Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 10832–10837, October 1996
Cell Biology

Association of a novel human FE65-like protein with the cytoplasmic domain of the β-amyloid precursor protein

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Communicated by Paul Greengard, Rockefeller University, New York, NY, July 2, 1996 (received for review May 13, 1996)

ABSTRACT **We identified a novel human homologue of the rat FE65 gene, hFE65L, by screening the cytoplasmic domain of β-amyloid precursor protein (βPP) with the "interaction trap." The cytoplasmic domains of the βPP homologues, APLP1 and APLP2 (amyloid precursor-like proteins), were also tested for interaction with hFE65L. APLP2, but not APLP1, was found to interact with hFE65L. We confirmed these interactions *in vivo* by successfully coimmunoprecipitating endogenous βPP and APLP2 from mammalian cells overexpressing a hemagglutinin-tagged fusion of the C-terminal region of hFE65L. We report the existence of a human FE65 gene family and evidence supporting specific interactions between members of the βPP and FE65 protein families. Sequence analysis of the FE65 human gene family reveals the presence of two phosphotyrosine interaction (PI) domains. Our data show that a single PI domain is sufficient for binding of hFE65L to the cytoplasmic domain of βPP and APLP2. The PI domain of the protein, Shc, is known to interact with the NPXYp motif found in the cytoplasmic domain of a number of different growth factor receptors. Thus, it is likely that the PI domains present in the C-terminal moiety of the hFE65L protein bind the NPXY motif located in the cytoplasmic domain of βPP and APLP2.**

Alzheimer disease is characterized by neuronal loss and a relatively high abundance of β-amyloid plaques and neurofibrillary tangles in the brains of affected individuals. β-amyloid plaques are primarily composed of an ≈4-kDa peptide called β-amyloid peptide (Aβ), which is a normal soluble proteolytic product derived from the β-amyloid precursor protein (βPP) (1, 2). Molecular investigations aimed at determining the pathogenic events leading to neurodegeneration in Alzheimer disease have demonstrated that either an increase in Aβ secretion (3, 4) or stoichiometric changes in the ratio of $A\beta_{1-42}/A\beta_{1-40}$ are brought about by familial Alzheimer disease-associated mutations in the APP and the recently identified presenilin (PS1 and PS2) genes (5, 44). Overexpression of βPP or the C-terminal moeity of βPP including the Aβ domain has been shown to be toxic to neurons (6–8). However, it is not clear in these experiments whether cell death is mediated directly by amyloidogenic Aβ or indirectly as a consequence of the overexpression of βPP/βPP C terminus. βPP is a member of a family of proteins that include the βPP-like proteins, APLP1 and APLP2 (9–11), that resemble type I cell surface receptors (12). Hence, the toxicity associated with overexpression of βPP and the βPP C terminus may be due to interference with normal βPP function.

βPP is transported to the cell surface following maturation in the Golgi complex and is then either secreted or reinternalized (13, 14). Two of the products of βPP processing are the secreted ectodomain of βPP and the Aβ peptide (15, 16). The interdependence of the processing pathways responsible for producing these βPP products has been illustrated by the observed decrease in the levels of Aβ generated when the secreted ectodomain of βPP is increased (17). The internalization of cell surface βPP occurs via the endocytic pathway and leads to the appearance of C-terminal fragments containing intact Aβ in lysosomes (18). The relationship between the internalization of βPP and the generation of Aβ is underscored by the finding that attenuation of Aβ release is accompanied by a reduction in βPP internalization (19). Internalization of βPP by clathrin-coated vesicles is required for entry of cell-surface βPP into the endosomal/lysosomal pathway, and this form of internalization of βPP uses the NPXY motif in the cytoplasmic domain of βPP (20, 21). Deletion of the YENPTY sequence of βPP not only impairs endocytosis of cell-surface βPP, but alters its intracellular trafficking by increasing its targeting into the endocytic pathway without first reaching the cell surface (22).

To identify the cellular factors and mechanisms responsible for mediating the intracellular trafficking and/or function of cell surface βPP, we screened for proteins that associate with the cytoplasmic domain of βPP. Using the interaction trap, we isolated one of three human homologues of the rat FE65 gene, which we have termed hFE65L. We demonstrate herein the interaction of hFE65L with βPP as well as its homologue, APLP2, by coimmunoprecipitation from mammalian cells.

EXPERIMENTAL PROCEDURES

Interaction Trap Screening. Screening for proteins that interact with the cytoplasmic domain of βPP was performed as described (23). DNA fragments coding for the cytoplasmic domains of human βPP (nt 2092–2235), APLP1 (5′-CCGGAATTCAGGAAGAAGCCCTACGGGGCTATC-3′ and 5′-CGCGGATCCTCAGGGTCGTTCCTCCAGGAAGCG-3′), and APLP2 (nt 2222–2365) were amplified using the polymerase chain reaction (PCR) with primers containing *Eco*RI and *Bam*HI sites on either end. The PCR products were subcloned into the pEG202 backbone and were sequenced to verify that the APP/APLP coding sequences were in-frame with the LexA coding sequence. We also confirmed the expression of these fusion proteins in yeast total protein extracts by Western blot analysis using an anti-LexA antibody (a gift from R. Brent, Massachusetts General Hospital, Boston). We initially transformed the yeast strain EGY48 that contained *LexAop-LEU2*, *LexAop-LacZ*, and LexA-βPP(649-695) with DNA from a galactose-inducible activation domain-cDNA fusion library constructed from 22-week-old human fetal brain [a gift from D. Krainc (Massachusetts General Hospital, Boston) and R. Brent] using the LiAc transformation protocol of Schiestl and Gietz (24). Approximately 2×10^6

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Abbreviations: Aβ, β-amyloid peptide; βPP, β-amyloid precursor protein; APP, β-amyloid precursor protein gene; HA, hemagglutinin; PI, phosphotyrosine interaction; CMV, cytomegalovirus.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U62325).

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yeast colonies were scraped, pooled, and frozen, and the library was plated at a multiplicity of 20× on galactose Ura$^-$ His$^-$ Trp$^-$ Leu$^-$ media. We isolated 68 Leu$^+$ colonies and streaked these to Ura$^-$ His$^-$ Trp$^-$ glucose plates and retested them on glucose Ura$^-$ His$^-$ Trp$^-$ Leu$^-$ glucose, Ura$^-$ His$^-$ Trp$^-$ Leu$^-$ galactose, Ura$^-$ His$^-$ Trp$^-$ glucose 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), and Ura$^-$ His$^-$ Trp$^-$ galactose X-Gal media to confirm that induction of the reporter genes was dependent on galactose. Of these 68 Leu$^+$ colonies, 39 showed galactose-dependent leucine prototrophy and blue color production on X-Gal medium. The inserts of rescued plasmids bearing the *TRP1* marker were amplified by PCR using the following primers: 5′-CTTGCTGAGTGGAGATGCC-3′ and 5′-GCCGACAACCTTGATTGG-3′; the products were classified by Southern blot analysis using the largest insert as a probe.

cDNA Cloning, Sequencing, and Computer Analyses. The cDNA insert of clone 4-2A capable of producing the highest β-galactosidase activity as a result of interaction with the cytoplasmic domain of βPP was sequenced using the modified Sequenase T7 DNA polymerase (United States Biochemical) for the dideoxy-chain termination method (25). To obtain a full-length open reading frame for the hFE65L gene, we screened a human kidney cDNA library constructed in λGT10 (CLONTECH) using the 4-2A insert as a probe and isolated the 61-19 clone. The cDNA insert from this clone was subcloned into the pSK+ Bluescript sequencing vector (Stratagene) and both strands were sequenced using oligonucleotide primers. Sequence comparisons were performed using the BLAST series of programs and protein motifs were identified using the ProfileScan program (ISREC WWW-Server). Similarities and identities reported are data obtained from BESTFIT analyses. We assembled hFE65 and hFE65L2 from nucleotide sequences recovered from the expressed sequence tag data base. hFE65 was assembled from GenBank accession nos. T09348, T31017, R67159, F07610, R87225, and D56274. hFE65L2 was assembled from GenBank accession nos. T54909 and R84592.

Northern Blot Analysis. The ^{32}P-labeled 4-2A *Eco*RI/*Xho*I cDNA fragment was used as a probe for hybrization to a nitrocellulose filter onto which 2 μg of poly(A)$^+$ RNA isolated from human tissues was immobilized (CLONTECH). The hybridization was performed at 42°C in the presence of 50% formamide and washed in 0.1× SSC and 0.1% SDS at 50°C.

Cell Transfections. The hFE65L sequence encoded by the entire 4-2A insert (C-terminal 203 amino acids) was expressed in mammalian cells under the control of the cytomegalovirus (CMV) promoter. To achieve this, we subcloned the *Hin*dIII/*Xho*I fragment of the 4-2A clone into the pCDNA3 vector (Invitrogen) such that the protein expressed is the product of the HA-tag-hFE65L cDNA fusion initially created in the construction of the activation domain-cDNA fusion library. As a negative control for coimmunoprecipitation experiments, we used a pCDNA3-derived plasmid, pTPR1HA, constructed with a human cDNA fragment that codes for an HA-tagged fusion protein that does not interact with the βPP cytoplasmic domain in the interaction trap. pTPR1HA [a gift from Anita Murthy (Massachusetts General Hospital, Boston) and Jim Gusella (Massachusetts General Hospital, Boston)] was constructed by subcloning the *Eco*RV/*Xho*I cDNA fragment of the activation domain-cDNA fusion library plasmid into the pCDNA3 backbone. Both of these constructs were transfected into COS7 African green monkey kidney cells, human H4 neuroglioma cells, H4 cells stably transfected with APP695 or APP751 (26), and human SY5Y neuroblastoma cells. Cells were transfected following the manufacturer's instructions with 10 μg of plasmid DNA using 50 μl of lipofectamine (GIBCO/BRL) per 100 mm culture dish and were harvested at ≈72 hr after transfection. Cells were routinely grown in DMEM supplemented with 25 mM Hepes, 4.5 mg/ml D-glucose, 2 mM glutamine, 20 units/liter penicillin, 100 mg/liter streptomycin, and 10% fetal bovine serum.

Immunoprecipitations and Western Blot Analyses. Cells were washed twice with cold PBS and directly lysed in 10 mM Tris (pH 8.0), 0.14 M NaCl, 0.025% NaN_3, 5 mM EDTA, and 1% Triton X-100 buffer containing 1 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. The lysates were centrifuged at 12,000 rpm for 10 min at 4°C in a microfuge. Total protein was estimated for the recovered supernatants with the BCA protein assay (Pierce) using BSA as a standard. Immunoprecipitations were performed on 250 μg of total protein using the anti-HA epitope tag monoclonal antibody, 12CA5 (Boehringer Mannheim). Immune complexes were collected with protein G–Agarose (Boehringer Mannheim), and then washed three times with lysis buffer. The immunoprecipitates were either heated at 55°C for 15 min or boiled for 5 min and resolved on a 7.5 to 15% gradient gel, transferred to polyvinylidene difluoride membrane (Pierce), and immunoblotted with the following antibodies: 22C11 (13), 369W (27), C7 (28), D2-I (29), and 12CA5 (30). The secondary antibody was either peroxidase-conjugated sheep anti-mouse Ig or peroxidase-conjugated donkey anti-rabbit Ig (Amersham). Immunoblotted proteins were detected with an Enhanced Chemiluminescence system (Amersham).

RESULTS

The βPP Cytoplasmic Domain Interacts with hFE65L. A homologue of the rat FE65 gene was isolated from a human fetal brain library using the interaction trap screen (23). We isolated 33 independent colonies showing that galactose-dependent transcriptional activation of the two reporter genes contained cDNA inserts of 1.1 to 1.3 kb that cross-hybridize to one another. The longest cDNA insert (clone 4-2A) codes for a 203 amino acid open reading frame that is 56% identical and 73% similar (Fig. 1) to the rat brain transcriptional activator, FE65 (31, 32).

To verify the specificity of this interaction, we transformed cells bearing the activation domain-hFE65L fusion with five lexA-fusion constructs containing cdc2, fus3, bicoid, the cys-rich region of βPP, and the GTPase activating protein-related domain of the neurofibromatosis I protein and found that transcriptional activation of the two reporters requires the expression of the cytoplasmic domain of βPP (data not shown).

hFE65L also Interacts with the Cytoplasmic Domain of APLP2. We also tested whether the activation domain-hFE65L fusion was able to activate transcription of both reporter genes in association with the cytoplasmic domains of APLP1 and APLP2. Our results indicate that hFE65L protein interacts with the cytoplasmic domain of APLP2 but not with that of APLP1 (data not shown).

Characterization of the hFE65L Gene and its Expression Pattern. We isolated a cDNA clone, 61–19, that overlaps with the insert of the 4-2A clone. This cDNA clone contains an insert of ≈2.3 kb. When assembled, these two clones represent 2.9 kb of hFE65L cDNA with a poly(A) tail on the 3′ end. There are three putative start methionines upstream of the FE65 homologous sequence (Fig. 1), with the third representing the best candidate for the translation initiation codon (33). However, we cannot exclude the possibility that the start methionine is positioned 5′ of the 61-19 sequence. The predicted open reading frame of clone 61-19 encodes 730 amino acids. Thus, the predicted hFE65L protein contains an extra 72 amino acids, 55 of these amino acids are N terminal to the start methionine of the rat FE65 protein (Fig. 1).

The predicted amino acid sequence of hFE65L is 51% identical and 70% similar to the overlapping 658 amino acids of the rat FE65 protein (Fig. 1). Searches of the GenBank data base with the open reading frame of the hFE65L revealed the

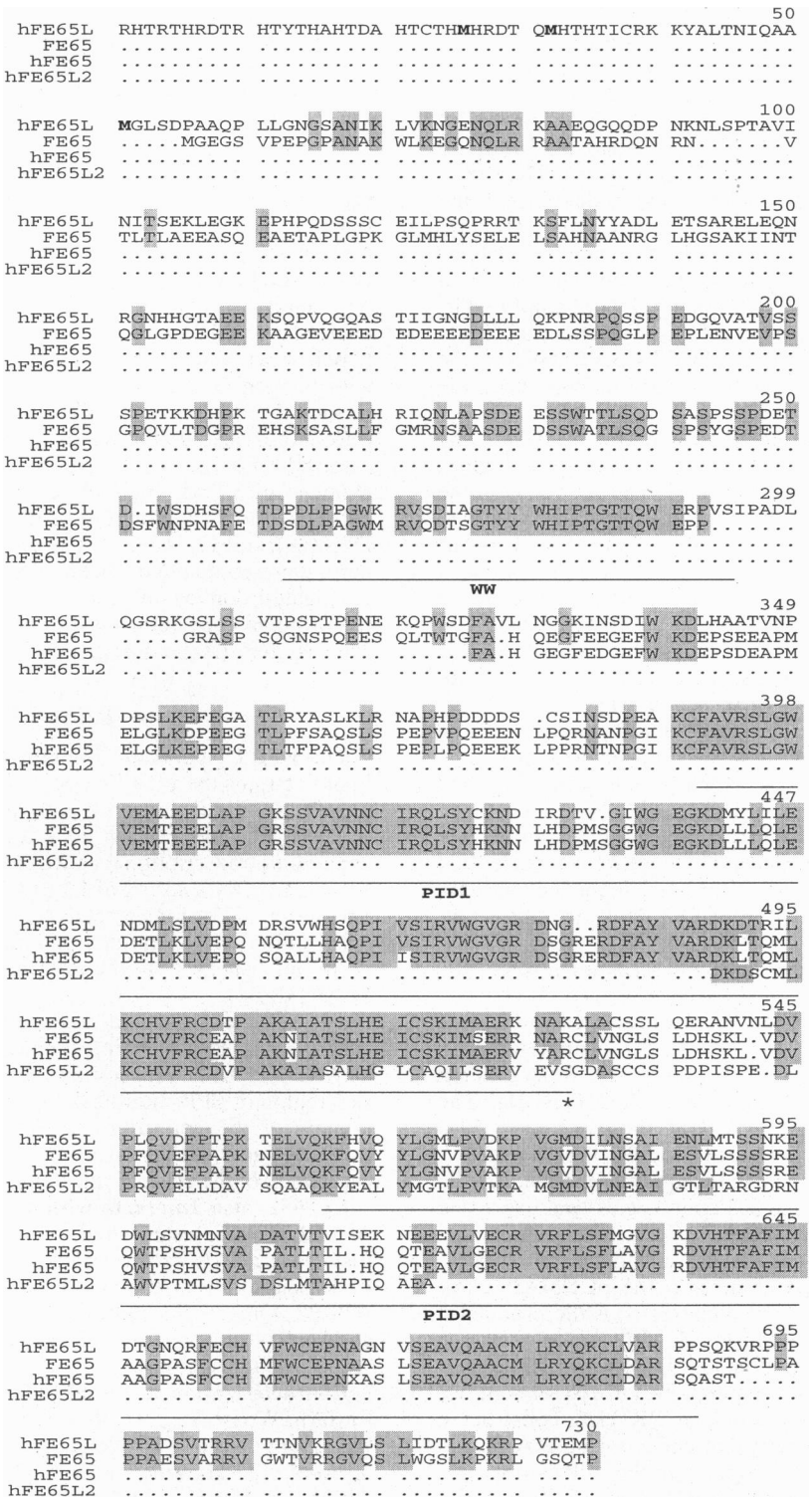

FIG. 1. Amino acid alignment of hFE65L with FE65 and two human homologues, hFE65 and hFE65L. Amino acid sequences were aligned using the PILEUP program. Shaded sequences represent FE65, hFE65, and hFE65L2 amino acids that are identical to those of the hFE65L predicted protein. The three putative start methionines are shown in boldface type. The WW and PI domains are underlined. The asterisk indicates the start of hFE65L coding sequence for the pCMV4-2A plasmid.

presence of several expressed sequence tags with conserved sequence. The assembled expressed sequence tags code for open reading frames of 366 and 129 amino acids, which we have named hFE65 and hFE65L2, respectively (Fig. 1). hFE65 codes for a polypeptide that, of the three, is most closely related to the rat FE65 protein with an amino acid identity of 95% and similarity of 96% over the 366 amino acids compared. hFE65L2 codes for a polypeptide that is 39% identical and 59% similar to rat FE65. This suggests that there is a family of three human genes homologous to the rat FE65 gene.

Interestingly, the hFE65L predicted protein contains several domains that provide clues to its function. These are a WW domain from amino acids 262 to 294, two phosphotyrosine interaction (PI) domains from amino acids 391-527 and 561-687, and a DNA-binding domain similar to a DNA-binding domain found in retroviral integrases from amino acids 436-

577. While these domains were initially identified in the rat FE65 protein (34, 35), the consensus sequences of these domains are conserved in the hFE65L predicted protein (Fig. 1).

To determine the expression pattern of the hFE65L gene and the size of the mRNA(s), we hybridized the human fetal brain cDNA insert from clone 4-2A to messenger RNA isolated from human tissues (Fig. 2). In contrast to the expression pattern for the rat FE65 gene for which a single transcript of 2.7 kb is detected (31), our results show that, with the exception of skeletal muscle that contains three additional transcripts sized 1.9, 4.4, and 5.4 kb, there are two major transcripts of ≈3.7 kb and 7.5 kb for the hFE65L gene in all human tissues tested. The latter pattern of expression was also observed in many different cell lines including COS7 and H4 cells (data not shown). In comparison, the rat FE65 transcript is brain specific (31).

βPP and APLP2 Can Be Coimmunoprecipitated with the C-Terminal 203 Amino Acids of hFE65L from Mammalian Cells. To further characterize the association between the hFE65L and the βPP family of proteins, we performed immunoprecipitation experiments on mammalian cells transiently transfected with an HA-tagged 4-2A cDNA insert under the control of the CMV promoter. Cell lysates obtained from COS7, H4, and SY5Y cells overexpressing the HA-tagged hFE65L C-terminal fusion protein were immunoprecipitated with the anti-HA antibody, 12CA5.

Western blot analyses of the immunoprecipitates were performed using the 22C11 antibody raised against the N-terminal portion of βPP. The 22C11 staining revealed proteins of approximately 140, 120, and 105 kDa in all three cell types examined (Fig. 3*A*) corresponding in size to mature βPP751, immature βPP751/mature βPP695 (unresolved in our gel system), and immature βPP695, respectively. As a negative control for the coimmunoprecipitation experiments, we used an HA-tagged fusion protein (Tpr1HA) that did not interact with the cytoplasmic domain of βPP in the interaction trap. Thus, coimmunoprecipitation of βPP is dependent on the expression of the HA-tagged hFE65L moiety. We simultaneously blotted this filter with the 12CA5 antibody to determine the amount of HA-tagged protein expressed and repeatedly observed that the level of HA-tagged protein produced was cell type-dependent with SY5Y > COS7 > H4 cells (Fig. 3*A*). Two 12CA5 immunoreactive bands of ≈40 kDa and ≈34 kDa (Fig. 3*A*) are produced from the pCMV4-2A plasmid bearing the HA-tagged hFE65L cDNA fusion. The full-length hFE65L fusion protein has a predicted molecular weight of 34 kDa. Thus, it is likely that the two first methionines in the open reading frame are being used since they are separated by ≈4 kDa. The pTPR1HA control reveals a single polypeptide of ≈44 kDa (Fig. 3*A*) and contains only the downstream of the two start methionines described for pCMV4-2A. The low levels of HA-tagged proteins in H4 cell extracts and differences in antibody affinities may explain the absence of endogenous βPP/APLP bands in the H4 lanes of Fig. 3 *B–D*.

The antibody, 369W, that recognizes the C-terminal fragment of βPP, also recognizes the full-length forms of βPP present in immunoprecipitates containing the HA-tagged FE65L fusion protein. Interestingly, we also observe the interaction of the 14 to 15 kDa C-terminal βPP fragment with the HA-hFE65L fusion protein (Fig. 3*B*) by coimmunoprecipitation.

Since both 22C11 and 369W recognize βPP and APLP2 (11), we also tested immunoprecipitates with C7, a βPP-specific (T.-W. Kim, R.E.T., and W. Wasco, unpublished results) rabbit polyclonal antibody directed against the C-terminal region of βPP. Using C7, we were able to confirm that βPP immunoreactivity is present in immunoprecipitates obtained from COS7 cells and SY5Y cells that contain the HA-tagged FE65L fusion protein (Fig. 3*C*). In addition, we tested immunoprecipitates with 6E10, a βPP-specific monoclonal antibody directed against the Aβ region of βPP, and similar data were obtained in the COS7 cells (data not shown). To assess whether APLP2 also interacts with the HA-tagged hFE65L fusion protein, we performed a similar experiment using an APLP2-specific antibody, D2-I. We are able to detect the interaction of APLP2 with the HA-FE65L fusion protein in SY5Y and COS7 cells, but not in H4 cells (Fig. 3*D*). Collectively, these results indicate that the C-terminal region of βPP and APLP2 indeed interact with the C-terminal region of hFE65L in mammalian cells.

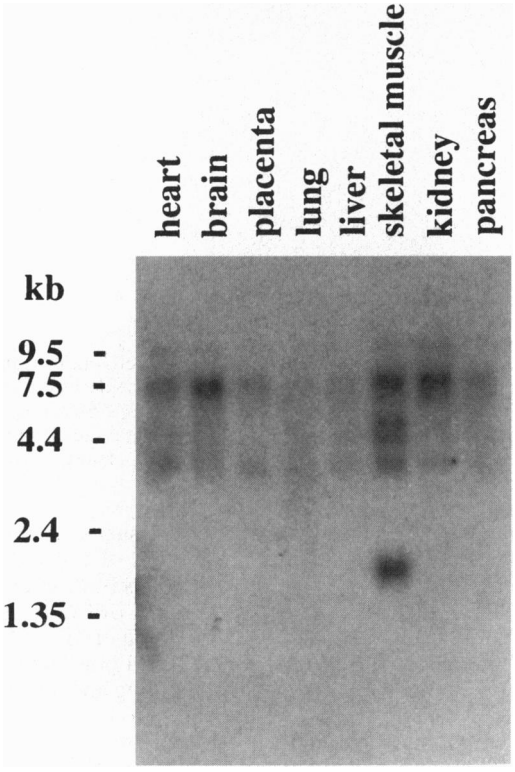

FIG. 2. Expression of the hFE65L gene in adult human tissues. A multiple tissue Northern blot (CLONTECH) with 2 μg of poly(A)$^+$ RNA per lane was hybridized to the ^{32}P-labeled 4-2A *Eco*RI/*Xho*I cDNA fragment. The hybridization was performed at 42°C in the presence of 50% formamide and washed in 0.1× SSC and 0.1% SDS at 50°C. RNA size markers in kb are shown on the right.

DISCUSSION

Our results demonstrate that the C-terminal moiety of the hFE65L protein interacts with the cytoplasmic domain of βPP and APLP2 in the yeast interaction trap system and in a variety of mammalian cells. We also report the sequence of a novel, ubiquitously expressed human gene, hFE65L, and the existence of two additional human homologues of the rat FE65 protein, indicating the presence of a novel gene family.

The observation that there is more than one human FE65-like protein raises the possibility of specific interactions among members of the βPP/APLP family and those of the hFE65L family. Our data obtained with the interaction trap suggest that the hFE65L protein interacts with the C terminus of both βPP and APLP2, but not with that of APLP1. As with the βPP and APLP2 gene products (10, 36), the hFE65L gene is ubiquitously expressed in all human tissues tested. It is of interest that both the rat FE65 protein and APLP1 are enriched in the brain (9, 31). However, unlike hFE65L, the rat FE65 protein was recently reported to interact with the C terminus of both βPP and APLP1 in the two-hybrid assay (37). In addition, the human homolog of the rat FE65 protein (hFE65) has also been

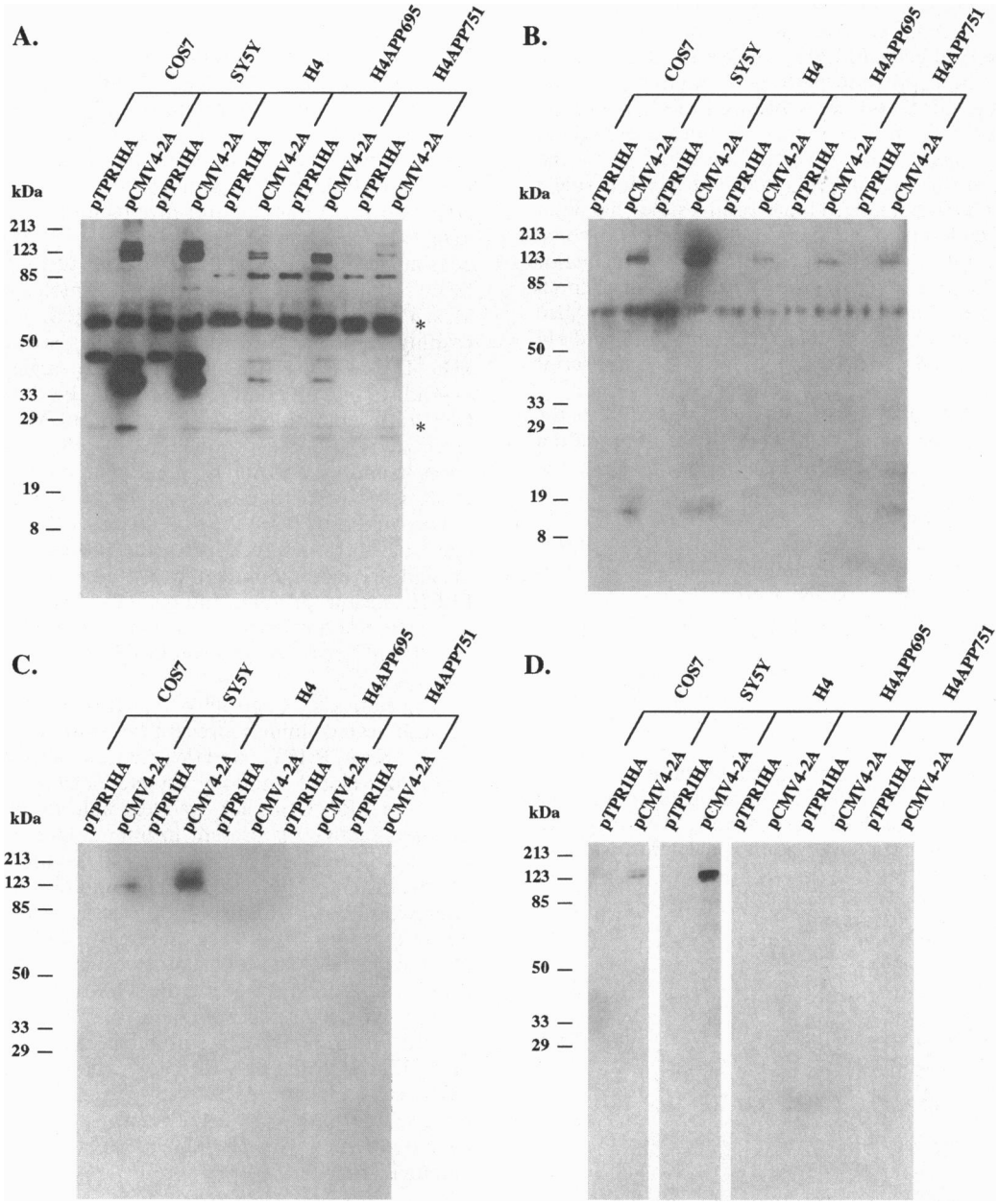

FIG. 3. Coimmunoprecipitation of βPP and APLP2 with the C-terminal region of hFE65L. COS7, SY5Y, H4, and H4 cells stably transfected with either APP695 or APP751 were transiently transfected with a pCMV construct expressing the HA-tag-hFE65L sequence derived from clone 4-2A or pTPR1HA as a negative control. Extracts obtained from cells harvested at 72 h were immunoprecipitated with the 12CA5 antibody directed against the HA-tag. Immunoprecipitated proteins were resolved on a 7.5–15% acrylamide gel and transferred to a nylon membrane. Immunoblot analysis was performed with the following primary antibodies: *A*, 22C11 and 12CA5; *B*, 369W; *C*, C7; *D*, D2-I. Immunoglobulin bands are indicated by asterisks in *A*. The molecular weight standards are shown on the left.

shown to interact with the cytoplasmic domain of βPP, APLP1 and APLP2 in the two-hybrid assay (45).

The predicted proteins encoded by the FE65/FE65L family of genes contain two direct tandem repeats of the PI domain. In the hFE65L protein, a PI domain is located in the C terminus within the region we have demonstrated interacts with the C termini of βPP and APLP2 *in vivo*. The rat FE65 PI domains have recently been shown to interact with the cytoplasmic domain of βPP *in vitro* (37). In the transforming protein, Shc, the PI domain recognizes tyrosine-phosphorylated NPXY motifs in a variety of growth factor receptors (38, 39). These data suggest that the NPTY motif found in the cytoplasmic domain of βPP and APLP2 may mediate the interaction of these molecules with the PI domain of hFE65L. It has been proposed that the NPTY motif of βPP also mediates its reinternalization from the cell surface via clathrin-coated vesicles (20, 21). In fact, deletion of the NPTY sequence from the βPP C terminus, or a substitution of NPTA for NPTY leads to increased secretion of βPPs (16, 17, 40). These data raise the possibility that binding of hFE65L to the NPXY motif in the βPP C terminus could potentially play a role in regulating the intracellular trafficking and/or secretion of βPP.

The PI domain of Shc has a structure similar to the pleckstrin homology domain and, like the pleckstrin homology domain, binds to acidic phospholipids (41), suggesting a role for the PI domain in recruiting hFE65L to membranes and increasing the probability of association of these molecules with βPP/APLPs at membranes.

Both the rat FE65 and the hFE65L proteins also contain a WW domain, a motif found in at least 17 proteins, some of

which are known to be involved in signaling or regulatory functions (42, 43). Like the SH3 domain, the WW domain interacts with proteins containing a proline-rich motif (43). If, in fact, the interaction of βPP with the hFE65L protein modulates βPP reinternalization, secretion, and/or function, it will be important to identify proteins that interact with the WW domain of hFE65L.

The PI domains overlap a region in the amino acid sequence of hFE65L that is similar to the retroviral integrase DNA-binding domain (31). FE65 was first described as a transcriptional activator based on the observation that the N-terminal half of the protein activates transcription (31). Presently, the function of the hFE65L protein is unclear, but the presence of several motifs believed to play roles in protein–protein interaction, signal transduction, and transcriptional activation provide a firm foundation for the design of future experiments. The results of such experiments should increase our understanding of the importance of the interactions among members of the FE65/FE65L and βPP/APLP families. It will be particularly interesting to determine the consequences of such associations on βPP trafficking, function, and generation of Aβ.

We thank Roger Brent and his group for providing the interaction trap reagents, Dimitri Krainc and Roger Brent for providing the human fetal brain activation-domain cDNA library, Wilma Wasco for the APLP1 and APLP2 clones, Anita Murthy and Jim Gusella for the pTPR1HA clone and the NFI bait construct, Gopal Thinakaran and Sam Sisodia for the D2-I antiserum, Sam Gandy for the 369W antiserum, and Dennis Selkoe for the C7 antiserum. We thank Steve Schmidt and Mia Liu for technical assistance. This research was supported by a grants from the American Health Assistance Foundation and the National Institutes of Health. R.E.T. is a Pew Scholar in the Biomedical Sciences.

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