Fe65L2: a new member of the Fe65 protein family interacting with the intracellular domain of the Alzheimer's β-amyloid precursor protein

Angela DUILIO[1], Raffaella FARAONIO[1], Giuseppina MINOPOLI, Nicola ZAMBRANO and Tommaso RUSSO[2]

Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli 'Federico II', CEINGE, Biotecnologie Avanzate s.c.r.l., Via S. Pansini 5, I-80131 Napoli, Italy

We previously demonstrated that Fe65 protein is one of the ligands of the cytoplasmic domain of β-amyloid precursor protein (APP). Another ligand of this molecule was recently identified; it is similar to Fe65, so it was named Fe65-like (Fe65L1). Herein we describe the cloning of another Fe65-like cDNA (Fe65L2), similar to Fe65 and to Fe65L1, which encodes a protein of approx. 50 kDa. Its cognate mRNA is expressed in various rat tissues, particularly in brain and testis. The three members of the Fe65 protein family share several structural and functional characteristics. The primary structures of the three proteins can be aligned in three regions corresponding to the protein–protein interaction domains of Fe65 [the protein–protein interaction domain containing two conserved tryptophan residues and the two phosphotyrosine interaction domain/phosphotyrosine binding (PID/PTB) domains], whereas the remaining sequences are poorly related. Like Fe65, Fe65L1 and Fe65L2 genes encode two different protein isoforms, derived from the alternative splicing of a very small exon of only six nucleotides, which results, within the N-terminal PID/PTB domain, in the presence or absence of two acidic/basic amino acids. Fe65L2 is able to interact, both *in vitro* and *in vivo*, with the intracellular domain of APP. Also, in the case of APP, another two closely related proteins exist, named β-amyloid precursor-like protein (APLP)1 and APLP2: by using the interaction trap procedure we observed that both Fe65 and Fe65L2 interact with APP, APLP1 or APLP2, although with different efficiencies.

INTRODUCTION

Alzheimer's disease (AD) is a severe neurodegenerative disorder that is characterized by the presence, in the affected brains, of the so-called senile plaques, resulting from the accumulation of insoluble aggregates of a 40–42 amino-acid-long peptide, named β-amyloid peptide. This derives from the proteolytic processing of a large membrane precursor, the Alzheimer's β-amyloid precursor protein (APP). Given its transmembrane topology, APP could be involved in extracellular signal transduction and/or it represents a precursor of a soluble signalling molecule (soluble APP). However, the functional role of APP, of soluble APP and of their processing is still unknown [1–3].

β-Amyloid peptide generation is closely related to the intracellular trafficking of APP, which is directed towards the cell surface, through a maturation pathway in the Golgi apparatus [4], and is internalized via an endocytic pathway [5]; several experimental results indicate that β-amyloid peptide is released in both these pathways [6,7]. One of the interesting aspects of APP physiology is represented by the emerging evidence that APP is part of a complex protein–protein interaction network, which is centred on the short cytosolic domain of APP. In fact, four different proteins have been found recently to bind to the cytodomain of APP. First, it was shown that a region of this domain from residues 657 to 676 binds to and activates oligomeric G_o protein [8,9]. This phenomenon takes place also *in vivo* and mutant APP proteins involved in the familial form of AD constitutively activate G_o [10]. Secondly, a 59 kDa protein, named APP-BP1, interacts both *in vitro* and *in vivo* with the C-terminal region of APP; it is similar to the product of the *Arabidopsis* auxin resistance gene AXR1 and to a *Caenorhabditis elegans* protein of unknown function [11]. The third factor interacting with the cytodomain of APP is the X11 protein, a neuron-specific protein of approx. 110–130 kDa, which is expressed in the cerebellum, the hippocampus and the spinal cord [12]. X11 possesses two protein–protein interaction domains, one phosphotyrosine interaction domain/phosphotyrosine binding (PID/PTB), involved in the binding to APP, and a PDZ domain, whose ligand is not known [12]. The fourth ligand of the APP cytosolic region is the Fe65 protein. The FE65 gene was originally isolated as a brain-specific gene, highly expressed in several districts of the nervous system and showing some characteristics of a transcription factor [13,14]. The presence, in the amino acid sequence deduced from the open reading frame (ORF) of FE65 cDNA, of three possible protein–protein interaction domains, a protein–protein interaction domain containing two conserved tryptophan residues (WW) [15] and two PID/PTB domains [16], suggested for this protein the further role of adapter molecule. We demonstrated that the most C-terminal of these PID/PTB domains (PID2) interacts with the intracellular domain of APP [17], and mutant APP proteins responsible for familial Alzheimer's disease, particularly the so-called Swedish mutant, showed a reduced binding to Fe65 [18]. The Fe65–APP interaction involves a 32 amino-acid-long region of the APP cytodomain, which contains an NPTY internalization motif. A Fe65-like protein (Fe65L1) was also identified with the in-

Abbreviations used: AD, Alzheimer's disease; APP, β-amyloid precursor protein; APLP, β-amyloid precursor-like protein; CMV, cytomegalovirus; EST, expressed sequence tags; GST, glutathione S-transferase; HA, haemagglutinin tag; ORF, open reading frame; PID/PTB, phosphotyrosine interaction domain/phosphotyrosine binding; WW, protein–protein interaction domain containing two conserved tryptophan residues.

[1] These authors contributed equally to the work.

[2] To whom correspondence should be addressed.

teraction trap screening of a human brain cDNA library [19]. This protein contains the same three protein–protein interaction domains and, like Fe65, it interacts both *in vitro* and *in vivo* with APP through the PID2 domain. This finding, together with the presence in the sequence data bank of expressed sequence tags similar to FE65 [19], suggested that Fe65 is a member of a multigene family.

Herein we describe the isolation and characterization of a third member of this Fe65 protein family (Fe65L2); compared with Fe65, it shows a different tissue-specificity but shares with the already known Fe65 proteins several structural and functional characteristics, including the ability to interact with APP.

MATERIALS AND METHODS

Library screening, PCR and generation of recombinant constructs

A 14–16-day-old Sprague–Dawley rat-brain cDNA library in the UniZAPTMXR vector (Stratagene) was screened using as probes two degenerated oligonucleotides designed on the basis of the strongest homologies observed among Fe65, Fe65L1 cDNAs and Fe65-like expressed sequence tags (EST) present in the DDBJ/EMBL/GenBank sequence data bank [19]. The two oligonucleotides encode the peptides LKCHVFRC (identical in Fe65, Fe65L1 and EST) and CFAVRSLGWVEM (identical in Fe65 and Fe65L1), and the degeneration was based on eukaryotic codon preference [20]. The hybridization was performed in Church buffer [0.5 M phosphate buffer (pH 7.2)/7 % (w/v) SDS/1 mM EDTA] at 45 °C, in the presence of 2×10^6 c.p.m./ml of oligonucleotides end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) by using T4 polynucleotide kinase (Boehringer). Positive clones were rescreened in Church buffer at 65 °C, using as probe the Fe65 cDNA labelled by random priming with [α-^{32}P]dATP (3000 Ci/mmol; Amersham). From the clones not hybridizing to the Fe65 probe, the pBluescript-derived plasmids containing the unidirectionally cloned cDNA inserts were recovered from lambda-ZAP phage by *in vivo* excision according to the manufacturer's instructions. Partial sequencing of these plasmids was performed by the dideoxy nucleotide method with the Sequenase kit (U.S. Biochemical). The longest cDNA insert (Fe65L2), different from both Fe65 and Fe65L1, was entirely sequenced on both strands using a series of oligonucleotide primers.

PCR amplifications were performed in a Perkin–Elmer 4800 Thermal Cycler with Taq DNA polymerase (Gibco-BRL) following the manufacturer's instructions. The Fe65L2 genomic fragment was amplified with oligonucleotide primers designed in the DNA region between nucleotides 858 and 972 of the Fe65L2 full-length cDNA: the human Fe65L1 cDNA fragment from nucleotides 1389 to 1514 of the published sequence [19] was obtained by amplification of a human fetal-brain cDNA library (Clontech). The resulting PCR fragments were cloned in pGEM-T vector (Promega) and sequenced.

The Fe65L2 coding region from nucleotides 288 to 1761 was obtained by PCR amplification of the Fe65L2 cDNA and cloned under the control of the cytomegalovirus (CMV) promoter in the pcDNAI-HA vector (kindly given by F. Fiore) for expression as a fusion protein with a haemagglutinin tag (HA) epitope (YPYDVPDYA). The Fe65L2 region from nucleotides 1044 to 1542, containing the PID2 region, was obtained by PCR amplification of the full-length cDNA, and cloned in the pGEX2TK vector (Pharmacia Biotech) for expression in *Escherichia coli* cells as a glutathione S-transferase (GST) fusion protein, and in the pGBT10 vector (Clontech) for expression in yeast cells as fusion protein with the GAL4 DNA binding domain. The cDNA region encoding the cytosolic region of the APLP2 protein (positions 2222–2365 of the published cDNA sequence) was obtained by amplification of the human fetal-brain cDNA library (Clontech), and cloned in the pGAD10 vector (Clontech) for expression in yeast cells as fusion protein with the GAL4 transcription activation domain.

Northern blot analysis, primer extension and RNase protection experiments

A ^{32}P-labelled PstI fragment corresponding to the 5′-end of the Fe65L2 cDNA sequence (see Figure 1) was used as probe for hybridization to a nylon membrane containing 2 μg of poly(A)$^+$ RNA isolated from Sprague–Dawley rat tissues (Clontech). The hybridization was performed with ExpressHyb solution (Clontech) for 2 h at 65 °C and washed in 0.1 × SSC/0.1 % (w/v) SDS at 50 °C (1 × SSC, 0.15 M NaCl/0.015 M sodium citrate). The same filter was rehybridized with a ^{32}P-labelled 5′-fragment of the rat Fe65 cDNA or with the β-actin cDNA. For the primer extension and RNase protection experiments, brain, liver and testis RNAs were isolated from 15–20-day-old Sprague–Dawley rats on caesium chloride gradients [21]. Primer extension analysis was performed as described in ref. [21], using as a primer an oligonucleotide complementary to the coding strand of the cDNA from nt 66 to 96 (see Figure 1) and as templates poly(A)$^+$ RNAs, prepared by using the Qiagen Oligotex kit.

The BglII fragment of the rat Fe65L2 cDNA (from nucleotides 533 to 1030; see also Figure 1) was cloned into pBluescript II KS+ vector, and the antisense RNA was synthesized by T7 RNA Polymerase (Promega) using as template 1 μg of the plasmid linearized with HindIII in the presence of [α-^{32}P]UTP (400 Ci/mmol; Amersham). Total RNA from each tissue (40 μg) was annealed to 2×10^5 c.p.m. of probe and the experiment was performed as described previously [13].

Cell transfections, extract preparation and co-immunoprecipitation assays

COS7 African green monkey kidney cells and the rat pheochromocytoma PC12 cell line were cultured in Dulbecco's modified minimal medium supplemented with 10 % (v/v) fetal-calf serum (COS7) or with 10 % fetal-calf serum and 5 % (v/v) horse serum (PC12) at 37 °C in an air/CO_2 (19:1) atmosphere; COS7 cells (3×10^6 cells per transfection) were transfected by electroporation at 250 μF and 220V with the pcDNAI-HA-Fe65L2 construct and/or with an expression vector carrying the wt APP 695 cDNA under the control of the CMV promoter. The total amount of transfected DNA was kept constant by the addition, where required, of pcDNAI-HA vector DNA. Immunoprecipitation was performed by using the anti-HA epitope Tag monoclonal antibody, 12CA5 (Boehringer), on cellular lysates (1 mg), prepared by using as lysis buffer 10 mM Tris/Cl (pH 7.5)/150 mM NaCl/0.1 mM EDTA/1 mM sodium orthovanadate/50 mM sodium fluoride/0.5 % (v/v) Nonidet P40/aprotinin (10 μg/ml)/leupeptin (10 μg/ml)/pepstatin (10 μg/ml). The immunocomplexes were collected with Protein A–Sepharose (Pharmacia Biotech), resolved on SDS/10 % (w/v)-PAGE gels and transferred to Immobilon-P membranes (Millipore). The filter was probed with a 1:2000 dilution of the anti-APP antiserum 369 [22] in TBS-T buffer [20 mM Tris/HCl/150 mM NaCl/0.05 % (v/v) Tween-20 (pH 7.5)] containing 1 % (w/v) non-fat dry milk for 1 h at room temperature, washed in TBS-T buffer and incubated with horseradish peroxidase-conjugated Protein A (1:5000, Amersham) in 1 % milk/TBS-T. Detection of the blots was by the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions.

Figure 1 Nucleotide sequence of the Fe65L2 cDNA

The amino acid sequence deduced from the longest ORF is reported. The fragment used as a probe in the Northern blot of Figure 2 is underlined. The primer used for the primer extension analysis is complementary to the sequence underlined by the arrow. The probe used for the RNase protection experiment reported in Figure 4 covers the cDNA region indicated by bold characters. The accession number of the reported sequence (DDBJ/EMBL/GenBank) is Y13413.

The GST-Fe65L2 fusion proteins containing the PID2 domain was obtained by isopropyl thiogalactoside induction of *E. coli* cells harbouring the corresponding construct in the pGEX2TK vector, and purified from the bacterial lysates with glutathione–Sepharose resin (Pharmacia Biotech) according to the manufacturer's instructions. For the pull-down experiments, purified fusion proteins were used to saturate glutathione–Sepharose beads (10 μl) and were challenged to PC12 cell extracts (250 μg/sample). Unbound proteins were removed by washing the beads with lysis buffer, while retained proteins were resolved by SDS/PAGE, electroblotted to Immobilon-P membrane and analysed by Western blot with the anti-APP 369 antibody.

Two-hybrid system assays

The yeast strain Hf7c [23] was grown in synthetic medium without uracil. Exponentially growing cells were transformed by the lithium acetate method [24] using pGBT–Fe65 or pGBT–Fe65L2 plasmids, encoding the Fe65 and Fe65L2 PID2 regions fused to the GAL4 DNA binding domain, in combination with the pGAD10-derived constructs encoding the APP [17], APLP1 [17] and APLP2 cytosolic regions fused to the GAL4 activation domain. Transformants were grown in synthetic medium plates lacking tryptophan and leucine (Trp^-/Leu^-); individual transformants were tested for the ability of the encoded proteins to form a functional GAL4 hybrid by plating replicas of the colonies on $His^-/Trp^-/Leu^-$ plates. The β-galactosidase assay was performed on all the co-transformants on Trp^-/Leu^- plates overlaid with nitrocellulose filters as described [18].

RESULTS AND DISCUSSION

Isolation of a cDNA similar to FE65, with a different pattern of tissue distribution

To identify Fe65-related genes, we screened a rat-brain cDNA library, using as probes degenerate oligonucleotides encoding conserved regions of Fe65 and Fe65L1 proteins (see the Materials and methods section). This screening led to the isolation of

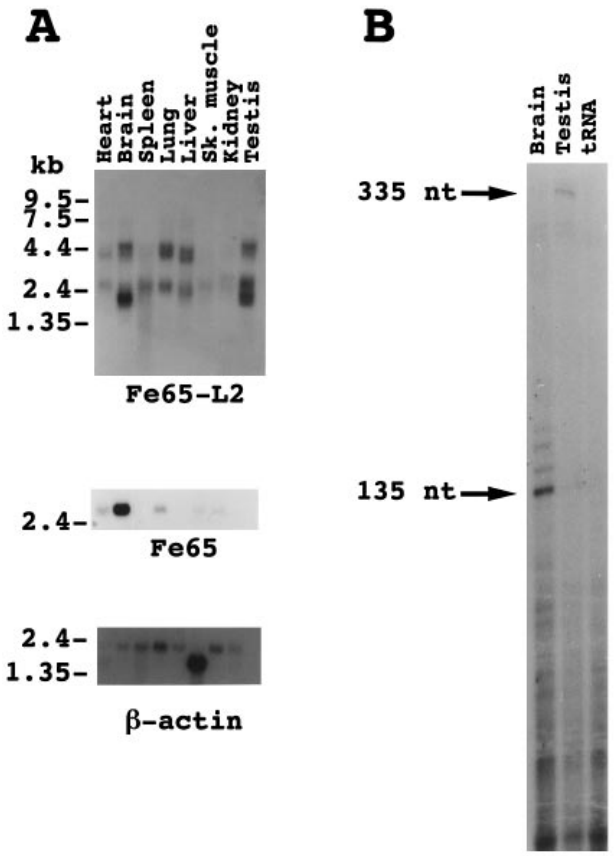

Figure 2 Tissue distribution of Fe65L2 mRNA

(Panel A) Northern blot analysis of RNA samples from various rat tissues hybridized to the Fe65L2 probe indicated in Figure 1. The same filter was rehybridized using as a probe a fragment of the Fe65 cDNA or the β-actin cDNA. The size markers are reported on the left side. Sk. muscle, skeletal muscle. (Panel B) Primer extension analyses of the Fe65L2 mRNA, performed by using the primer indicated in Figure 1. The arrows indicate the extension products.

numerous clones that were screened again by using the Fe65 cDNA as a probe under stringent conditions. The cDNAs not hybridizing to Fe65 cDNA were partially sequenced: three of them show sequences that significantly diverge from both Fe65 and Fe65L1 cDNAs. The longest cDNA, named Fe65L2, was completely sequenced on both strands (AC number: Y13413) and resulted in 2024 nucleotides in length, including a short poly(A) tail (see Figure 1). The 5′-untranslated region of this cDNA was used as a probe for the Northern blot shown in Figure 2(A). As expected, the Fe65L2 mRNA is present in the RNA from rat brain, but its tissue-specificity is significantly different from that of Fe65 mRNA (Figure 2A). In fact, a transcript of approx. 2000 nucleotides, compatible with the length of the isolated cDNA, is present in the brain and also in the testis, whereas, as already known [13], Fe65 mRNA is mainly represented in the brain, and only a long exposure of the filter shows small amounts of the transcript in heart and lung RNA samples (see Figure 2A). Several longer transcripts, hybridizing to the Fe65L2 probe, are visible in other RNA samples of the Northern blot (heart, brain, spleen, lung, liver, testis); they can be unprocessed forms of the Fe65L2 mRNA or Fe65L2-related transcripts.

The primer extension analysis reported in Figure 2(B) shows that, by using the primer reported in Figure 1, the most abundant Fe65L2 mRNA species present in rat brain starts 41 nt upstream of the 5′-end of the cloned cDNA. The same mRNA species is also present in testis; another mRNA is approx. 200 nt longer, and it seems to be more abundant in testis than in brain.

Fe65L2 shares several structural characteristics with Fe65 and Fe65L1 proteins

Figure 3 shows the alignment of the amino acid sequences derived from the ORF of Fe65, Fe65L1 and Fe65L2. Three structural elements are extremely conserved among the three sequences, and they correspond to the three protein–protein interaction domains WW, PID1 and PID2. In contrast, the spacer regions between these elements are completely unrelated. We tentatively identify a starting methionine codon (see Figure 1), on the basis of the presence in the same ORF of preceding stop codons, which allows us to predict that the molecular mass of the Fe65L2 protein would be approx. 50 kDa. Therefore the length of Fe65L2 is significantly shorter than that of the other two members of the family, because of the absence of the long N-terminal region preceding the WW domain in Fe65 and Fe65L1 proteins. Another difference between Fe65L2 and both Fe65 and Fe65L1 is represented by the C-terminal sequence following the PID2 domain. These regions of Fe65 and Fe65L1 are significantly similar, whereas they are completely unrelated to that of Fe65L2, except for the presence of several basic residues. The conservation between Fe65 and Fe65L1 is higher than that between Fe65 and Fe65L2 and between Fe65L1 and Fe65L2. In fact, the amino acid identity between Fe65 and Fe65L1 is 72% for PID1 and 55% for PID2, whereas between Fe65 and Fe65L2 it is 55% and 47% respectively.

We demonstrated that at least two Fe65 mRNAs exist as a consequence of an alternative splicing of a short exon of only six nucleotides [13]; these two mRNAs encode two proteins differing by only two amino acids. The sequence of the cloned Fe65L2 cDNA contains the nucleotides encoding these two amino acids, whereas the sequence of the Fe65L1 cDNA does not. The RNase protection experiment reported in Figure 4 was designed to evaluate the possible existence of a Fe65L2 mRNA isoform lacking six nucleotides, as observed for the Fe65 gene. An antisense Fe65L2 probe was synthesized *in vitro*: it covers the region of the Fe65L2 mRNA from nt 533 to 1030, so that the annealing of the Fe65L2 mRNA containing the six nucleotides fully protects the antisense probe, whereas the mismatches caused by the absence of the six nucleotides would result in the protection of two smaller probe fragments. As shown in Figure 4, the RNAs from brain and testis contain not only a mRNA that completely protects the antisense probe, but also a mRNA that protects two smaller bands of 373 and 118 nt respectively. In contrast, the RNA from rat liver contains only the short (without the six nucleotides) Fe65L2 mRNA, which protects the two small fragments.

By using two couples of oligonucleotides, designed on the basis of the Fe65L1 and Fe65L2 sequences flanking the region containing the six nucleotides, we amplified a human fetal-brain or a rat-brain cDNA library respectively. The amplification products were cloned, and the nucleotide sequence of several clones demonstrated that for both Fe65L1 and Fe65L2 two different cDNAs exist, with or without six nucleotides. In the case of Fe65 and of Fe65L1 the exons are identical and encode glutamic acid and arginine, whereas in the case of Fe65L2 the mini-exon encodes aspartic acid and arginine. The same oligonucleotide couples were used to amplify human or rat genomic DNA respectively. Only the Fe65L2 oligonucleotides amplified a 500 bp region, whose sequence is reported in Figure 5, in which

Figure 3 Amino acid alignment of Fe65, Fe65L1 and Fe65L2

The amino acid sequences of rat Fe65 (AC: X60468) and Fe65L2 (AC: Y13413) and of human Fe65L1 (AC: U62325), derived from the translation of the ORF of the corresponding cDNAs, were aligned by using the PILEUP program, and the similarity was maximized by manual adjustment to take into account the already known structural features of the WW and PID domains. Amino acid identity or similarity (R-K; T-S; F-Y; L-I-V-M) is reported in bold letters. The three protein–protein interaction domains (WW, PID1 and PID2) are underlined. The asterisks indicate the amino acids encoded by the alternatively spliced exon.

the alternatively spliced exon was found. The Fe65L1 oligonucleotides failed to amplify the human genomic DNA, probably because of the bigger size of the introns included between the two primers used for the amplification. In conclusion, these results demonstrated that all the members of the Fe65 gene family encode two protein isoforms differing only by the presence/absence of a couple of amino acids (Glu/Asp-Arg). The conservation of this alternative splicing in the three genes suggests an important functional role for the presence or absence of these two amino acids. No evident structural motif is generated by the presence/absence of these amino acids; it could be hypothesized that they create a tyrosine phosphorylation signal at the level of the Tyr residue C-terminal to the splicing site in Fe65 and Fe65L1 proteins, but this hypothesis is conflicting with the presence of a Phe residue, instead of Tyr, in the corresponding position of the Fe65L2 protein (see Figure 3). The two amino acids are located in the PID1 elements and the comparison with the sequence of the PID2 elements of the three proteins indicates that they are not present in any of the PID2 structures of Fe65 family members. The identification of the ligand(s) of the PID1 elements of the Fe65 family will provide experimental clues to the understanding of the functional roles of these different isoforms.

Fe65L2 interacts both *in vitro* and *in vivo* with APP

The high similarity between the structures of the Fe65 family members suggests a possible conservation of their functions. In fact, it was demonstrated that Fe65 and Fe65L1 bind to the intracellular domain of APP [17,19]. To evaluate if also Fe65L2 interacts with APP we expressed and purified from *E. coli* a GST-Fe65L2 fusion protein containing the PID2 domain from amino acids 252 to 418. Extracts from PC12 cells were used in the pull-down experiment reported in Figure 6A; the proteins interacting with the GST–Fe65 and GST–Fe65L2 fusion proteins, containing the PID2 domain, were analysed by Western blot with an anti-APP antibody. The result of this experiment indicates that, like Fe65, Fe65L2 does interact with APP.

To evaluate whether this interaction also takes place *in vivo* between the native full-length proteins, an expression vector (CMV–HA/Fe65L2) was constructed containing the CMV promoter, which drives the transcription of a hybrid message encoding an HA fused in-frame to the Fe65L2 cDNA coding sequence lacking the starting methionine. This construct was transfected in COS7 cells together with the CMV–APP expression vector [18], and the extracts from these cells, harvested 72 h after the transfection, were immunoprecipitated by using an anti-HA antibody. The immunocomplexes obtained were analysed by Western blot using an anti-APP antibody. Figure 6(B) shows that APP is co-immunoprecipitated by the anti-HA antibody in the extracts from COS7 cells co-transfected with the CMV–HA/Fe65L2 and CMV/APP vectors, whereas no signal is present in the immunoprecipitated proteins from cells transfected only with CMV–APP vector and with the empty pcDNAI–HA vector.

As far as the interaction between Fe65 proteins and the members of the APP family is concerned, the interaction-trap procedure was exploited. The yeast strain Hf7c was co-trans-

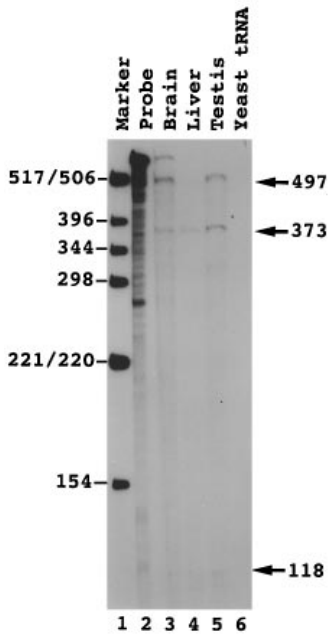

Figure 4 Analysis of two alternatively spliced isoforms of Fe65L2 mRNA

RNase protection experiment performed by using total RNA from rat brain, liver and testis. The RNA samples were incubated to an antisense probe encompassing the region that is expected to contain the alternatively spliced mini-exon (see Figure 1). When the probe is annealed to the mRNA containing the six nucleotides of the mini-exon, the whole antisense probe is protected (497 nucleotides), whereas two fragments are protected (373 and 118 nt respectively) when the probe is annealed to the mRNA lacking the six nucleotides. Lane 1, size marker; lane 2, undigested probe. The probe was annealed to total RNA from rat brain (lane 3), liver (lane 4) and testis (lane 5), or to yeast tRNA (lane 6). The arrows indicate the protected bands.

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.........:.........:.........:.........:.........:
CCTCTGGTTCACATCCGTGTATGGGGTGTGGGCAGCTCCAAGGGCCGGTG   50
P  L  V  H  I  R  V  W  G  V  G  S  S  K  G  R
AGGACTTCTGCATTCCCCCAACTCTGTGAGAGTTGTTTCCCCTTCCCCTT 100

GCAGGCTGTGATCCCCACTGATGCTGAACCCTTGGACACAGATCCTCTGA 150

TGTGGACCCAGCACGGCCTTACCCTGCCTGGTTCATCCTGGTTGGGGTGG 200

GTGGGACCCAACCCAGCTCAGCAGGGACAGATAGGGAGAGTCTGAGAGAC 250

TGGCTGATTTCTCCTGTGCTCCACATGCTCTCAACAGTGACAGGTATGTG 300
                                    D  R
ATAACTGGTCTCTAAGGCCCTATCCCTGCCTCTCCCCATTCCCAACGGTG 350

CCATATCCTTAAACCTAACTGGGTGAAGGCCTCTCACAGGCACTTAGAAA 400

CCTCTGCCCCTAACAGCTTTGTCTCCGGCCCCTGCCAGGGACTTTGCTTT 450
                                          D  F  A  F
TGTTGCGGGTGACAAAGACAGCTGTATGCTTAAATGTCATGTGTTTCGCT 500
 V  A  G  D  K  D  S  C  M  L  K  C  H  V  F  R  C
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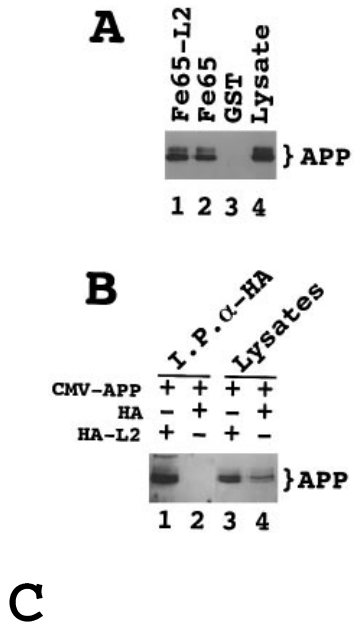
Figure 5 Partial nucleotide sequence of the rat Fe65L2 gene shows the existence of a six-nucleotide-long mini-exon

The sequence corresponding to the Fe65L2 cDNA is underlined and the translation is reported.

formed with vectors containing either the cDNA sequences encoding the PID2 domain of Fe65 or that of Fe65L2 fused to the GAL4 DNA binding domain, in combination with three different constructs driving the expression of the GAL4 activation domain fused to the intracellular domain of APP, APLP-1 or APLP-2. The His$^+$ and β-gal$^+$ phenotypes of several individual



	p GAD 10	p GAD-APP	p GAD-APLP1	p GAD-APLP2	time min
Fe65 (PID2)	-	+	-	++	45
	-	++	+	+++	150
Fe65-L2 (PID2)	-	+	-	++	45
	-	++	+	+++	150

Figure 6 Fe65L2 binds to the cytodomain of APP

(Panel A) Pull-down assay carried out on extracts from PC12 cells (250 μg) incubated with the fusion proteins GST–Fe65L2 (lane 1) or GST–Fe65 (lane 2), or with wild-type GST (lane 3). The proteins associated with the GST proteins were analysed by Western blot with anti-APP 369 antibody. The lysate not incubated with GST protein (20 μg) was analysed in lane 4. (Panel B) Co-immunoprecipitation experiments of extracts from COS7 cells transfected with CMV–APP expression vector and with vectors driving the expression of HA–Fe65L2 (lane 1) or HA alone (lane 2). Proteins were immunoprecipitated with an anti-HA (I.P. α-HA) antibody and analysed by Western blot with the anti-APP 369 antibody. Lanes 3 and 4, control Western blot of the lysates from transfected cells (10 μg each) with anti-APP antibody. (Panel C) Analysis of the interaction between Fe65 or Fe65L2 with APP, APLP1 and APLP2 by the two-hybrid system in yeast. The yeast strain Hf7c was transformed with pGBT-Fe65 or pGBT-Fe65L2 plasmids, containing the PID2 element of the two proteins, in combination with pGAD–APP, pGAD–APLP1 or pGAD–APLP2, containing the cytodomain of the three proteins. Control experiments were performed using pGAD10 wild-type vector. Co-transformants were tested for β-galactosidase activity after 45 min and 150 min. $-$, colourless colonies; $+$, faint blue colonies; $++$, blue colonies; $+++$, dark blue colonies.

transformants derived from the six co-transformations were analysed: no difference was observed between Fe65 and Fe65L2 expressing clones, in terms of growth in a medium lacking histidine. As shown in Figure 6(C), the evaluation of the β-galactosidase activity allowed us to observe that the darkest blue signals are given by the clones expressing APLP-2, whereas the faintest signals are given by the clones expressing APLP-1; for these last clones a 150-min-long incubation is necessary to render the colour visible.

These observations suggest a common role for all the members of the Fe65 protein family. The apparent redundancy of these functions can be explained by considering that: (i) there are

several significant differences between the three Fe65 proteins, namely those concerned with the N-terminal regions flanking the WW domain of Fe65 and Fe65L1, which seems to be completely absent in Fe65L2; (ii) the tissue-specific distribution of the three mRNAs is significantly different, and a cell-specific or a district-specific distribution of the three proteins can be hypothesized in the brain; (iii) it can be speculated that the three proteins have a different intracellular compartmentalization.

The relevance of the Fe65 protein family in the generation of the Alzheimer phenotype is under examination. We demonstrated previously that the interaction of the mutated forms of APP, found in several cases of AD, with Fe65 is decreased and in the case of the so-called Swedish mutation this interaction is almost completely abolished [18]. The significance of this observation requires the understanding of the functions of Fe65 and of its interaction with APP. The structural characteristics of Fe65 suggest that this protein could function as an adapter by interacting with several proteins through its three protein–protein interaction domains. An important step towards the elucidation of the Fe65 functions will be the identification of the ligands of the PID1 and of the WW domains of Fe65. The involvement of this complex network in the functions and in the normal and pathological processing of APP is to be explored, also in the light of the existence of the other two members of the Fe65 protein family, Fe65L1 and Fe65L2.

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