Regulation of β-Amyloid Secretion by FE65, an Amyloid Protein Precursor-binding Protein*

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The principal component of Alzheimer's amyloid plaques, Aβ, derives from proteolytic processing of the Alzheimer's amyloid protein precursor (APP). FE65 is a brain-enriched protein that binds to APP. Although several laboratories have characterized the APP-FE65 interaction in vitro, the possible relevance of this interaction to Alzheimer's disease has remained unclear. We demonstrate here that APP and FE65 co-localize in the endoplasmic reticulum/Golgi and possibly in endosomes. Moreover, FE65 increases translocation of APP to the cell surface, as well as both αAPP, and Aβ secretion. The dramatic (4-fold) FE65-dependent increase in Aβ secretion suggests that agents which inhibit the interaction of FE65 with APP might reduce Aβ secretion in the brain and therefore be useful for preventing or slowing amyloid plaque formation.

Amyloid plaques are one of the major hallmarks of Alzheimer's disease (AD) pathology. The plaque core is largely composed of an approximately 4-kDa peptide referred to as Aβ (1, 2). Because mutations linked to AD have been shown to increase secretion of Aβ (3), secreted Aβ is believed to play a causative role in AD etiology. The precursor to Aβ is the Alzheimer's amyloid protein precursor (APP) (4, 5). APP is a type I integral membrane protein, the majority of which is found in the ER/Golgi (6). A fraction of APP is transported to the plasma membrane, then routed through the endosomal/lysosomal system (6–12).

At least three unidentified proteases, known as the α-, β-, and γ-secretases, process APP (7, 13–16). The combination of β and γ cleavages generates Aβ. α-Secretase cleaves APP within the Aβ domain, releasing aAPP, the large extracellular domain. While aAPP is generated primarily at or en route to the plasma membrane (17), Aβ is formed in both the secretory and endocytic pathways (8). It has been suggested that the majority of secreted Aβ is made in the endocytic pathway (8).

Several studies have shown that the cytoplasmic tail of APP is important for the regulation of APP metabolism and localization. The carboxyl terminus of APP contains the sequence YENPTY. NPXY is a consensus sequence for endocytosis of low density lipoprotein receptors (18). Deletion of portions of APP that contain the YENPTY sequence results in increased secretion of APP, and decreased secretion of Aβ (8, 19–22). The effects of these deletions are thought to be the result of increased APP at the cell surface. Mutation of the second tyrosine in the YENPTY sequence to alanine also increases APP secretion but has no effect on Aβ secretion (23). These observations suggest that secretion of Aβ and APP may be regulated independently by signals in the cytoplasmic tail of APP.

FE65 is a brain-enriched protein of unknown function (24) that binds to the cytoplasmic domain of APP. FE65 contains two types of protein-protein interaction domains: a WW domain in the amino terminus and tandem phosphotyrosine interaction domains (PIDs) in the carboxyl terminus. WW domains recognize poly-proline sequences (25), whereas PIDs typically recognize phosphorylated NPXY sequences (26).

FE65 was first shown to interact with APP in the yeast two-hybrid system (27, 28). Subsequently, several studies have shown that FE65 binds directly to the YENPTY sequence in the cytoplasmic domain of APP through its carboxyl-terminal-most PID (28–31). Although it was previously thought that PIDs bind to tyrosine–phosphorylated sequences, the FE65-APP interaction is phosphorylation-independent (29, 31). Because NPXY sequences are known to be involved in molecular targeting and FE65 binds to the YENPTY sequence in APP, we hypothesized that FE65 is an important regulator of Aβ secretion. In this study, we have investigated the effects of FE65 on the metabolism and trafficking of APP.

EXPERIMENTAL PROCEDURES

FE65 Constructs—Rat FE65 was subcloned into pcDNA3 (Invitrogen, Carlsbad, CA), a mammalian expression vector, by polymerase chain reaction. The 5' polymerase chain reaction primer encoded an amino-terminal FLAG epitope tag.

Antibodies—Polyclonal antibodies to FE65 were raised by immunizing rabbits with the WW domain of FE65 (25) fused to glutathione S-transferase. The antibodies were tested for their specificity in immunoblots and immunoprecipitations by competition with antigen. Two polyclonal antibodies demonstrating high affinity and specificity (170 and 173) were combined and affinity purified on a CNBr-activated Sepharose-WW domain column (Amersham Pharmacia Biotech). Fig. 1, a and b show the specificity of the antibodies for immunoblottting and immunoprecipitation, respectively. In both cases, the antibodies recognized a band around 100 kDa that was greatly increased in intensity.
upon transfection with FE65 cDNA. S3A/107 (8), a mixture of monoclonal APP antibodies used for the immunofluorescence studies, were a generous gift of E. H. Koo. 369, a polyclonal antibody, was used to immunoprecipitate holoAPP (32). Monoclonal Aβ antibodies 6E10 and 4G8 were used for immunoprecipitation and ELISA (33).

Stable Cell Lines—To determine the effects of FE65 on APP, a cell line was needed that expressed low levels of endogenous FE65, therefore permitting manipulation of FE65 protein levels by overexpression. For this purpose, Madin-Darby canine kidney cells (MDCK), which overexpress the 695-amino acid isoform of APP (MDCK-695) were stably transfected with FE65 cDNA. MDCK-695 cells were a generous gift of C. Haass (34). Cells were stably transfected with FE65 in 10-cm diameter plates using the calcium phosphate transfection system (Life Technologies, Inc., Grand Island, NY), essentially following the manufacturer instructions. Each plate was transfected with 15 μg of FE65 in pcDNA3 and 5 μg of pPUR (CLONTECH), a selection vector containing a puromycin resistance gene. After selection with 2.5 μg/ml puromycin, individual clones were isolated using cloning rings. Clonal cell lines with high expression of FE65 (MDCK-695/FE65) were identified by immunoblotting (Fig. 1a) and immunoprecipitation (Fig. 1b) with FE65 antibodies. These cells were maintained in Dulbecco’s Modified Eagle’s medium (DMEM) containing 200 μg/ml G418 (Life Technologies, Inc.) and 1 μg/ml puromycin. Fig. 1c shows APP expression in representative MDCK-695 and MDCK-695/FE65 cell lines. Levels of APP expression did not vary with FE65 expression. Control cell lines, obtained by transfection of MDCK-695 cells with vector, were simultaneously prepared by puromycin selection. No significant differences were seen in these control cell lines compared with MDCK-695 cells (data not shown). In parallel, cell lines were prepared that stably overexpressed FE65 but not APP (MDCK-FE65).

Transient Transfection—MDCK and MDCK-FE65 cells were transiently transfected with APP-751 cDNA. The transfections were performed with LipofectAMINE (Life Technologies, Inc.) as described in the manufacturer instructions. Briefly, a 10-cm diameter plate of cells was transfected with 10 μg of APP in pcDNA3 for 8–12 h. Cells were assayed approximately 48 h post-transfection.

Metabolic Labeling and Immunoprecipitation—Metabolic labeling and immunoprecipitation were performed essentially as described (33). Briefly, cells were plated at a density of 5.0 × 10⁵ cells/cm² and then grown for approximately 16 h. After washing, they were incubated with [35S]methionine (NEN Life Science Products) in methionine-free DMEM for 2 h at 37°C, followed by a 2-h chase at 37°C in complete DMEM. αAPP was immunoprecipitated from the chase medium with 6E10, and Aβ with 6E10 plus 4G8, followed in both cases by ag罗斯e-linked goat anti-mouse IgG (American Qualex, La Mirada, CA). Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and immunoblotting. Whole cellular holoAPP was determined by lysing cells with 1% Nonidet P-40 in PBS immediately after labeling, followed by immunoprecipitation with 369 and Sepharose-linked protein A (Amersham Pharmacia Biotech) (32). Values obtained for αAPP, and Aβ were normalized to this total labeled APP in each experiment.

ELISA—For the sandwich ELISA, cell medium was changed to fresh serum-free DMEM. After incubation for 4 h at 37°C, conditioned medium was collected and subjected to a sandwich ELISA (35) for Aβ using 6E10 as the capture antibody. For detection, samples were incubated with biotinylated 4G8 followed by alkaline phosphatase-labeled antibion. Aβ was quantified with a Dynatech plate reader. The values obtained were normalized to total cellular holoAPP determined by immunoblotting with 369 and [125I]protein A (Amersham Pharmacia Biotech) followed by PhosphorImager quantification.

Immunofluorescence—Cells were plated at 3.5 × 10⁶ cells/cm² on glass coverslips. Except where indicated, the cells were briefly pretreated with 0.02% saponin in 80 mm PIPES/KOH, pH 7.0, 1 mM MgCl₂, 1 mm EDTA, and 30% glycerol and then fixed in 4% paraformaldehyde in PBS with 0.12 μm sucrose for 10 min at 4°C. The coverslips were then incubated in PBS with 0.2% Triton X-100 for 2 min at room temperature, rinsed in PBS, and blocked with 10% bovine serum albumin in PBS. Primary antibody was incubated for 1 h at room temperature or overnight at 4°C, and secondary antibodies were added for 1 h at room temperature in PBS with 1% bovine serum albumin. The secondary antibodies used were goat anti-mouse IgG and goat-antirabbit IgG for APP and FE65, respectively, conjugated to rhodamine red-X and Oregon green-488 (Molecular Probes, Eugene, OR). Coverslips were mounted with DABCO in polyvinyl alcohol. For surface immunofluorescence, cells were blocked and incubated with primary antibody before fixation. In all cases, immunofluorescence was eliminated by omission of primary antibody or by competition with antigen (data not shown). Immunofluorescence was examined by deconvolution light microscopy (Deltavision) and confocal laser scanning microscopy (Zeiss LSM510).

Subcellular Fractionation—Iodixanol gradients were formed by layering successively less concentrated solutions of Optiprep (Life Technologies, Inc.) in a centrifuge tube and then placing the tube on its side for 3–4 h essentially as described in the manufacturer instructions. Optiprep was chosen because it is an iso-osmotic medium; it resolves ER, Golgi, and lysosomes extremely well; and continuous gradients can be made very easily and reproducibly without high speed centrifugation. MDCK-695/FE65 cells were gently scraped from 15-cm diameter plates and collected by centrifugation. They were then homogenized in 10 mM Hepes, pH 7.4, 1 mM EDTA, 0.25 M sucrose with a metal Dounce homogenizer. Nuclei, unbroken cells, large pieces of plasma membrane, and heavy mitochondria were removed by centrifugation at 3000 × g. The supernatant (PNS) was then centrifuged at 17,000 × g for 15 min. The 17,000 × g pellet was loaded on a preformed 10–30% continuous iodixanol gradient and centrifuged at 100,000 × g for 1 h. The gradients were unloaded by aspiration of 1-ml fractions from the top of the gradient. After centrifugation of the fractions at 100,000 × g to collect the membranes and remove the iodixanol, the 100,000 × g fractions were resuspended, boiled in sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Surface Biotinylation—MDCK cells were plated at 3.0 × 10⁴ cells per 10-cm diameter dish. After washing twice with Hanks’ balanced salt solution (HBSS, Sigma), cells were incubated with 0.5 mg/ml NHS-LC-biotin (Pierce) in HBSS at 4°C for 30 min. Unreacted biotin was eliminated by two washes with DMEM containing 10% fetal calf serum and two washes with HBSS. The cells were then lysed with 1% Nonidet P-40, and equal amounts of protein were subjected to immunoprecipitation with the APP antibody, 369. The immunoprecipitated material was then immunoblotted with anti-biotin monoclonal antibody (Sigma) and [125I]protein A. Cell surface APP was quantified by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

FE65 Alters the Proteolytic Processing of APP—When measured by sandwich ELISA, Aβ secretion increased 4.2 ± 0.5-fold (mean ± S.E., n = 12, p < 0.0001) in MDCK-695/FE65 cells when compared with MDCK-695 cells (Fig. 2a). Qualitatively similar results were observed by pulse-chase labeling followed...
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Fig. 2. FE65 causes an increase in secretion of Aβ from MDCK-695 cells (−FE65) and MDCK-695/FE65 cells (+FE65). a, conditioned medium (4 h) was subjected to a sandwich ELISA for Aβ using 6E10 as the capture antibody and 4G8 as the detection antibody. The data represent means ± S.E. (n = 12). b, cells were pulsed with [35S]methionine for 2 h followed by a 2-h chase to look at processing of newly synthesized Aβ. Aβ was immunoprecipitated from the chase medium with a 4G8/6E10 mixture and visualized by PhosphorImager. The autoradiogram represents results typical of those observed in three experiments with one FE65-expressing clone and two experiments with an additional FE65-expressing clone. *, p < 0.0001.

by immunoprecipitation (Fig. 2b). In all cases, values were normalized to holoAPP; therefore, the observed differences were not because of variation in total APP levels.

Immunoprecipitation of aAPP from conditioned medium of [35S]methionine pulse-labeled MDCK-695 and MDCK-695/FE65 cells demonstrated that overexpression of FE65 increased secretion of aAPP. During a 2-h chase, MDCK-695/FE65 cells secreted 1.7 ± 0.1-fold (n = 11, p < 0.0005) more aAPP than did MDCK-695 cells (Fig. 3a). Stable overexpression of FE65 enhanced release of aAPP from an independent MDCK cell line (MDCK-FE65) transiently transfected with the 751-amino acid isoform of APP by 2.2 ± 0.1-fold (n = 6, p < 0.0001), as compared with MDCK cells similarly transfected with APP-751 (Fig. 3b). Therefore, FE65 regulates APP metabolism independent of APP isoform.

Large increases in aAPP secretion are often accompanied by decreases in Aβ secretion, probably because of substrate depletion (33, 36–38). In contrast, lesser increases in aAPP can be accompanied by an increase in Aβ (38). In the present study, FE65 increased both aAPP and Aβ secretion. To determine whether substrate depletion occurred upon overexpression of FE65, we determined the approximate percentage of APP molecules secreted as either aAPP or Aβ. Over the time course of these studies, approximately 20% of APP labeled during the chase was converted to aAPP, and less than 10% to Aβ, consistent with the idea that holoAPP substrate was not rate-limiting.

APP and FE65 Co-localize in Perinuclear Organelles—FE65 and APP co-immunoprecipitate from homogenates of MDCK-695/FE65 cells (data not shown; and see Ref. 39) and other cell types (28, 31). To determine where FE65 and APP interact in intact cells, MDCK-695/FE65 cells were double labeled with a mixture of polyclonal FE65 antibodies, 170 and 173, and a mixture of APP monoclonal antibodies, 5A3 and 1G7 (8), and then examined by confocal microscopy. The two proteins co-localized in perinuclear organelles (Fig. 4) that may be ER/Golgi compartments and/or endosomes. The strong co-localization of APP and FE65 supports the hypothesis that APP and FE65 interact in vivo.

If the binding of FE65 to APP has a true physiological role in the brain, then FE65 and APP should co-localize in neural-derived cells that express both proteins at endogenous levels. H4 human neuroglioma cells were examined with the same antibodies that were used for the MDCK cells. FE65 and APP fluorescence again overlapped in juxta-nuclear organelles and at some edges of H4 cells (Fig. 5). These data suggest that the effects of FE65 on APP processing are mediated by the interaction of APP and FE65 in either the ER/Golgi or in the recycling pathway. They also suggest that the interaction may be an important regulator of APP processing in neurons, which express high endogenous levels of both proteins.

To further confirm that the interaction of FE65 and APP is likely to occur in MDCK cells in vivo and to characterize the compartments in which they interact, MDCK-695/FE65 PNS was separated into a 17,000 × g pellet and supernatant followed by separation on iodixanol gradients. The 17,000 × g pellet contains Golgi, lysosomes, light mitochondria, peroxisomes, and ER, whereas the 17,000 × g supernatant contains small vesicles, endosomes, and soluble proteins. FE65 and APP co-localized in the least dense organelles of the 17,000 × g pellet (Fig. 6a and b). There was a strong, sharp peak of both APP and FE65 in fraction 1, with a smaller, broader peak in fractions 3–6. Fraction 1 contains relatively pure Golgi (40, 41).
and was highly enriched in rab6, a Golgi marker (data not shown). Fractions 3–6 are highly enriched in ER (40, 41). Most of the APP seen in these fractions appeared to be immature as would be expected for ER-associated APP (Fig. 6a).

APP and FE65 also co-localized in the least dense fractions from the 17,000 × g supernatant (Fig. 6, c and d). Although these gradients have not been characterized well in the literature, fraction 1 was highly enriched in both rab11 and EEA1 (data not shown). Rab11 localizes to secretory vesicles, endosomes, and TGN (42), whereas EEA1 is an early endosomal marker (43). Therefore, fraction 1 contained endosomes. It may have also contained other membranes, such as small vesicles derived from the TGN, although it was not rab6 immunoreactive. Together our data suggest that there is a strong co-localization of APP and FE65 in the ER/Golgi and possibly in endosomes.

**FE65 Alters the Subcellular Localization of APP**—To visualize APP at the cell surface, MDCK-695 and MDCK-695/FE65 cells were labeled with the APP antibodies for immunofluorescence without prior permeabilization. FE65 overexpression caused an increase in high intensity surface APP labeling (Fig. 7). To ensure that the plasma membranes were intact, the cells were also labeled with the FE65 antibodies 170/173. No fluorescence was observed (data not shown), indicating that the APP labeling was indeed extracellular. FE65 overexpression did not alter the APP immunostaining patterns in permeabilized MDCK-695 and MDCK-695/FE65 cells (data not shown). Thus the FE65-dependent changes in the amount of APP localized to the plasma membrane were specific to that pool of APP.

To quantify the effects of FE65 on surface APP, MDCK-695 and MDCK-695/FE65 cells were surface biotinylated. After biotinylation, total APP was immunoprecipitated. The fraction of this APP that was biotinylated at the cell surface was identified by immunoblotting with anti-biotin antibodies. FE65 overexpression caused a 2.4 ± 0.37-fold (n = 9) increase in APP at the cell surface (Fig. 8). These data suggest that an FE65-dependent translocation of APP to the plasma membrane was responsible for the observed changes in the proteolytic processing of APP.

**DISCUSSION**

We have shown here that FE65 and APP are present in the same subcellular compartments and that FE65 expression increases the amount of APP at the plasma membrane. This
FIG. 7. FE65 causes translocation of APP to the plasma membrane. MDCK-695 (a) and MDCK-695/FE65 (b) cells were labeled with APP monoclonal antibodies 5A3/1G7 without prior permeabilization to label only APP at the cell surface. The images were colorized using a Zeiss glow scale table, which colors pixels in decreasing order of intensity as white, yellow, red, and black. The images shown are representative of multiple randomly selected fields from three experiments and were collected by confocal laser scanning microscopy.

translocation of APP to the cell surface is associated with a dramatic increase in $\alpha$-secretion concomitant with a smaller increase in secretion of $\alpha$APP$\beta$

Our data suggest that the observed increase in APP metabolism may be because of an increased flux of APP through the secretory pathway. Such an increase in trafficking would result in a larger fraction of APP reaching the plasma membrane. Increased flux of APP through the secretory pathway to the cell surface is predicted to result in increased $\alpha$-secretase cleavage of APP and, therefore, secretion of $\alpha$APP$\beta$. In addition, routing of more APP to the cell surface increases the amount of APP available for endocytosis. Because much of the $\alpha$-secreted by cultured cells is generated in the endocytic pathway (8), the observed increase in APP at the plasma membrane is a plausible source of the increased $\alpha$-secretion.

Many studies have shown that deletion of the YENPTY sequence of APP results in increased APP$\beta$ secretion and decreased $\alpha$-secretion (8, 19, 20, 22), whereas we have shown that overexpression of FE65, which binds to this sequence, can increase secretion of both APP$\alpha$ and $\alpha$-secretion. The difference in the effects of deletion of the YENPTY sequence versus FE65 overexpression is easily explained because the deletion is expected to inhibit efficient endocytosis of APP. If the cell surface APP is endocytosed at a decreased rate, less $\alpha$-APP will be produced. If FE65 does not interfere with endocytosis, its overexpression would not be expected to affect $\alpha$-secretion in the same way as the deletion. It is interesting to note that mutation of the second tyrosine in the YENPTY sequence, which does not affect FE65 binding, also does not affect $\alpha$-secretion (23).

FE65 may produce its effects on secretion of proteolytic fragments of APP by targeting of some other molecule to APP through its other protein-protein interaction domains. In addition to the PID that binds to APP, FE65 contains a WW domain and another, more amino-terminal, PID (27). One of several proteins that binds to the WW domain has recently been identified as mena (44), a member of the abl tyrosine kinase pathway that is known to localize to focal adhesions (45). It will be interesting to determine whether mena or other FE65 binding partners affect APP processing as well.

The results of the present study suggest that agents that inhibit the interaction of FE65 with APP might decrease $\alpha$-secretion in the brain. Such a reduction in $\alpha$-secretion might then result in decreased accumulation of amyloid plaques. Thus, discovery of an agent that inhibits the interaction of FE65 with APP could provide a novel approach to slowing or preventing the progression of Alzheimer’s disease.

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FIG. 8. FE65 increases APP at the cell surface. a, immunoprecipitation and immunoblotting of cell surface biotinylated APP from MDCK-695 cells (−FE65) and MDCK-695/FE65 cells (+FE65). b, quantification of APP at the plasma membrane by PhosphorImager. The data represent means ± S.E. (n = 9). *p = 0.0013.
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