

Interaction of the Phosphotyrosine Interaction/Phosphotyrosine Binding-related Domains of Fe65 with Wild-type and Mutant Alzheimer's β -Amyloid Precursor Proteins*

(Received for publication, October 9, 1996, and in revised form, December 23, 1996)

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The two tandem phosphotyrosine interaction/phosphotyrosine binding (PID/PTB) domains of the Fe65 protein interact with the intracellular region of the Alzheimer's β -amyloid precursor protein (APP). This interaction, previously demonstrated *in vitro* and in the yeast two hybrid system, also takes place *in vivo* in mammalian cells, as demonstrated here by anti-Fe65 co-immunoprecipitation experiments. This interaction differs from that occurring between other PID/PTB domain-containing proteins, such as Shc and insulin receptor substrate 1, and activated growth factor receptors as follows: (i) the Fe65-APP interaction is phosphorylation-independent; (ii) the region of the APP intracellular domain involved in the binding is larger than that of the growth factor receptor necessary for the formation of the complex with Shc; and (iii) despite a significant similarity the carboxyl-terminal regions of PID/PTB of Fe65 and of Shc are not functionally interchangeable in terms of binding cognate ligands. A role for Fe65 in the pathogenesis of familial Alzheimer's disease is suggested by the finding that mutant APP, responsible for some cases of familial Alzheimer's disease, shows an altered *in vivo* interaction with Fe65.

The β -amyloid precursor protein (APP)¹ is an integral membrane protein from which the β -amyloid peptide is generated. The β -amyloid peptide forms the extracellular insoluble aggregates characteristic of Alzheimer's disease. The function of APP and the regulation of the proteolytic events generating the β -amyloid peptide are still unknown. APP was expected to be involved in signal transduction processes, because of its transmembrane topology. Three main isoforms of APP exist, generated by alternative splicing (APP₇₇₀, APP₇₅₁, and APP₆₉₅) and all possessing the same intracellular domain (reviewed in Ref.

1). Although little is known about the putative extracellular ligand(s) for APP, several results describe the interaction of its intracellular domain with other proteins. These include the interaction with the heterotrimeric G protein Go (2), a 59-kDa ubiquitously expressed protein named APP-BP1 (3), the X11 protein (4), the neuron-abundant Fe65 protein, and an Fe65-like protein (4–6). It was shown that intact APP binds to oligomeric Go protein and that the intracellular region of APP spanning residues 657–676 activates Go (2, 7). Furthermore, the interaction of APP with a monoclonal antibody directed against its extracellular domain mimics a ligand-receptor binding that triggers Go activation (7). APP-BP1 interacts both *in vitro* and *in vivo* with the carboxyl-terminal region of APP, which represents its intracellular domain. This protein is homologous to the product of the *Arabidopsis* auxin resistance gene *AXR1* and to a *Caenorabditis elegans* protein of unknown function (3).

The *Fe65* gene is mainly expressed in the neurons of specific regions of the mammalian nervous system (8, 9) and encodes a protein containing two different types of protein-protein interaction domains: the WW domain (reviewed in Ref. 10) and the phosphotyrosine interaction/phosphotyrosine binding (PID/PTB) domain (reviewed in Ref. 11). The latter was found in the oncoprotein Shc (12, 13), in its relatives ShcB-Sck and ShcC (14), in other apparently unrelated proteins, such as Numb, X11, and Dab (15), and in insulin receptor substrate 1 (IRS-1) and IRS-2 (16, 17). The PID/PTB domains interact with phosphotyrosine residues located in the intracellular domains of growth factor receptors, such as EGF-R, trkA, and platelet-derived growth factor receptor in the case of Shc (13) and insulin receptor and interleukin 4 receptor in the case of IRS-1 (16). In contrast, the Fe65 region containing the two PID/PTB domains was demonstrated to interact with the intracellular domain of APP (5).

All the PID/PTB domains present in the Shc family, IRS-1, and Fe65 interact with intracellular regions of membrane proteins containing the consensus motif Φ XNPNXY (where Φ is hydrophobic and X is any amino acid). However, Fe65 possesses at least two unique characteristics: (i) although all the known members of the PID/PTB family contain only one PID/PTB element (13), Fe65 is an exception, because its sequence interacting with APP shows two consecutive PID/PTB domains; and (ii) although the Tyr present in the consensus sequence of all the growth factor receptors must be phosphorylated to allow the binding to the PID/PTB domain of Shc and IRS-1 (18), no experimental result supports the possibility that

* This work was supported by grants from the Associazione Italiana per la Ricerca sul Cancro, the Ministero dell'Università e Ricerca Scientifica e Tecnologica 40% and 60%, and Consiglio Nazionale delle Ricerche, Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: APP, amyloid precursor protein; PID/PTB, phosphotyrosine interaction/phosphotyrosine binding; IRS, insulin receptor substrate; EGF-R, epidermal growth factor receptor; FAD, familial Alzheimer's disease; wt, wild-type; CHO, Chinese hamster ovary; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; TBS-T, Tris-buffered saline and Tween 20.

the Tyr of the consensus motif present in the intracellular domain of APP can be phosphorylated.

Another important aspect of APP is the interaction between Fe65 and the mutant forms of APP found in some forms of familial Alzheimer's disease (FAD). These point mutations involve residues in the juxtamembrane and transmembrane regions of APP (19), and it can be hypothesized that they could cause conformational changes of the APP intracellular domain or defects in the transduction phenomena involving APP, which in turn could affect the interaction with Fe65.

In this report we show that the PID/PTB region of Fe65 significantly diverges from the structurally related domains present in the family of the growth factor receptor-binding proteins, including Shc and IRS-1. Furthermore, we document that some mutant forms of APP, found in FAD, interact poorly *in vivo* with Fe65, suggesting the involvement of this molecule in the genesis of the Alzheimer's disease phenotype.

EXPERIMENTAL PROCEDURES

Generation of the Recombinant Constructs—The various Fe65 cDNA fragments used in this study were obtained by amplification of the FE65 cDNAs described previously (8, 20) by polymerase chain reactions. The fragment corresponding to the Fe65 cDNA encoding both PID/PTB domains (Fe65-PID1.2, codons 312–612) (5), as well as the deletion mutants Fe65-PID1 (codons 312–479), Fe65-ΔC1 (codons 312–508), Fe65-ΔC2 (codons 312–577), Fe65-PID2 (codons 484–612), and Fe65-ΔN1 (codons 377–612) and the Shc PID/PTB domain (Shc-PID, codons 46–232) were obtained by direct amplification of the FE65 or Shc cDNAs with specific oligonucleotide primers (CEINGE). The FE65 fragments containing internal deletions, *i.e.* Fe65-ΔSp. (codons 312–456 and 484–612) and Fe65-ΔSp. C3 (codons 312–424 and 484–612), as well as the Fe65-Shc chimeric constructs, *i.e.* Fe65-ΔC1-Shc (codons 312–508_{Fe65} and 84–232_{Shc}), Fe65-ΔC2-Shc (codons 312–577_{Fe65} and 154–232_{Shc}), Shc-ΔC-PID1 (codons 46–193_{Shc} and 445–457_{Fe65}), and Shc-ΔC-PID2 (codons 46–193_{Shc} and 600–612_{Fe65}), were obtained by overlap extension of the corresponding polymerase chain reaction-generated fragments containing complementary sequences (21). The recombinant constructs were obtained by ligation of the polymerase chain reaction fragments digested with appropriate restriction enzymes and purified from agarose gels with the QIAEX gel extraction kit (Qiagen) in the yeast pGBT10 vector (Clontech) and/or in pGEX vectors (Pharmacia Biotech Inc.) by standard cloning procedures and sequenced by using the Sequenase kit (U. S. Biochemical Corp.).

Cell Culture, Metabolic Labeling, and Extract Preparation—PC12 cells were cultured in Dulbecco's modified minimal essential medium (ICN) supplemented with 10% fetal calf serum (Hyclone), 5% horse serum (Life Technologies, Inc.), and 1% penicillin/streptomycin mixture (ICN). For the metabolic labeling, exponentially growing PC12 cells were starved of methionine and cysteine for 30 min and then incubated in the presence of 80 μ Ci/ml [³⁵S]methionine/[³⁵S]cysteine mixture (Promix; Amersham Corp.; 1000 Ci/mol) for 3 h.

COS cells were cultured in Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum and transfected by electroporation with the Fe65 cDNA cloned into the pRc/CMV vector (Invitrogen). A431 cells were cultured in Dulbecco's modified minimal essential medium supplemented with 5% fetal calf serum and 1% antibiotic mixture. For EGF stimulation, confluent cultures of A431 cells were starved from serum for 72 h, and then EGF (100 ng/ml, Boehringer Mannheim) was added for 5 min at 37 °C. The cell lines expressing the wild-type (wt) and mutant APP were derived from a Chinese hamster ovary (CHO) line stably transfected with the wt APP₇₅₁ and the following natural APP mutants containing the indicated missense mutations: E693Q, V717I, and K670N/M671L. These lines were gifts from Dr. Eddie Koo (Harvard Medical School, Boston, MA) and were cultured in Dulbecco's modified minimal essential medium, 10% fetal calf serum, and 1% antibiotics.

For the preparation of the cellular extracts, monolayer cultures were washed twice with cold phosphate-buffered saline and lysed at 4 °C in lysis buffer as described (5). The extracts were then clarified by centrifugation, and their protein concentration was determined by the Bio-Rad protein assay following the manufacturer's instructions.

Antibodies, Immunoprecipitations, and Western Blots—The anti-APP monoclonal antibody used for the immunoprecipitations was 6E10 (22), whereas polyclonal antibody 369 (23) was used for the detection of APP in immunoblots; the 421 monoclonal antibody (Oncogene Science),

specific for the p53 protein, was used as a control in the immunoprecipitations. The anti-Fe65 polyclonal antibody was raised in rabbits by using a glutathione *S*-transferase GST-Fe65 fusion protein (Fe65 residues 201–236) as an immunogen. For the immunoprecipitations, the cellular extracts were incubated with appropriate dilutions of the different antibodies for 1 h at 4 °C, and then protein A-Sepharose resin (Pharmacia, 30 μ l/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% bromphenol 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 antibody for 1 h at room temperature. The excess antibody was removed by sequential washing of the membranes in TBS-T, and then a 1:2000 dilution of horseradish peroxidase-conjugated protein A (Amersham) was added to the filters for 1 h at room temperature. Filters were washed and the signals detected by chemiluminescence using the ECL system (Amersham).

Expression in *Escherichia coli* of the Recombinant Proteins, Pull-down and Peptide Competition Assays, and Surface Plasmon Resonance—The constructs obtained by the cloning of the polymerase chain reaction fragments in the pGEX vectors were used to transform *E. coli* cells (BL21 strain). The resulting colonies were grown in LB broth, and the synthesis of the various GST fusion proteins was achieved by the induction of exponentially growing cultures with 0.25 mM isopropyl-1-thio- β -D-galactopyranoside for 3 h at 30 °C. The cells were harvested by centrifugation and lysed by sonication in phosphate-buffered saline (1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, 27 mM KCl, 137 mM NaCl). The recombinant proteins were purified with glutathione-Sepharose resin (Pharmacia) following the instructions of the manufacturer.

For the pull-down assay, glutathione-Sepharose beads (Pharmacia, 10 μ l/sample) were saturated with appropriate amounts of the recombinant proteins in phosphate-buffered saline containing 1 mM dithiothreitol and incubated with the cell extracts (500 μ g/sample) for 2 h at 4 °C. Unbound proteins were removed by washing the beads with lysis buffer, whereas the bound proteins were eluted and resolved by SDS-PAGE. Labeled proteins were visualized by fluorography of the gels with Amplify (Amersham) following the manufacturer's instructions, whereas unlabeled proteins were revealed by specific antibodies in Western blot experiments.

For the pull-down competition assay, ³⁵S-labeled proteins from PC12 extracts (500 μ g/sample) were incubated with GST or GST-Fe65 fusion protein (10 μ g) bound to glutathione-Sepharose beads in the presence (100-fold molar excess) or the absence of the competing peptides. After washing the resin, the protein samples were resolved on an 8% SDS-PAGE gel and visualized by autoradiography.

Surface plasmon resonance experiments were carried out on a BIAcore Biosensor apparatus (Pharmacia), and analysis was carried out with BIAevaluation software, version 2.0 (Pharmacia). A peptide corresponding to the carboxyl-terminal-most 49 amino acids of the APP cytoplasmic domain was immobilized by a terminal cysteine. All binding experiments were performed at 25 °C. GST fusion proteins were cleaved from the GST with thrombin (10 cutting units/400 μ l of resuspended beads, Sigma), the thrombin and GST were removed, and the proteins were then used for binding. Either PID1 or PID2 was added at concentrations of 0.31, 0.625, 1.25, and 2.5 μ M in 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.05% P-20 surfactant. The association, dissociation, and equilibrium constants were determined based on the assumption that the kinetics were first order.

Yeast Transformation and β -Galactosidase Assay—Yeast cells, Hf7c strain (24), were grown under standard conditions in synthetic medium without uracyl. Hf7c competent cells were prepared from exponentially growing cultures by the lithium acetate method (25) and co-transformed with the various Fe65 PID/PTB domain mutants cloned in the GAL4 DNA binding domain-containing vector pGBT10 and the plasmid pL1, containing a cDNA fragment encoding for the carboxyl-terminal, intracellular region of the human β -amyloid precursor protein (residues 664–695) fused to the GAL4 activation domain (5). The pGBT10-derived plasmids carry the Trp-selective marker, whereas the pL1 construct carries the Leu-selective marker; on co-transfection of each of the pGBT10-derived plasmids with pL1, the transformants harboring both plasmids were selected on Trp⁻/Leu⁻ plates, and the ability of the encoded proteins to form a functional GAL4 hybrid was tested by plating replicas of the colonies on His⁻/Trp⁻/Leu⁻ plates. The β -galactosidase assay was performed on all of the above described co-transfor-

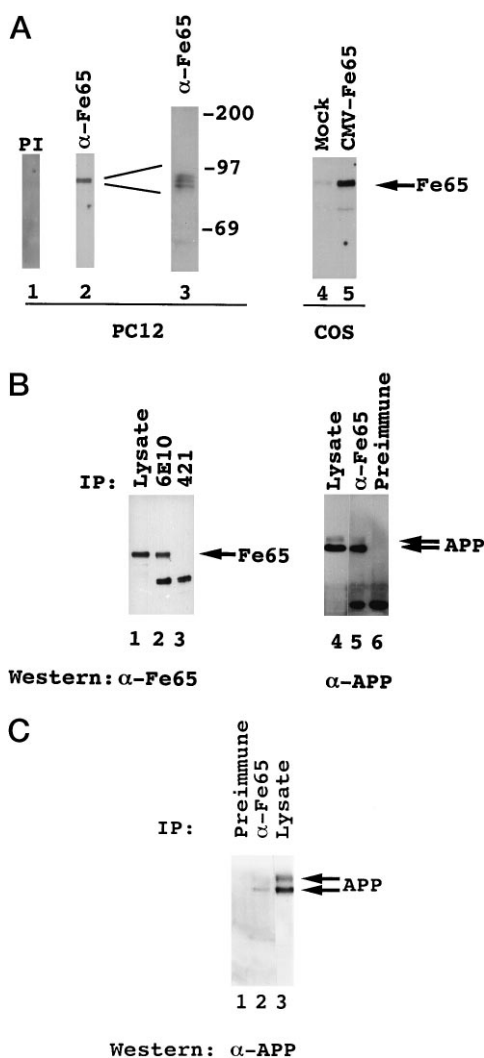


FIG. 1. Western blot and co-immunoprecipitation. *A*, Western blot of protein extracts from PC12 and COS cells using the anti-Fe65 antibody. *Lanes 1 and 2*, Western blot of PC12 cell extracts (10 μ g) stained by preimmune serum (PI) or by anti-Fe65 antibody (α -Fe65). *Lane 3*, Western blot of a long-migrated PC12 cell extract (100 μ g) stained by anti-Fe65 antibody. *Lanes 4 and 5*, Western blot of COS cell extracts mock transfected or transfected with the rat Fe65 cDNA under the control of the cytomegalovirus (CMV) promoter, stained by anti-Fe65 antibody. *B*, co-immunoprecipitation experiments of proteins from CHO cells stably transfected with APP cDNA and transiently transfected with Fe65 cDNA. *Lanes 1 and 4*, 10 μ g of lysates from the transfected cells were run as a control and probed with the anti-Fe65 and anti-APP antibodies, respectively. Equal amounts of lysates were immunoprecipitated (IP) with the anti-APP monoclonal antibody 6E10 (*lane 2*), with a control monoclonal antibody (antibody 421, *lane 3*), with the anti-Fe65 antibody (*lane 5*), or with the preimmune serum (*lane 6*). The lysates and the immunoprecipitates were analyzed by Western blot with anti-Fe65 antibody (*lanes 1–3*) or with anti-APP antibody 369 (*lanes 4–6*). *C*, co-immunoprecipitation experiment of PC12 cell lysates. Equal amounts of extracts were immunoprecipitated (IP) with the preimmune serum (*lane 1*) or with the anti-Fe65 antibody (*lane 2*). 20 μ g of PC12 lysate were run as a control (*lane 3*). The samples were analyzed by Western blot with the 369 anti-APP antibody.

mants on Trp⁻/Leu⁻ plates overlaid with nitrocellulose filters as described (5).

RESULTS AND DISCUSSION

Specific Anti-Fe65 Antibodies Demonstrate the Interaction between Fe65 and APP in Vivo—The screening of a cDNA library from human brain using the two hybrid system in yeast and pull-down experiments using GST-Fe65 fusion proteins demonstrated that the region of Fe65 from amino acid 312 to amino acid 612, which contains two motifs with sequences that

can be aligned to the Shc PID/PTB domain, interacts with the intracellular domain of APP and APLP1 (5). To evaluate whether this interaction also takes place with the native full-length proteins in intact cells, we first generated an anti-Fe65 antibody directed against a Fe65 fragment from amino acid 201 to amino acid 236, synthesized in *E. coli*. Fig. 1A shows that the Western blot of protein extracts from PC12 cells with this antibody visualizes a thick protein band of about 90 kDa. The Western blot of a “long-migrating gel” demonstrates four bands with molecular masses that range between 85 and 95 kDa. The same four bands were observed in COS cells transfected with the Fe65 cDNA (Fig. 1A, *lane 5*), thus suggesting that the protein heterogeneity is due to posttranslational modifications and not to alternative splicing or the cross-reaction of the antibody with related proteins.

Immunoprecipitation experiments were performed in CHO cells, stably expressing the full-length APP751 cDNA and transiently transfected with Fe65 cDNA, under the control of a strong housekeeping promoter. Protein extracts from these cells were incubated with a monoclonal antibody directed against the extracellular domain of APP, and the immunocomplexes were precipitated and analyzed by Western blot using the Fe65-specific antibody. Fig. 1B shows that Fe65 can be found in the complexes immunoprecipitated by the anti-APP antibody and not in the proteins immunoprecipitated by an unrelated monoclonal antibody (antibody 421, directed against the p53 protein). Similarly, APP is present in the proteins co-immunoprecipitated with Fe65 by the anti-Fe65 antibody and not in the control immunoprecipitation performed with the preimmune serum.

Fig. 1C shows that the interaction between Fe65 and APP also occurs at physiological concentrations of these proteins in PC12 cells. In fact, the Western blot with anti-APP antibody 369 identified this protein in the immunocomplexes containing the Fe65 protein.

Fe65 Interacts with APP in a Phosphorylation-independent Fashion, and the Interaction Motif Is Longer than the Φ XNPXY Consensus—It was clearly demonstrated by competition experiments that Shc binds, through its PID/PTB domain, to activated growth factor receptor in a tyrosine phosphorylation-dependent manner (18). Synthetic peptides covering different regions of the APP intracellular domain have been tested for their ability to compete for the binding between Fe65 and APP. Fig. 2 shows that neither an 11-amino acid-long peptide (residues 681–691 of APP₆₉₅) nor a 20-amino acid-long peptide (residues 675–694 of APP₆₉₅) is able to compete for the Fe65-APP interaction, whereas the 32-amino acid-long peptide, covering the intracellular domain of APP₆₉₅ from residues 664–695, corresponding to the sequence encoded by the pL1 cDNA clone isolated in the two hybrid system screening (5), efficiently competes for the binding of the GST-Fe65 fusion protein with PC12 ³⁵S-labeled proteins.

In the case of the Fe65-APP interaction the tyrosine phosphorylation of the Φ XNPXY motif has no effect; as shown in Fig. 2, the tyrosine-phosphorylated 11- and 32-mer peptides show the same behavior as the corresponding unphosphorylated versions. In fact, the 11-mer phosphopeptide is unable to compete, whereas the 32-mer phosphopeptide does, on the contrary, compete.

Therefore, in contrast to what was observed for the other PID/PTB domains (26), the interaction of Fe65 and APP is not dependent on the phosphorylation of the Tyr present in the Φ XNPXY motif of APP. This observation could be explained in light of the results obtained from NMR analysis of the Shc- Φ XNPXY peptide complex (27), which allowed the identification of three positively charged amino acids (Arg⁶⁷, Lys¹⁶⁹, and

Arg¹⁷⁵, *double underlined* in Fig. 3) interacting with the negatively charged phosphate moiety of the phosphotyrosine. These three residues are conserved in all the members of the PID/PTB Shc family (27), whereas (see the alignment of Fig. 3) they are not conserved in the PID/PTB domains of Fe65. Similarly, in other sequences related to Fe65, present in the sequence data banks as expressed sequence tags, these basic residues are not conserved, supporting the hypothesis that the proteins encoded by these Fe65-like genes also interact with unphosphorylated ligands.

Binding Efficiency of the Two PID/PTB Domains of Fe65—The most evident difference between Fe65 and the other pro-

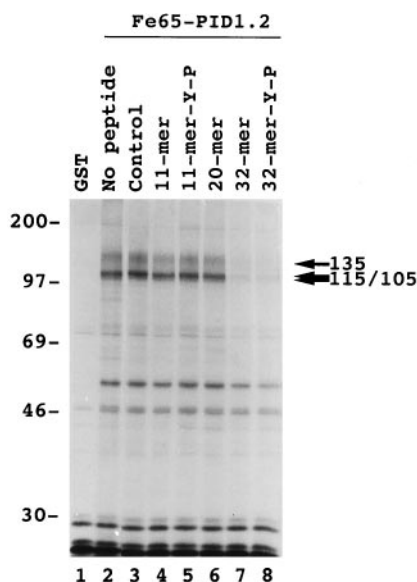


FIG. 2. The Fe65-APP interaction does not require the tyrosine phosphorylation of the APP carboxyl-terminal region. Pull-down assay showing the interference of various unphosphorylated and phosphorylated synthetic peptides corresponding to the carboxyl-terminal region of APP with the formation of complexes between ³⁵S-labeled proteins from PC12 cells and the Fe65 protein. The labeled extracts were incubated with the glutathione-Sepharose-bound proteins GST (lane 1) or GST-Fe65 (Fe65-PID1.2, lanes 2–8) in the presence of a 100-fold molar excess of the synthetic peptides indicated at the top. Arrows, positions of the ³⁵S-labeled APP isoforms of 105, 115, and 135 kDa specifically interacting with Fe65. Sequences of the peptides: control peptide, LGQQQPFPPQPPY; 11-mer, GYENPTYKFFE, residues 681–691 of APP₆₉₅; 11-mer-Y-P, GYENPTYpKFFE; 20-mer, SKMQQNGYENPTYKFFEQMQ, residues 675–694 APP₆₉₅; 32-mer, DAAVTPEERHLSKMQQNGYENPTYKFFEQMQN, residues 664–695 of APP₆₉₅; 32-mer-Y-P, DAAVTPEERHLSKMQQNGYENPTYpKFFEQMQN.

teins possessing a PID/PTB domain is that two consecutive segments of Fe65 can be aligned to the PID/PTB module of Shc. These segments are located at the carboxyl-terminal half of Fe65 from amino acid 312 to amino acid 612 and are separated by 27 amino acids. We evaluated the possible redundancy of one of the two elements by constructing GST-Fe65 fusion proteins containing only the PID1 or the PID2 element (the amino- and carboxyl-terminal, respectively). The pull-down experiments reported in Fig. 4A show that PID1 is unable to interact with APP from PC12 cells, compared with the complete PID/PTB region of Fe65. In contrast, PID2 alone efficiently interacts *in vitro* with APP as the complete PID1.2 region of Fe65. This result was confirmed by testing the activity of PID1 and PID2 fused to the DNA binding domain of GAL4 in the two hybrid system in yeast, in which only PID2 is able to interact with the intracellular domain of APP fused to the activation domain of GAL4 (see Fig. 4B), although at a lower efficiency compared with the complete PID1.2 region of Fe65.

The binding of Fe65 to APP was further explored by testing the efficiency of GAL4-Fe65 constructs lacking small fragments at the amino terminus or the carboxyl terminus to bind APP. As shown in Fig. 4B, the deletions at the carboxyl terminus (Fe65-ΔC1 and Fe65-ΔC2) are sufficient to completely prevent the interaction, whereas the amino-terminal deletion of PID1 resulted in a decreased efficiency of interaction. Interestingly, the internal deletion removing the 27-amino acid-long spacer resulted in a mutant protein (Fe65-Δ Sp.), which completely retains the ability to interact with APP. A longer internal deletion, involving the PID1 carboxyl terminus (Fe65-Δ Sp. C3) again resulted in a phenotype suggesting a decreased interaction with APP (see Fig. 4B).

Surface plasmon resonance analysis of the binding of the PID/PTB domains of Fe65 to the cytoplasmic tail of APP support the aforementioned observations that: (i) PID2 is sufficient for binding to APP; (ii) PID1 is unable to bind to APP; and (iii) tyrosine phosphorylation of APP is not required for the interaction (see Fig. 5). In these experiments, measurements were taken of the binding of recombinant Fe65 PID1 or PID2 to an immobilized synthetic peptide corresponding to the last 50 amino acids of APP. Although PID1 does not bind at any concentration tested (see Fig. 5A), the interaction of PID2 with APP is dose-dependent (see Fig. 5B). The *k*, *k_d*, and *K_d* values for PID2 binding to APP were determined to be $9.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, $3.3 \times 10^{-2} \text{ s}^{-1}$, and 488 nm, respectively.

The PID/PTB Domains of Fe65 Do Not Share Any Functional Similarity with Shc—The above-reported data suggest that the PID/PTB domains of Shc and Fe65 are significantly divergent. According to these functional observations, the

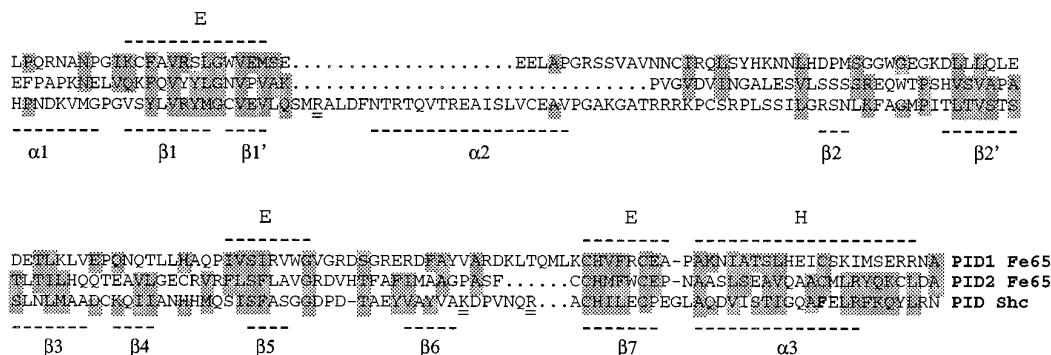


FIG. 3. Alignment of the Shc and Fe65 proteins at the level of the respective PID/PTB domains. The secondary structure of the Shc PID/PTB domain as defined from the NMR structure by Zhou *et al.* (27) is indicated by the dashed lines below the Shc sequence; for the definition of the Fe65 PID1 and PID2 domains, several programs for protein secondary structure prediction were used (33), and the dashed lines above the PID1 and the PID2 sequences indicate the extended (E) and helical (H) regions assigned with high scores by all the programs used. The sequence similarities between the three PID/PTB domains are indicated by the shaded areas; the double underlined residues indicate the Shc charged residues making contacts with the phosphotyrosine of the ΦXNPXpY motif (27) that are absent from the Fe65 PID1 and PID2 domains.

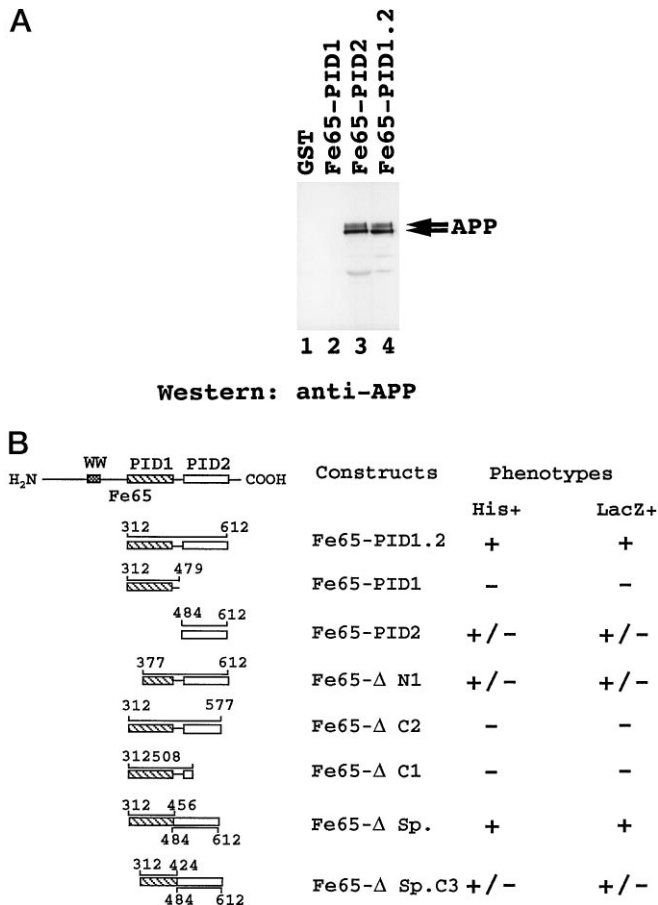


FIG. 4. Analysis of APP-Fe65 interaction. *A*, pull-down assay carried out on PC12 cell extracts incubated with the GST protein (lane 1) or with the single PID/PTB domains of Fe65 (Fe65-PID1 and Fe65-PID2, lanes 2 and 3, respectively) or with the GST-Fe65 fusion proteins corresponding to the tandem Fe65 PID/PTB domains (Fe65-PID1.2, lane 4). *B*, results of the analysis of the interaction of the Fe65 PID/PTB mutants with APP by the two hybrid system in yeast. The plasmid pL1, encoding a hybrid protein GAL4 activation domain-APP intracellular domain (residues 664–695; see Ref. 4) was used to transform the yeast strain Hf7c in the presence of each of the plasmids expressing the Fe65 cDNA fragments listed at the left fused to the GAL4 DNA binding domain in the pGBT10 plasmid. The co-transformants were selected on Trp⁻/Leu⁻ plates, and their phenotypes were analyzed for the ability to grow on selective Trp⁻/Leu⁻/His⁻ plates and for the ability to express the β -galactosidase gene. In fact, when a successful interaction of the two hybrid proteins takes place inside the cell, a functional GAL4 molecule is reconstituted that is able to transactivate the reporter genes *HIS3* and *LacZ* controlled by the GAL4 cis-elements. The phenotypes obtained from the single co-transformants are indicated on the right in *B*.

alignment reported in Fig. 3 shows that large segments of Fe65 and Shc are very poorly conserved, largely because PID1 and PID2 domains of Fe65 are shorter than Shc, so that, for example, it is impossible to find in both of the Fe65 PID/PTB structures any sequence corresponding to the region including the α_2 helix of Shc, as determined by NMR analysis (27). The strongest similarity among Shc and Fe65 PID/PTB domains is restricted at the level of the sequence corresponding to the α_3 structure of Shc. This sequence similarity is confirmed by the computer-assisted analysis of the Fe65 secondary structure, which predicts, with a very high score, three extended structures and, in the region aligned to the α_3 region of Shc, a helical structure (see Fig. 3). To evaluate whether at least this last region could be interchangeable between Shc and Fe65, we constructed chimeric proteins in which the carboxyl-terminal region of Fe65 PID2 is substituted by the corresponding carboxyl-terminal region of Shc, and, vice versa, the carboxyl-

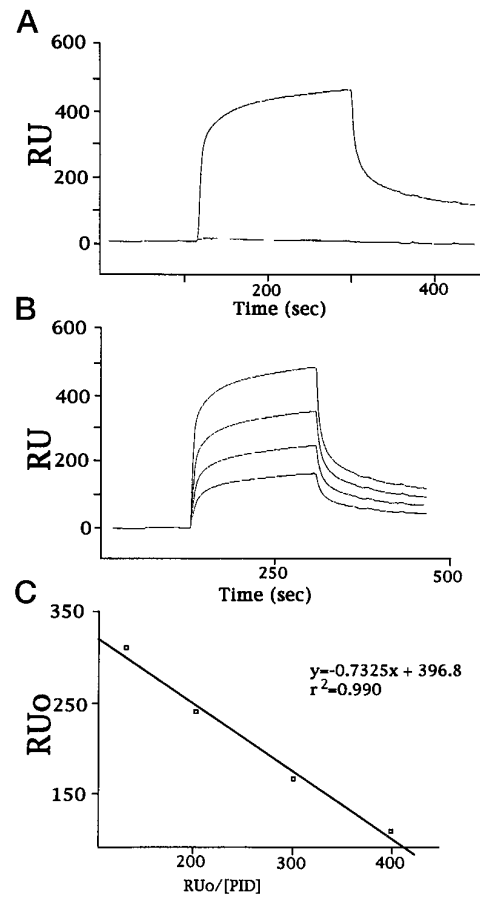


FIG. 5. Surface plasmon resonance analysis of the kinetics of Fe65 PID1 and PID2 binding to APP. *A*, response functions (sensorgrams) illustrate specific binding and dissociation profiles obtained after Fe65-PID1 or PID2 was added to the buffer flowing over a biosensor chip coated with a synthetic 50-amino acid peptide corresponding to the cytoplasmic tail of APP. Either PID1 (lower trace) or PID2 (upper trace) was added at 2.5 μ M. Although PID2 bound, PID1 did not. *B*, sensorgrams of the dose dependence of PID2 binding to APP. Concentrations used were 2.5, 1.25, 0.625, and 0.31 μ M (top to bottom). *C*, linearity over a range of concentrations implies a simple bimolecular interaction. *RU*, response units; *RU_o*, response units 5 s after termination of injection of Fe65-PID2.

terminal structure of Shc is substituted by the carboxyl-terminal part of either Fe65-PID1 or PID2. These chimeric proteins were tested for their ability to interact with APP or EGF receptor, respectively. In the first case, as shown in Fig. 6A, the yeast two hybrid system assay showed no interaction between the intracellular domain of APP fused to the GAL4 transactivation domain and two Fe65-Shc chimeras fused to the GAL4 DNA binding domain. Similarly, the interaction of Shc and the EGF receptor is prevented by the presence of either PID1 or PID2 carboxyl-terminal regions in the place of that of Shc. In fact, the proteins from A431 cells treated with EGF, co-precipitated with GST-Shc wild-type, Shc- Δ C-PID1, or Shc- Δ C-PID2 (see Fig. 6B), were analyzed by Western blot using an anti-phosphotyrosine antibody; this experiment showed that EGF-R interacts only with GST-Shc wild-type protein.

Although the two PID/PTB domains of Fe65 share the same differences with Shc, their nucleotide sequence homology and the degree of amino acid sequence similarity are very low, therefore, they are probably not repeated sequences generated by an internal duplication of the gene. However, there are some structural characteristics that distinguish the PID/PTB domains of Fe65 from those of the Shc family. These include the already mentioned absence of the basic residues involved in the interaction with the phosphate of the phosphotyrosine, a cys-

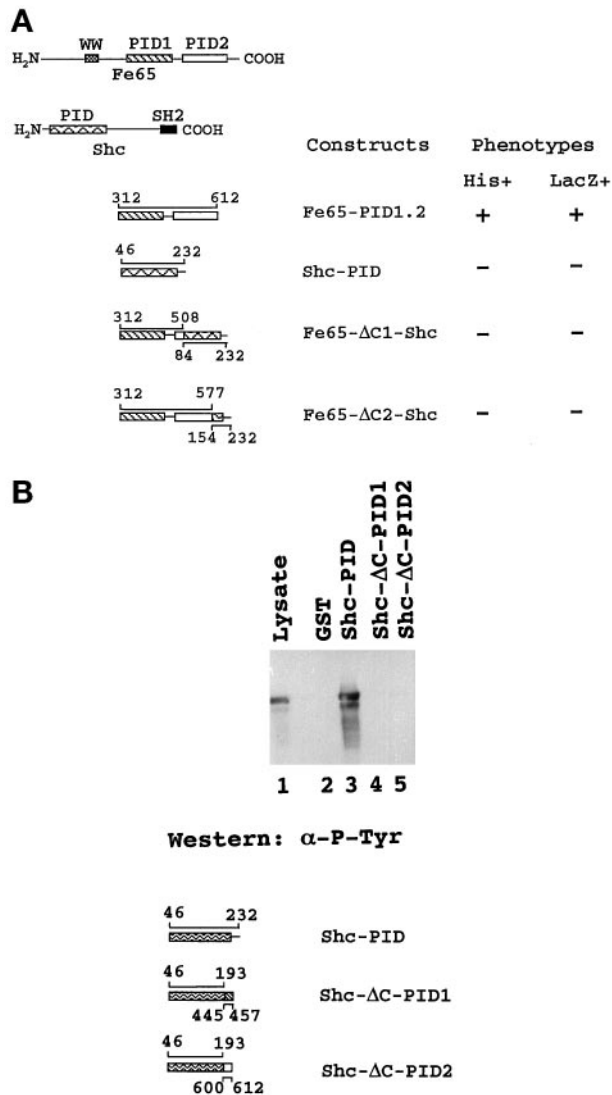


FIG. 6. The carboxyl-terminal regions of the Shc and Fe65 PID/PTB domains are not interchangeable. A, chimeric fragments, in which the Fe65 cDNAs ΔC2 and ΔC1 were fused to the region of the Shc cDNA corresponding to the deleted regions of Fe65, were analyzed in the two hybrid system as described in the legend of Fig. 4B. B, association of the EGF-R from EGF-stimulated A431 cells with GST-Shc or GST-Shc-Fe65 chimeras. Extracts from A431 cells stimulated with EGF (200 μg/sample) were incubated with glutathione-Sepharose-bound GST protein (lane 2), GST-Shc fusion protein (Shc-PID, residues 46–232; lane 3) or the Shc-Fe65 chimeras Shc-ΔC-PID1 (lane 4) and Shc-ΔC-PID2 (lane 5), in which residues 194–232 of the Shc PID/PTB domain were substituted with the corresponding residues of Fe65-PID1 (residues 445–457) and Fe65-PID2 (residues 600–612). 10 μg of the EGF-treated A431 extract were loaded on lane 1 as a control of migration. A schematic representation of the chimeric constructs is shown at the bottom.

teine that substitutes in both the PID/PTB domains of Fe65 and in the Fe65-like sequences (6) present in the Sequence Data Bank (accession numbers R67159 and T54909), and the Phe¹⁹⁸ of Shc, which is conserved in all the members of the Shc PID/PTB family (see Ref. 15; Fig. 3, bold). The mutation of this Phe¹⁹⁸ of Shc impairs both *in vitro* and *in vivo* the binding activity of Shc (15), and this is confirmed by the results of the experiments showing that the two Shc-Fe65 chimeras, in which the carboxyl-terminal structure of Shc is substituted by the carboxyl-terminal sequence of either PID1 or PID2 of Fe65, are completely unable to bind the EGF-R, despite a significant degree of similarity among the three sequences in this region. Similarly, the Fe65-Shc chimera, in which the carboxyl-termi-

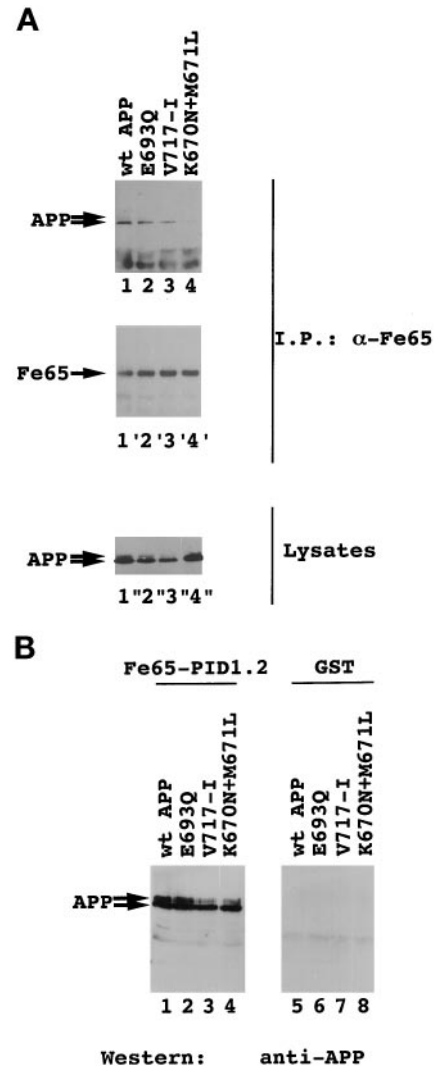


FIG. 7. Interaction of Fe65 with APP mutant forms of familial Alzheimer's disease. A, extracts prepared from the wild-type (lane 1) or mutant APP-expressing CHO cells indicated at the top (lanes 2–4) were immunoprecipitated with the Fe65-specific antibody and resolved on an 8% SDS-PAGE gel, blotted to a polyvinylidene difluoride membrane, and stained with the anti-APP antibody. The same blot was stained by using the anti-Fe65 antibody (lanes 1'–4'). Equal amounts of extracts from the same cell lines were examined to evaluate the amount of stably expressed wt or mutant APP by Western blot with the 369 antibody (lanes 1'–4'). B, extracts prepared from the wild-type (lanes 1 and 5) or the mutant APP-expressing cells (lanes 2–4 and 6–8) were analyzed for the interaction with glutathione-Sepharose-bound GST-Fe65 fusion protein (Fe65-PID1.2, lanes 1–4) or with the GST protein as a control (lanes 5–8) in pull-down experiments carried out with 200 μg of extract/sample. The proteins were resolved on an 8% SDS-PAGE gel, and the blots were stained by using anti-APP antibody 369. Arrows, migration of the different APP isoforms.

nal segment of PID2 is deleted and substituted by the corresponding carboxyl-terminal sequence of Shc, loses the ability to interact with APP, thus suggesting that the carboxyl-terminal region of PID2 plays an important role in the interaction with APP.

In Vivo Interaction of Fe65 with APP Mutant Forms Found in Alzheimer's Patients—The familial Alzheimer's mutants of APP are represented by missense mutations of the Val⁷¹⁷ residue changed to Ile, Phe, or Gly residues, Glu⁶⁹³, or Lys⁶⁷⁰ and Met⁶⁷¹ (APP₇₇₀) (19). Although these mutants are responsible for only a few cases of FAD, they represent a clear molecular link between β-amyloid deposition and the FAD genotype. To gain information about the molecular basis of FAD, we ad-

addressed the question of whether Fe65 and mutant APPs interact. To this end, CHO cells, stably expressing wt or mutant APP, were transiently transfected with the Fe65 cDNA, and 48 h after the transfection, the cell extracts were incubated with the anti-Fe65 antibody. The immunoprecipitated proteins were assayed by Western blot using the anti-APP and anti-Fe65 antibodies. The result reported in Fig. 7A shows that, despite the very similar amount of Fe65 in the immunoprecipitates from the different cell lines, the mutant forms of APP associate with Fe65 with a lower efficiency, compared with that shown by the wt APP. This is particularly evident in the case of the Lys⁶⁷⁰-Met⁶⁷¹ (APP₇₇₀) double mutation, also known as the Swedish mutation.

The results of the experiments aimed at the definition of the APP sequence interacting with Fe65 indicate that all the examined APP mutations are located outside the sequence involved in the interaction (*i.e.* from residue 664 to residue 695). This is in agreement with the observation that the mutant forms of APP expressed in CHO cells are able to associate and co-precipitate *in vitro* with a GST-Fe65 fusion protein with the same efficiency as the wt form (see Fig. 7B).

The decreased interaction efficiency between Fe65 and the Alzheimer's mutant forms of APP is particularly intriguing. The examined APP₇₇₀ mutations (V717F, E693Q, and K670N/M671L) take place at the level of the juxtamembrane and transmembrane domains of the protein, and it can be speculated that, as in the case of other integral membrane proteins, such as EGF-R (28) and insulin-like growth factor I receptor (29), a point mutation in this region is able to alter the transduction mechanisms. There are some data suggesting a role for APP in transducing extracellular signals through the activation of the G protein Go, and very recently it was observed that the APP mutants (V642I/F/G) are constitutively active Go-linked receptors (30). The activation of Go involves the segment of the APP intracellular domain from His⁶⁵⁷ to Lys⁶⁷⁶ (APP₆₉₅) probably through a direct interaction with the oligomeric G protein (2). The comparison of these data with our results suggests that the interaction domains of APP with Go protein and with Fe65 probably overlap. In fact, the region of the intracellular domain of APP interacting with Fe65 is longer than the simple ΦXNPXY element that is sufficient to mediate the interaction of Shc or IRS-1 with growth factor receptors. The peptides extended up to -6 (APP681-691) and -12 (APP675-694) residues from the Tyr of the ΦXNPXY element of APP are unable to significantly compete for the binding to Fe65; therefore, the shortest sequence that efficiently binds to Fe65 is that encoded by the L.1 clone previously isolated by the yeast two hybrid screening and spanning the APP intracellular domain from residue 664 to residue 695 (5). This means that there are about 12 amino acids of APP common to the Fe65 and the Go interaction domains.

An intriguing scenario concerns APP trafficking between the axonal membrane and the intracellular vesicular compartments (31). It is important to note that the NPXY motif, contained in the APP-Fe65 interaction sequence, has been known

for a long time as an internalization signal for membrane proteins (32), and Fe65, possessing two protein-protein interaction domains (WW and PID/PTB), is a good candidate to be an adaptor molecule between APP and the cytoskeletal structures.

Acknowledgments—We thank A. Di Donato for helpful discussion, E. Koo for the kind gift of the CHO lines expressing wt and mutant APP, and P. G. Pelicci for the Shc cDNA.

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Interaction of the Phosphotyrosine Interaction/Phosphotyrosine Binding-related Domains of Fe65 with Wild-type and Mutant Alzheimer's β -Amyloid Precursor Proteins

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J. Biol. Chem. 1997, 272:6399-6405.
doi: 10.1074/jbc.272.10.6399

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