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The Fe65 Adaptor Protein Interacts through Its PID1 Domain with the Transcription Factor CP2/LSF/LBP1*

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Nicola Zambrano, Giuseppina Minopoli, Paola de Candia, and Tommaso Russo‡

From the Dipartimento di Biochimica e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, via S. Pansini 5, 80131 Napoli, Italy

The neural protein Fe65 possesses three putative protein-protein interaction domains: one WW domain and two phosphotyrosine interaction/phosphotyrosine binding domains (PID1 and PID2); the most C-terminal of these domains (PID2) interacts *in vivo* with the Alzheimer's β-amyloid precursor protein, whereas the WW domain binds to Mena, the mammalian homolog of *Drosophila*-enabled protein. By the interaction trap procedure, we isolated a cDNA clone encoding a possible ligand of the N-terminal PID/PTB domain of Fe65 (PID1). Sequence analysis of this clone revealed that this ligand corresponded to the previously identified transcription factor CP2/LSF/LBP1. Co-immunoprecipitation experiments demonstrated that the interaction between Fe65 and CP2/LSF/LBP1 also takes place *in vivo* between the native molecules. The localization of both proteins was studied using fractionated cellular extracts. These experiments demonstrated that the various isoforms of CP2/LSF/LBP1 are differently distributed among subcellular fractions. At least one isoform, derived from alternative splicing (LSF-ID), is present outside the nucleus; Fe65 was found in both fractions. Furthermore, transfection experiments with an HA-tagged CP2/LSF/LBP1 cDNA demonstrated that Fe65 interacts also with the nuclear form of CP2/LSF/LBP1. Considering that the analysis of Fe65 distribution in fractionated cell extracts demonstrated that this protein is present both in nuclear and non-nuclear fractions, we examined the expression of Fe65 deletion mutants in the two fractions. This analysis allowed us to observe that a small region N-terminal to the WW domain is phosphorylated and is necessary for the presence of Fe65 in the nuclear fraction.

Alzheimer's disease (AD)[1] is a neurodegenerative disorder in which the main pathological traits are neuronal loss and the presence of neurofibrillary tangles and senile plaques in affected tissues (1, 2). These plaques are formed by extracellular deposits of a 4-kDa peptide, known as β-amyloid (Aβ), which is derived from the proteolytic processing of the β-amyloid precursor protein (APP). APP is an integral membrane protein ubiquitously expressed at the level of plasma membrane and of several intracellular compartments (3). It shows a large extracellular/intraluminal domain, a single transmembrane tract, and a small cytosolic (CY) domain.

Familial forms of AD are linked in a few cases to mutations of the APP gene, probably directly affecting the processing of APP and therefore Aβ generation. In other cases, mutations of presenilin 1 and 2 genes occur, which produce a phenotype that includes Aβ overproduction and deposition (4, 5) and, as suggested by recent results, could be a consequence of a direct interaction between APP and PS2 (6). Very little is known about APP and presenilin functions; consequently, our understanding of molecular mechanisms regulating APP processing is limited.

The transmembrane topology of APP suggests that it could be involved in the transduction of signals through the membrane. Although the possible ligands of the extracellular/intraluminal domain of APP are still unknown, recent results showed that a complex protein network seems to be centered on the APP CY domain. In fact, at least four proteins have been demonstrated to interact with this 50-amino acid-long cytosolic tail. The first is the G_o protein, which is activated both *in vitro* and *in vivo* by direct interaction with APP (7). Mutant forms of APP constitutively activate G_o (8) and provoke various effects, including DNA fragmentation (9). The second protein found to interact with APP was named APP-BP1. It is similar to the auxin resistance gene product AXR1 of *Arabidopsis* and to a protein in *Caenorabditis elegans* of unknown function (10). The X11 protein is neuron specific, and it interacts with APP through a phosphotyrosine interaction/phosphotyrosine binding (PID/PTB) domain and possesses other putative protein-protein interaction domains (11). The fourth member of this family of APP CY domain ligands is Fe65. It is a 90-kDa adaptor protein expressed in neurons of several regions of the mammalian nervous system (12, 13). It possesses three putative protein-protein interaction domains: one WW domain and two PID/PTB domains. We have demonstrated that the most C-terminal of these domains (PID2) binds both *in vitro* and *in vivo* to APP (14, 15). Furthermore, two other proteins similar to Fe65, named Fe65-L1 and Fe65-L2, were identified and found to interact with APP (16, 17).

For interaction with Fe65 to occur, the last 32 C-terminal residues of APP are needed. These residues contain an NPXY motif, which was demonstrated to be the element of growth factor receptors recognized by PID/PTB domains of Shc and IRS1. However, contrary to observations of Shc and IRS1 behavior (18, 19), the Fe65-APP interaction is phosphorylation independent (11, 15). Mutations of APP responsible for familial forms of AD significantly affect the Fe65-APP *in vivo* interaction, especially in the case of the so-called Swedish mutant whose binding to Fe65 is almost completely abolished (15).

The possible role of the Fe65 adaptor protein was confirmed by the finding that several proteins interact with Fe65 through

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‡ To whom correspondence should be addressed. Tel.: 39-81-7463131; Fax: 39-81-7463650; E-mail: russoto@unina.it.

[1] The abbreviations used are: AD, Alzheimer's disease; Aβ, β-amyloid peptide; APP, β-amyloid precursor protein; CMV, cytomegalovirus, CY, cytosolic; HA, hemagglutinin; PID, phosphotyrosine interaction domain; PTB, phosphotyrosine binding; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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its WW domain. One of these ligands was identified as Mena, the mammalian homolog of the *Drosophila* enabled protein (20). To understand more about the function of Fe65, we addressed the point of the role of the third possible protein-protein interaction domain of Fe65, the N-terminal PID/PTB domain (PID1). Herein, we report that the PID1 domain of Fe65 interacts with the CP2/LSF/LBP1 protein, previously described as a transcription factor involved in the regulation of several genes (21–23). Studies of the compartmentalization of Fe65 and CP2/LSF/LBP1 within the cell suggest that these proteins are involved in a complex intracellular trafficking process.

MATERIALS AND METHODS

Two-hybrid System-based Screening—The yeast two-hybrid system (24) was used to identify the Fe65 PID1 ligands and was performed as described in Ref. 25. The amino acid region chosen as a bait was from residue 365 to residue 533 of Fe65. This peptide contains the entire PID1 element, 141 amino acids long, extremely conserved between rat and man; only five residues out of 141 are different in the human protein (16). A PCR fragment from nucleotide 1167 to nucleotide 1673 of the rat FE65 cDNA coding region (26) was subcloned, using standard techniques, into pGTB10, a yeast expression vector carrying the Trp selective marker. The obtained plasmid was used to transform the Hf7c yeast strain (27) generating a clone that constitutively expressed the above-mentioned Fe65 amino acid region fused to the DNA binding domain of the yeast GAL4 transcription factor. The Hf7c strain contains the HIS3 reporter gene and the *lacZ* reporter gene under the control of the GAL4 transcription factor cis-element (28). The human brain cDNA library cloned into the pGAD10 vector (CLONTECH), which carries the Leu selective marker, was used to transform the Hf7c Fe65-expressing strain. 5×10^6 transformants were obtained on $Ura^-Trp^-Leu^-$ plates, harvested in 20 ml of 65% glycerol, 10 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$ and stored in 1-ml aliquots at −80 °C. The transformants were then plated on $Ura^-Trp^-Leu^-His^-$ plates; after 3 days, the His^+ colonies were isolated. On these colonies, the β-galactosidase assay was also performed as described in Ref. 25. The plasmid DNAs from positive clones were then rescued and introduced by electroporation into *Escherichia coli* HB101 competent cells. The cDNA inserts of the library plasmids were analyzed by digestion with *Eco*RI and by nucleotide sequence with T7 sequencing kit (Amersham Pharmacia Biotech).

Generation of the Recombinant Constructs—The various FE65 cDNA fragments used in this study were obtained by amplification of the FE65 cDNA previously described (26); the Δ-N191-C665-Δ-spacer deletion mutant of Fe65 was obtained by overlapping PCR according to Ref. 15; the resulting construct consists of a mutant Fe65 cDNA (Δ-N191-C665) bearing an internal deletion of the spacer region joining the WW and the PID1 domains (from residue 292 to residue 364). The LSF and LSF-ID coding regions were obtained by reverse transcriptase-PCR of HeLa cell RNA using Superscript reverse transcriptase and *Taq* DNA polymerase (Life Technologies, Inc.) according to the instructions of the manufacturer with the following pair of primers (CEINGE): LSF-2F, 5′-ATAGGATCCGCCTGGGCTCTGAAGCT; LSF-Rev, 5′-ATACCCCGGGCTACTTCAGTATGATATGATAG. The recombinant constructs were obtained by ligation of the PCR fragments digested with appropriate restriction enzymes (Boehringer Mannheim) and purified from agarose gels with the QIAEX gel extraction kit (Qiagen) in the pcDNAI-HA vector (a kind gift of Francesca Fiore, European Institute of Oncology) for expression as a fusion protein with a hemagglutinin tag epitope (YPYDVPDYA). The sequence and the reading frame of the recombinant constructs were checked by nucleotide sequencing.

Cell Culture, Transfections, and Extract Preparation—The COS7 African green monkey kidney cells and the rat pheochromocytoma PC12 cells were cultured in Dulbecco's modified minimal medium supplemented with 10% fetal calf serum (COS7) or with 10% fetal calf serum and 5% horse serum (PC12) at 37 °C in a 5% CO_2 atmosphere; COS7 cells (3×10^6 cells per transfection) were transfected by electroporation at 250 microfarads and 220 V with the pcDNAI-derived constructs. For cotransfection experiments, the CMV-Fe65 expression construct (15) was used in electroporation experiments in COS7 cells with the HA-tagged LSF and LSF-ID expression vectors or with a CMV-APP expression vector carrying the wild-type APP 695 cDNA.

For the preparation of the cellular extracts, monolayer cultures were harvested in cold phosphate-buffered saline and sonicated in lysis buffer (50 mM Tris·HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 0.4 mM EDTA, 50 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin). The extracts were clarified by centrifugation at 16,000 × *g* at 4 °C, and the protein concentration was determined by the Bio-Rad protein assay according to manufacturer's instructions.

Fractionated extracts were prepared according to Ref. 29; 1×10^7 cells were harvested in cold phosphate-buffered saline and resuspended in lysis buffer (10 mM Hepes, 1 mM EDTA, 60 mM KCl, 0.2% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml each of aprotinin, leupeptin and pepstatin, pH 7.9). Nuclei were recovered by centrifugation at 3,000 × *g* at 4 °C for 5 min; the supernatant was clarified by centrifugation at 15,000 × *g* for 15 min at 4 °C and used as the cytosol/membrane (CM) fraction. Pelleted nuclei were washed in lysis buffer without Nonidet P-40 and further purified on a 30% sucrose cushion by centrifugation at 9,000 × *g* for 15 min at 4 °C. The nuclear extract was prepared by freeze and thaw extraction (3 cycles) in a buffer containing 250 mM Tris·Cl, 60 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin, pH 7.8.

Antibodies, Immunoprecipitations, Western Blots, and Phosphatase Treatment—The anti-Fe65 antiserum used in this study has been previously described (15); the anti-LSF serum (30) and its corresponding pre-immune serum were a kind gift of R. Roeder and C. Parada (the Rockefeller University). Anti-YY1 antibody was from Santa Cruz Biotechnology, the anti-mbh1 serum was a kind gift of E. Ziff (31), the anti-HA monoclonal antibody 12CA5 was purchased from Boehringer Mannheim, and the anti-HA polyclonal antibody, HA probe, was from Santa Cruz Biotechnology. The anti-APP monoclonal antibody 6E10 used for immunoprecipitation was a precious gift of Joseph Buxbaum (Mount Sinai School of Medicine), and the 421 monoclonal antibody (Oncogene Science), specific for the p53 protein, was used as a negative control.

For the immunoprecipitations, the cellular extracts were incubated with appropriate dilutions of the antibodies for 1 h at 4 °C, and then protein A-Sepharose resin (Pharmacia Biotech, 30 μl per sample) was added to the extracts for collection of the immunocomplexes. The proteins were eluted with a buffer containing 50 mM Tris·Cl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, and 0.01% bromophenol blue, resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes (Millipore) according to the manufacturer's instructions. For the Western blot experiments, the filters were blocked in 2% nonfat dry milk in TBS-T solution (20 mM Tris·HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.5) and incubated with appropriate dilutions of the primary antibodies for 1 h at room temperature. The antibody in excess was removed by sequential washing of the membranes in TBS-T, and then a 1:5000 dilution of either horseradish peroxidase-conjugated protein A or horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham) was added to the filters for 1 h at room temperature. Filters were washed, and the signals were detected by chemiluminescence using the ECL system (Amersham).

For alkaline phosphatase treatment, 500 μg of protein extract prepared from COS7 cells were immunoprecipitated with the anti-Fe65 antibody; the immunoprecipitated proteins were equilibrated in 20 mM ammonium bicarbonate buffer, pH 8.5, and treated for 4 h at 37 °C in the presence or the absence of 1 μg of alkaline phosphatase (Sigma) in a volume of 20 μl. After treatment, the proteins were resolved on 8% SDS-PAGE gels and analyzed in Western blot experiments with the anti-Fe65 antibody.

RESULTS AND DISCUSSION

Two-hybrid System Screening of the Fe65 PID1 Ligands—To isolate cDNAs coding for proteins able to form complexes with the region of Fe65 containing the PID1 motif, we employed the interaction trap procedure. The region of Fe65 from amino acid 365 to amino acid 533, including the whole PID1 element (see Ref. 15), was cloned into the pGBT10 vector and used as a bait to screen a human brain cDNA library by the two-hybrid system in yeast. Two positive clones were isolated; the cDNA inserts present in the pGAD vector were identical, and their nucleotide sequence encodes the C-terminal 195 amino acid of the already known transcription factor CP2/LSF/LBP1 (21–23). To evaluate whether the interaction observed in yeast between recombinant fragments of the two proteins also takes place *in vivo* between the native molecules, we explored the existence of Fe65-CP2/LSF/LBP1 complexes in PC12 cells. Fig. 1 shows the Western blot of the proteins immunoprecipitated from PC12 cell extracts. The immunoprecipitation was performed with an anti-CP2/LSF/LBP1 polyclonal antibody or with the corresponding preimmune serum and analyzed with the anti-Fe65

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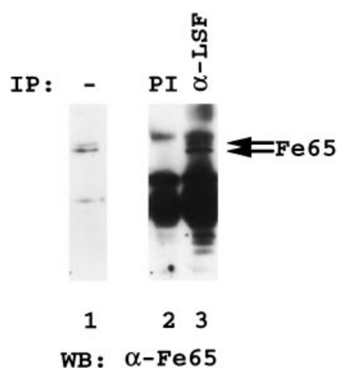

FIG. 1. ***In vivo* analysis of the Fe65-CP2/LSF/LBP1 complex.** Equal amounts of PC12 cell extracts were immunoprecipitated with preimmune serum (PI, *lane 2*) or with the anti-CP2/LSF/LBP1 antiserum (*lane 3*). 10 μg of PC12 lysate were run as a migration control (*lane 1*). The samples were analyzed by Western blot with the anti-Fe65 antibody.

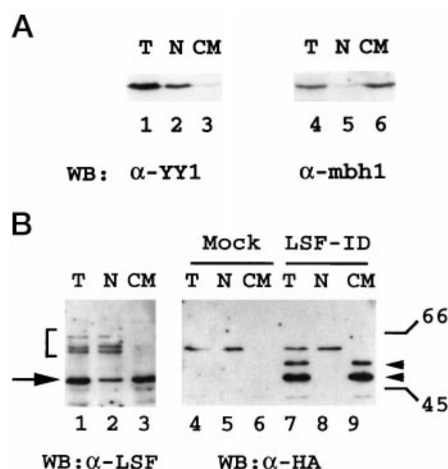

FIG. 2. **CP2/LSF/LBP1 is also present in the non-nuclear fraction.** *A*, Western blot of unfractionated and fractionated COS7 cell extracts with anti-YY1 (*lanes 1–3*) or anti-mbh1 (*lanes 4–6*) antibodies. *B*, in *lanes 1–3*, Western blot of unfractionated and fractionated COS7 cell extracts with anti-CP2/LSF/LBP1 are shown; the *bracket* and the *arrow* indicate the migration of the different CP2/LSF/LBP1 isoforms. *Lanes 4–9*, Western blot with HA probe (Santa Cruz) of unfractionated and fractionated extracts from COS7 cells transfected with CMV-LSF-ID vector. *Lanes 4–6*, mock-transfected cells; *lanes 7–9*, CMV-LSF-ID-transfected cells. *Arrowheads* indicate the protein bands appearing only in the extracts from CMV-LSF-ID-transfected cells; the size of the lowest and most abundant band is that expected on the basis of the nucleotide sequence of the transfected cDNA (HA tag + LSF-ID cDNA). Its migration is slightly slower than the 50-kDa band recognized by the anti-LSF antibody in untransfected cell extracts (*lane 3*) because of the presence of the HA tag. *T*, total extract; *N*, nuclear fraction; *CM*, cytosol/membrane fraction.

antibody. The result of the experiment clearly demonstrates that, by using the specific antibody, Fe65 co-immunoprecipitates with CP2/LSF/LBP1.

The first interesting observation regarding the findings reported in this paper is the absence of any ΦXNPXY motif in the CP2/LSF/LBP1 amino acid sequence. This element was found in all of the proteins interacting with PID/PTB domains, both in the case of growth factor receptors, in which it must be tyrosine phosphorylated to interact with Shc or IRS1 (19, 20), and in the case of the members of the APP family, whose interaction with the members of the Fe65 family and with X11 is, on the contrary, phosphorylation independent (11, 15). Several results have already suggested that the ΦXNPXY motif is not strictly required for Fe65-APP interaction. In fact, a 32-amino acid-long peptide, covering the C-terminal part of the APP cytodomain and containing the YENPTY motif, efficiently

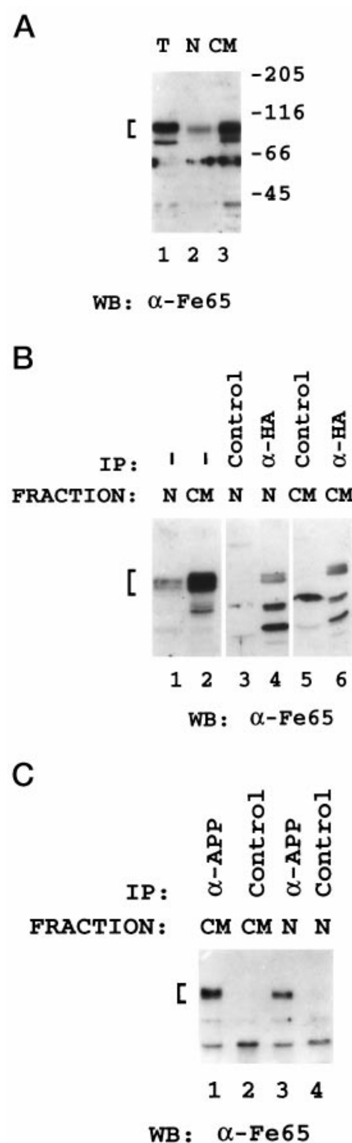

FIG. 3. **The Fe65/LSF complex is present in both nuclear and non-nuclear fractions.** *A*, Western blot with anti-Fe65 antibodies of unfractionated and fractionated extracts from COS7 cells transfected with the CMV-Fe65 expression vector. The *bracket* indicates the position of the different Fe65 isoforms. *B*, co-immunoprecipitation of protein fractions from COS7 cells transfected with CMV-Fe65 and CMV-HA-LSF expression vectors. Cellular fractions were prepared from transfected cells and immunoprecipitated with the control antibody 421 (*lanes 3* and *5*) or with the anti-HA antibody 12CA5 (*lanes 4* and *6*). The samples were resolved on 8% SDS-PAGE gel, blotted to polyvinylidene difluoride membrane, and stained with the anti-Fe65 antibody to detect the presence of Fe65 in the immunocomplexes. 10 μg of proteins from each fraction were loaded as a migration control in lanes 1 and 2 of the gel. The *bracket* indicates the migration of the Fe65 protein. *C*, co-immunoprecipitation of protein fractions from COS7 cells transfected with CMV-Fe65 and CMV-APP expression vectors. Nuclear (*N*, *lanes 3* and *4*) and non-nuclear (*CM*, *lanes 1* and *2*) fractions were prepared from transfected cells and immunoprecipitated with the control antibody 421 (*lanes 2* and *4*) or with the anti-APP antibody 6E10 (*lanes 1* and *3*). The samples were resolved on 8% SDS-PAGE gel, blotted to polyvinylidene difluoride membrane, and stained with the anti-Fe65 antibody to detect the presence of Fe65 in the immunocomplexes. The *bracket* indicates the migration of the Fe65 protein. *T*, total extract; *N*, nuclear fraction; *CM*, cytosol/membrane fraction.

competes for the binding of Fe65, whereas neither an 11-amino acid-long peptide nor a 20-amino acid-long peptide, both containing the YENPTY sequence, are able to compete with APP for the interaction with Fe65 (15). Furthermore, site-directed mutagenesis of the YENPTY sequence of APP demonstrated

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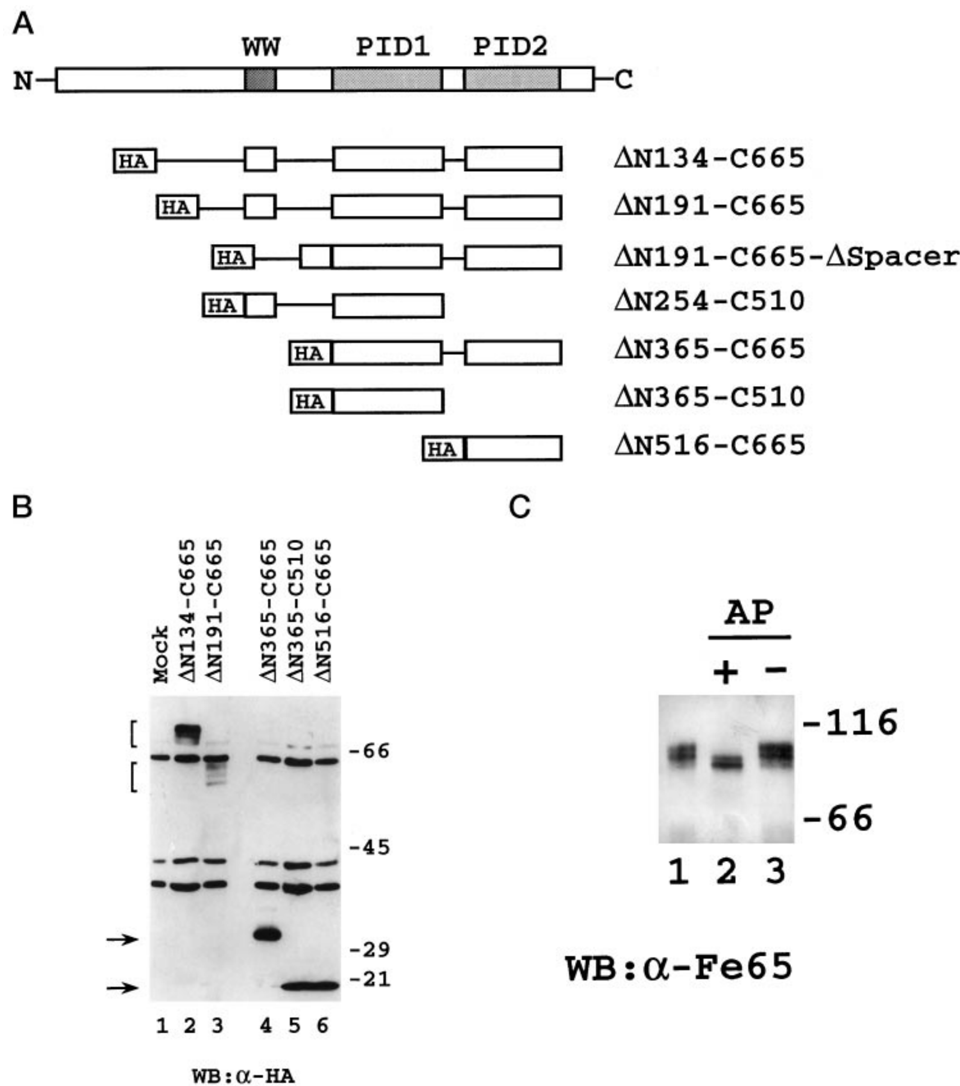

FIG. 4. **Analysis of *in vivo* expression of Fe65 deletion mutants and of Fe65 phosphorylation.** *A*, schematic representation of the Fe65 protein and of the HA-tagged Fe65 deletion mutants used in this study. The cDNA regions corresponding to the indicated residues of the Fe65 protein were obtained by PCR amplification or by overlapping PCR (see "Materials and Methods") of the full-length Fe65 cDNA and cloned in frame with the HA epitope in the pcDNAI-HA vector. *B*, Western blot of cell lysates from COS7 cells transfected with constructs driving the expression of the Fe65 deletion mutants. Total extracts (5 μg/lane) from COS7 cells transfected with the indicated constructs (*lanes 2–6*) were analyzed in Western blot experiment with the anti-HA 12CA5 antibody. *Lane 1*, mock-transfected cells. *C*, immunoprecipitated Fe65 protein from COS7 cell extracts was incubated for 4 h in alkaline phosphatase buffer in the presence (*lane 2*) or the absence (*lane 3*) of alkaline phosphatase; after incubation, samples were analyzed by Western blot with the anti-Fe65 antibody. *Lane 1*, untreated sample.

that the mutation of asparagine into alanine or of the C-terminal tyrosine into alanine had no effect on the binding of Fe65 to APP (11). Therefore, it seems conceivable that the Fe65 PID/PTB domains can recognize protein motifs significantly different from those interacting with the same domains in Shc or in IRS1 proteins. We examined the possible similarities between the CY domains of APP family proteins and CP2/LSF/LBP1, but no significant alignment was found.

CP2/LSF/LBP1 Is Also Present Outside the Nucleus—The observation that the transcription factor CP2/LSF/LBP1, whose expected intracellular localization is the nucleus, forms complexes with Fe65, which, as a partner of the cytosolic domain of the membrane protein APP and of the cytoskeleton-associated protein *Mena* (20, 32), is expected to by cytosolic or associated with membranes, prompted us to explore the subcellular compartmentalization of the two proteins. To do this, we examined the presence of CP2/LSF/LBP1 in two cellular fractions obtained by differential centrifugation. Nuclei and nuclear extracts (fraction N) were prepared from COS7 cells as described under "Materials and Methods." The supernatant of the nuclear pellet was then clarified by centrifugation to eliminate debris and the Nonidet P-40-resistant membranes. A fraction such as this is expected to contain cytosolic proteins and the membrane proteins soluble in 0.2% Nonidet P-40 (fraction CM). Given the procedure used for the cell fractionation, the nuclear extract is expected to contain, besides nucleoplasmic proteins, proteins present in perinuclear membranes. To control the efficiency of this fractionation, we analyzed the distribution in the fractions N and CM of two marker proteins, the transcription factor YY1 (33) and the cytosolic protein mbh1, mostly associated to the cytoskeleton (31, 34). Fig. 2*A* shows the Western blot analyses with the antibodies directed against YY1 and mbh1, showing that YY1 is present only in the nuclear fraction and mbh1 is present almost exclusively in the fraction CM. The extracts were then analyzed by Western blot using the anti-CP2/LSF/LBP1 antiserum. Fig. 2*B* shows that, in the total cell extract, this antibody recognized at least four protein bands ranging between 66 and 50 kDa. The distribution of these bands among the two fractions is not homogeneous. In fact, the nuclear fraction contains almost exclusively the four

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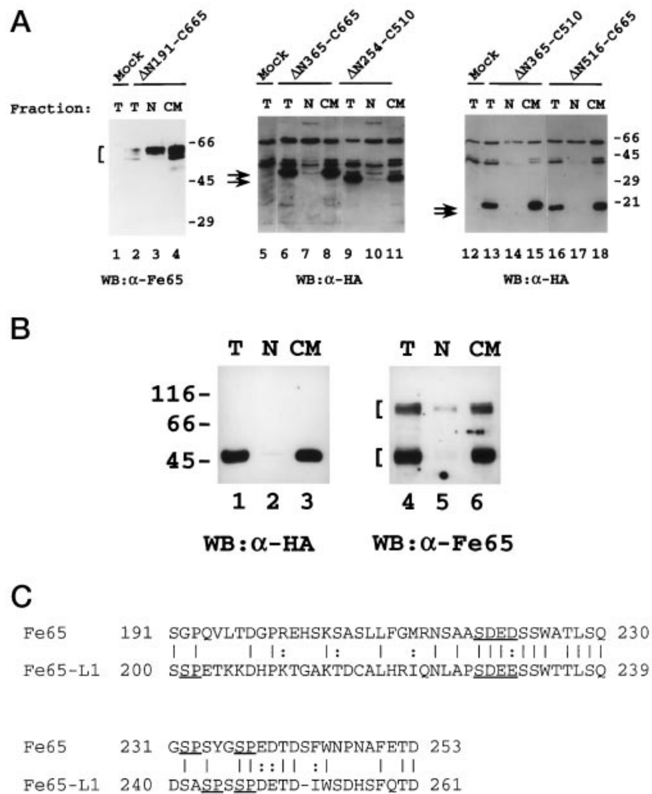

FIG. 5. **Subcellular distribution of the Fe65 deletion mutants.** *A*, extracts from COS7 cells transfected with the indicated Fe65 deletion constructs were analyzed in Western blot experiment with the anti-Fe65 (*lanes 1–4*) or with the anti-HA 12CA5 antibody (*lanes 5–18*). *Lanes 1*, *5*, and *12*, extracts from mock-transfected cells; *lanes 2–4*, *6–11*, and *13–18*, extracts or fractions from cells transfected with the indicated HA-Fe65 deletion mutants. The *bracket* and the *arrows* indicate the position of the Fe65 mutant proteins. *T*, total extract; *N*, nuclear fraction; *CM*, cytosol/membrane fraction. *B*, extracts from COS7 cells transfected with the Δ-N191-C665-Δ-spacer construct were analyzed by Western blot with the HA probe (Santa Cruz) (*lanes 1–3*) or with the anti-Fe65 antibody (*lanes 4–6*). The *brackets* indicate the positions of the endogenous Fe65 protein and of the transiently expressed deletion mutant. *C*, alignment of the Fe65 and Fe65-L1 proteins in the region containing the putative signal(s) for nuclear targeting; the *underlined sequences* indicate the potential phosphorylated serine residues followed by a proline or by an acidic stretch characteristic of mitogen-activated protein kinase and casein kinase II consensuses, respectively.

slowest bands, which have been described to correspond to various phosphorylated forms of the protein (35), whereas the CM fraction contains mostly the lowest band of 50 kDa, whose size is compatible with that of the CP2/LSF/LBP1 isoform encoded by the alternatively spliced mRNA LSF-ID (22). This CP2/LSF/LBP1 form is unable to bind the DNA, and therefore its presence outside the nucleus is not surprising. To further analyze this point, we transfected COS7 cells with a vector in which the CMV promoter drives the expression of the LSF-ID cDNA tagged with the HA epitope. As shown in Fig. 2*B*, the Western blot of fractionated cell extracts demonstrated that the trasfected HA-LSF-ID is present only in the CM fraction and absent from the nuclear fraction. This result suggests a role for the region missing from LSF-ID and present in the unspliced form of CP2/LSF/LBP1 in the subcellular distribution of the two isoforms.

CP2/LSF/LBP1 Interacts with Fe65 Both in Nuclear and Non-nuclear Fractions—A plausible simple explanation for the existence of an Fe65-CP2/LSF/LBP1 complex is, therefore, that Fe65 interacts in the extranuclear compartment with the alternatively spliced LSF-ID form, which is present mostly outside the nuclear compartment. We cannot directly test this hypothesis by immunoprecipitating cell extracts with the anti-Fe65 antibody and analyzing the immunoprecipitated proteins with the anti-CP2/LSF/LBP1 antibody, because of the comigration of the CP2/LSF/LBP1 bands with the immunoglobulin heavy chain band. However, it is possible that the nuclear form of CP2/LSF/LBP1 interacts with Fe65. In fact, the distribution of Fe65 among the two fractions, represented in Fig. 3*A*, shows that most of the cellular Fe65 is contained in the fraction CM, but a significant part of Fe65 is also present in the nuclear fraction. Therefore, we evaluated the interaction of Fe65 with the nuclear, unspliced form of CP2/LSF/LBP1. To accomplish this, we transfected COS7 cells with an expression vector in which an HA-tagged CP2/LSF/LBP1 cDNA (unspliced form) is under the control of the CMV promoter. Fig. 3*B* shows that both in nuclear and in non-nuclear fractions, Fe65 co-immunoprecipitates with the HA-tagged CP2/LSF/LBP1. This means that the Fe65 present in the nuclear fraction is able to form a complex with the nuclear, unspliced form of CP2/LSF/LBP1.

The presence of Fe65-APP complexes was also found in both fractions (see Fig. 3*C*); it would thus be very interesting to demonstrate the existence of a ternary complex APP-Fe65-CP2/LSF/LBP1. To address this point, we analyzed the proteins immunoprecipitated with anti-CP2/LSF/LBP1 from PC12 cells or with anti-HA from COS7 cells transfected with the vector encoding HA-CP2/LSF/LBP1 by Western blot with anti-APP antibodies. In both cases, no co-immunoprecipitation was observed (data not shown). The simplest explanation of these results is that the ternary complex APP-Fe65-CP2/LSF/LBP1 does not exist, but, considering the poor efficiency of this experimental approach, to clearly define this point, further experiments are required. In any case, a possibility to be considered is that Fe65 and CP2/LSF/LBP1 form complexes within the nucleus or associated with the nuclear envelope independent from the already demonstrated Fe65-APP and Fe65-Mena complexes. This possibility must be further examined, also considering previous observations which suggest that Fe65 could function as a transcription factor (12).

A Region Flanking the WW Domain Is Phosphorylated and Contributes to the Regulation of Fe65 Distribution between Nuclear and Non-nuclear Fractions—An important point to be addressed concerns with the molecular basis of the distribution of Fe65 between the nuclear and non-nuclear fractions. To address this point, we generated various deletion mutants of the Fe65 cDNA, described in Fig. 4*A*, that were cloned in an expression vector under the control of the CMV promoter. These fragments were tagged with an in-frame 5′ sequence encoding the amino acid sequence recognized by the anti-HA antibody. The HA-tagged deletion mutants were expressed in COS7 cells by transient transfection, and their expression was analyzed by Western blot using the anti-HA antibody. As shown in Fig. 4*B*, all of the fragments are expressed in COS7 cells, and their size is in agreement with the expected masses. The most interesting observation that can be made with this experiment concerns the heterogeneity of the bands. In fact, as previously observed (15), the anti-Fe65 antibody recognizes multiple Fe65 bands. The band heterogeneity cannot be explained on the basis of the existence of alternatively spliced isoforms of the Fe65 mRNA or of similar proteins cross-reacting with the antibody, as the transient expression of the Fe65 cDNA in various cell lines leads to the appearance of the same electrophoretic pattern. Therefore, it is conceivable that the band heterogeneity is the result of post-translational modifications of the protein. Very interestingly, among all of the deletion mutants, only the Δ-N134-C665 and Δ-N191-C665 proteins show a heterogeneous pattern of bands, similar to that of the wild-type Fe65, whereas all of the other constructs are expressed as single bands. This means that only the Δ-N134-

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C665 and Δ-N191-C665 proteins still contain the target region of the post-translational modifications and/or still possess the conformation suitable for these modifications.

A possible modification that could occur at the level of the region of Fe65 present in the wild-type molecule and in the two longest mutants, but absent from the other mutants, is phosphorylation because of the presence of several serines, contained in the consensus (S/T)P*XX* motif and in the consensus $(S/T)(D/E)_3$, that are known to be phosphorylated by MAPK and casein kinase, respectively (36, 37). To test this hypothesis, total extracts from PC12 cells were incubated with alkaline phosphatase for various times. As shown in Fig. 4*C*, the treatment with phosphatase results in the shift of the bands toward faster migrating forms, which supports the hypothesis that Fe65 is phosphorylated.

To evaluate the possible role of the various Fe65 domains in the distribution of the proteins between the two fractions described above, we analyzed the presence of the various deletion mutants in the two fractions. As shown in Fig. 5, the Δ-N191-C665 mutant protein is present in both fractions, like the wild-type Fe65 and the Δ-N134-C665 protein (not shown), whereas the shorter mutants are significantly present only in the cytosolic/membrane fraction. This result suggests that the presence of Fe65 in the nuclear fraction is regulated. This does not seem to be dependent on the association of Fe65 with membranes, through its binding to APP, because the shorter mutant containing the PID2 motif (Δ-N365-C665, see Fig. 4), which is restricted to the CM fraction, can be co-immunoprecipitated with APP (not shown). Furthermore, it is interesting to note that the pattern of band heterogeneity of wild-type and mutant Fe65 molecules in the nuclear fraction is shifted toward the slower (*i.e.* phosphorylated) bands (see Fig. 3*A*, *lane 2*, and Fig. 5*A*, *lane 3*), thus suggesting that the phosphorylated forms of Fe65 are preferentially associated with the nuclear fraction.

To evaluate the role of the small region flanking the WW domain at its N-terminal side, in which the phosphorylated residues are probably present, we generated a deletion mutant of the Fe65 cDNA, similar to the Δ-N191-C665 but for an internal deletion abolishing the 72 residues present between the WW and the PID1 domains (see Fig. 4*A*, Δ-N191-C665-Δ-spacer). The protein encoded by this mutant construct is expressed in transiently transfected COS7 cells at significant levels and is present only in the CM fraction (see Fig. 5*B*), therefore indicating that the small region flanking the WW domain at its N-terminal side is necessary but not sufficient to target Fe65 to the nuclear fraction.

The analysis of the three members of the Fe65 protein family shows that Fe65 and Fe65-L1 possess a long N-terminal sequence flanking the WW domain, whereas in Fe65-L2 it is significantly shorter, considering that its putative starting methionine is located 28 residues upstream of the WW domain (17). The similarity between Fe65 and Fe65-L1 is very high in regions containing the three protein-protein interaction domains, and, very interestingly, the remaining parts of the two proteins are unrelated except for a small stretch of amino acids that is included in the region of Fe65 and that seems to be responsible for the observed compartmentalization of the protein (see Fig. 5*C*). This region is not conserved in Fe65-L2.

Our finding supports the hypothesis that this region of Fe65 contains regulatory information. The analysis of its sequence indicates that the most interesting motif that could be involved in this regulation is represented by the (S/T)P*XX* elements present in the N-terminal part of Fe65 flanking the WW domain. These elements were found in many DNA binding proteins and were suggested to be a key structure in nonspecific DNA binding sequences (38). In CP2/LSF/LBP1 itself, several of these elements are present and phosphorylated by mitogen-activated protein kinase (39). They are also found in the lamin B receptor, an integral protein of the nuclear envelope inner membrane (40), and in the NDC-1 protein, also found in the nuclear envelope membrane (41). Interestingly, both presenilins have recently been demonstrated to be present at the level of the nuclear membrane (42), and both contain several (S/T)P*XX* motifs (43). Furthermore, APP is phosphorylated by cdc2 at Thr-668, which is followed by a P*XX* motif (44).

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Nicola Zambrano, Giuseppina Minopoli, Paola de Candia and Tommaso Russo

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