptors.

**DISCUSSION**

We report that the cytoplasmic adaptor protein FE65 interacts with the apoE receptor, ApoEr2 (Figs. 2, 3, and 8), through the NPXY sequence in exon 18 of ApoEr2 (Fig. 5). The interaction involves the PTB1 domain of FE65 (Fig. 6), similar to the interaction of FE65 with another apoE receptor, LRP1 (24). Co-immunoprecipitation data suggest that FE65 forms a cytoplasmic link between ApoEr2 and APP (Fig. 9), again similar to findings with LRP1 (19). FE65 increased \( \alpha \)-cleavage of both APP (Fig. 1) and ApoEr2 (Fig. 7) in COS7 cells. The lack of FE65 in knock-out mouse brains was associated with decreased levels of ApoEr2 CTF, supporting the importance of this interaction in vivo (Fig. 8). Interestingly, the effects of FE65 on APP and ApoEr2 processing require both FE65 PTB domains (Figs. 7 and 10), suggesting that the cytoplasmic link between APP and ApoEr2 provided by FE65 is important for the changes in processing.

Studies have differed in the observed effects of FE65 family members on APP processing. We found in COS7 cells that FE65 increased APP \( \alpha \)-cleavage and decreased Aβ production (Fig. 10), perhaps by increasing the presence of the molecules on the cell surface (Fig. 7). FE65 family members increased surface APP in Madin-Darby canine kidney cells (11) and H4 cells (25). In contrast, FE65 decreased APP \( \alpha \)-cleavage in CHO cells (26), which we confirmed (Fig. 1). The reduction in \( \alpha \)-cleavage was not found when we used the FE65 construct lacking the WW domain (data not shown), suggesting that the effect may depend on a protein interacting with the WW domain. The increase in \( \alpha \)-cleavage in COS7 cells by FE65 did not depend on the WW domain (Fig. 7), demonstrating that the effects in different cell types depend on different interacting proteins. Some may also be due to the formation of a newly recognized form of cleaved FE65, lacking the N terminus but retaining the two PTB domains (27). FE65 can also be phosphorylated (28), which could alter its effects on APP and ApoEr2 metabolism in cell type-specific ways.

This work contributes to the developing model for a role for ApoEr2, FE65, and APP in cell migration. FE65 interacts with molecules important in cytoskeleton remodeling through its WW domain (6) and promotes movement of neuronal growth cones (29) and cell motility (7). Mice lacking FE65 and its homologue FE65L1 display lissencephalies indicative of developing neurons migrating into the superficial layers of the cortex.
ApoEr2 and FE65

A.

COS7

|       | ER2 | APP | sAPP | Total APP | APP CTF |
|-------|-----|-----|------|-----------|---------|
| 110  | -   | +   | 110  | +         | +       |
| 15   | -   | +   | 15   | +         | +       |
| 110  | -   | +   | +    | +         | +       |

B.

FE65  |    | 1   | 2   | 3   | 4

|       | APP/ER2 | sAPP | APP CTF | Total APP | Total ER2 |
|-------|----------|------|---------|-----------|-----------|
| 110  | +        | 110  | +       | +         | +         |
| 15   | +        | 15   | +       | +         | +         |
| 110  | +        | +    | +       | +         | +         |

C.

FIGURE 10. Full-length FE65 increased α-cleavage of APP and decreased ApoEr2. A, COS7 cells were transfected with APP and ApoEr2 in the absence or presence of the FE65 constructs from Fig. 6 (indicated across the top of the film). Secreted APP was measured in conditioned media (15 μl) with antibody 6E10, and APP CTF was detected from cell lysates (20 μg) with antibody C1/6.1. Full-length FE65 (construct 4) and the construct containing PTB1 and PTB2 domain of FE65 (construct 1) increased secreted APP and APP CTF (upper panel); FE65 constructs containing either PTB1 (construct 2) or PTB2 (construct 3) did not alter APP proteolytic fragments. Immunoblots showed similar levels of total cellular ApoEr2 and APP (lower panels). B, COS7 cells were transfected with APP and FE65 in the absence or presence of the ApoEr2. Secreted APP was measured in conditioned media (15 μl) with antibody 6E10; APP CTF was detected from cell lysates (20 μg) with antibody C1/6.1. ApoEr2 increased secreted APP and APP CTF in the presence of FE65. C, COS7 cells were transfected with ApoEr2, APP, and the indicated FE65 constructs. Ab42 levels in the conditioned media were determined by enzyme-linked immunosorbent assay. Full-length FE65 and the construct containing PTB1 and PTB2 domains significantly decreased secreted Ab42 levels (by 75 and 86%, respectively, p < 0.01).

extracellular matrix proteins that affect neuronal migration interact with APP (F-spondin) and ApoEr2 (F-spondin and Reelin). Binding of extracellular molecules to the transmembrane APP and ApoEr2 proteins could affect cytoskeleton remodeling through effects on FE65.

The increase in APP-ApoEr2 co-precipitation in the presence of FE65 in COS7 cells and in CHO LRP−/− cells (Fig. 9) suggests that FE65 forms an intracellular link between APP and ApoEr2. It is possible that the presence of FE65 actually increases a direct interaction between APP and ApoEr2 by changing their trafficking or removing some other protein that prevents their interaction. The connections between APP and ApoEr2 are another example of links between APP and apoE receptors. APP interacts with the apoE receptor LRP1 extracellularly through the KPI domain of APP (15, 16). There is also an intracellular link between APP and LRP (18, 19), as first suggested by the finding that FE65 could bind LRP through its PTB1 domain (13). APP interacts through cytoplasmic domains with LRP. We have reported a link between the APP and ApoEr2 extracellular domains through the extracellular matrix molecule, F-spondin (1). F-spondin binds APP (32) through an N-terminal domain and ApoEr2 through its C-terminal thrombospondin domain (1). The data in this study suggest that there is also an intracellular link via FE65. Thus, apoE receptors, and perhaps apoE itself, affect APP trafficking and proteolysis.

Our findings with LRP-containing and LRP-lacking cells suggest that ApoEr2 and LRP compete for binding of FE65 (Fig. 9). The presence of LRP in CHO cells would bind the overexpressed FE65, preventing it from binding to ApoEr2 and thus complexing intracellularly with APP. Neurons express each of these proteins (34) and thus individual FE65 molecules in neurons could bind to LRP or ApoEr2. The basal levels of APP-ApoEr2 co-precipitation differed between LRP+ and LRP− cells (Fig. 9). It is possible that APP and apoE receptors form larger complexes on the cell surface, and thus the presence of LRP has complex effects on trafficking of these proteins. The coordinated functions of APP and apoE receptors is underscored by the similarities of intracellular binding proteins (e.g. FE65, Dab-1, and JIP-1).

FE65 bound to the cleaved C terminus of APP is important to several signaling mechanisms of APP as the complex is transported from the cell membrane (27). FE65 bound to ApoEr2 could also affect signaling processes through cleavage of its C terminus (22) or prevent translocation of the APP C terminus to the nucleus. Binding of FE65 to LRP decreases the transcriptional regulation associated with FE65 binding to the APP intracellular domain (35); it seems likely that FE65 binding to ApoEr2 similarly affects this signaling. Binding of FE65 to LRP versus ApoEr2 would also be expected to greatly affect the intracellular trafficking of FE65. LRP and ApoEr2 have very different rates of internalization at the cell surface, with LRP recycling in 30 s and ApoEr2 recycling in 8 min (36). Thus, the binding of FE65 to ApoEr2 rather than LRP could greatly affect its subcellular location between the cell membrane and endosomal compartments.

In summary, we found that FE65 binds ApoEr2 and alters its trafficking and processing. Understanding the competing bind-
ing of intracellular adaptor proteins will be vital in describing the normal regulation of transmembrane proteins like apoe receptors and APP.

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**Note Added in Proof**—Subsequent experiments in FE65 knock-out adult brain tissue did not confirm the decrease in ApoEr2 CTF (Fig. 8C).

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