The Regions of the Fe65 Protein Homologous to the Phosphotyrosine Interaction/Phosphotyrosine Binding Domain of Shc Bind the Intracellular Domain of the Alzheimer’s Amyloid Precursor Protein*

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Fe65 is a protein mainly expressed in several districts of the mammalian nervous system. The search of protein sequence data banks revealed that Fe65 contains two phosphotyrosine interaction (PID) or phosphotyrosine binding (PTB) domains, previously identified in the Shc adaptor molecule. The two putative PID/PTB domains of Fe65 were used to construct glutathione S-transferase-Fe65 fusion proteins. Co-precipitation experiments demonstrated that the Fe65 PID/PTB domains interacted with several proteins of apparent molecular mass 135, 115, 105, and 51 kDa. The region of Fe65 containing the PID/PTB domains was used as a bait to screen a human brain cDNA library in yeast by the two-hybrid system. Three different cDNA clones were isolated, two of which contain overlapping segments of the cDNA encoding the COOH terminus of the Alzheimer’s β-amylloid-precursor protein (APP), that represents the short intracellular domain of this membrane protein. The third clone contains a cDNA fragment coding for the COOH terminus of the human counterpart of a mouse β-amylloid-like precursor protein. The alignment of the three APP encoding cDNA fragments found in the screening suggests that the region of APP involved in the binding is centered on the NPTY sequence, which is analogous to that present in the intracellular domains of the growth factor receptors interacting with the PID/PTB domain of Shc.

The interaction of tyrosine kinase receptors with cytoplasmic and membrane-associated proteins plays a key role in the membrane signal transduction mechanisms (1). This complex protein-protein interplay is based on several protein modules that are distributed, in single or multiple copies either alone or in combination, in a wide range of proteins (2-4).

Shc is one of the members of this machinery; it is involved in the coupling of the activated tyrosine kinase growth factor receptors to the Ras activation pathway (5, 6), through at least two other proteins, Grb2, which binds to tyrosine-phosphorylated Shc (6), and Sos, which, upon binding to Grb2, acts on Ras as a GDP/GTP exchange factor (7). Shc contains a Src homology 2 domain at its COOH terminus, which interacts with tyrosine-phosphorylated epidermal growth factor receptor (5). However, another domain was found at the NH₂ terminus of Shc, named PID (for phosphotyrosine interaction domain) or PTB (phosphotyrosine binding) domain, which binds to an unidentified 145-kDa protein (8) and to several autophosphorylated growth factor receptors (9). Other results demonstrated that the PID/PTB domain of Shc interacts with the insulin receptor (IR) (10), which also binds another factor (IRS-1) showing some similarities with the amino-terminal domain of Shc (10, 11). The analysis of sequence data banks for proteins showing similarities with this PID/PTB domain of Shc allowed the identification of a group of proteins sharing with Shc a region of about 160 amino acids with common predicted structural features and several conserved motifs (12).

This group of proteins includes the Fe65 protein. The Fe65 cDNA was isolated, together with many other expressed sequence tags, through a differential screening of a rat brain cDNA library (13). The corresponding mRNA is in fact mainly expressed in the brain and in several districts of the nervous system, including the ganglia of the somatic and visceral nervous system and the ganglionic structures of sense organs (14). It encodes a protein of about 90 kDa, present both in the cytoplasm and in the nucleus, which contains a domain highly homologous to the retroviral integrases (15, 16). The COOH-terminal region of the Fe65 protein can be aligned from amino acid 312 to 457 and from amino acid 484 to 612, respectively, with the PID/PTB domain of Shc (12).

Herein we report that the COOH-terminal region of the Fe65 protein, containing the two PID/PTB domains, binds several proteins in both neuronal and non-neuronal cells. The screening of a human brain cDNA expression library in yeast, based on the two-hybrid system, allowed us to demonstrate that this region binds the intracellular domain of the Alzheimer’s β-amylloid precursor protein (APP).

MATERIALS AND METHODS

Cell Culture, Labeling, and Extract Preparation—PC12 rat pheochromocytoma cells were cultured in Dulbecco’s modified Eagle’s medium (ICN) supplemented with 10% fetal bovine serum (HyClone), 5% horse serum (Life Technologies, Inc.) and 1% antibiotics; Rat2 fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum and 1% antibiotics. For 35S-labeling, methionine-starved PC12 and Rat2 cultures (80% confluence) were incubated for 3 h in medium containing 80 μCi/ml [35S]methionine (1150 Ci/mmol, ICN). Harvested cells were 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 diithothreitol, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each aprotinin, leupeptin, and pepstatin). The extracts were clarified by centrifugation at 16,000 × g at 4°C and the protein concentration determined by the Bio-Rad pro-

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tein assay according to manufacturer's instructions.

DNA Constructs and Co-precipitation Assays—Various Fe65 cDNA fragments were obtained by PCR amplification of the full-length cDNA (15, 16) and subcloned in the pGEX-2TK plasmid (Pharmacia Biotech Inc.) using standard techniques. Glutathione S-transferase (GST)-Fe65 fusion proteins were expressed and purified on glutathione-Sepharose columns (Pharmacia) according to manufacturer's instructions.

GST-Fe65-PID is a fusion between the vector-encoded GST protein and the fragment of the Fe65 protein (residues 312-612) that contains the NH$_2$- and COOH-terminal PID/PTB domains (see Fig. 1). The GST-Fe65-PID protein is a slightly shorter version of the GST-Fe65-PID protein; in fact it derives from an alternatively spliced transcript in which the codons encoding the amino acids 409-410 are lacking (15).

pET APP4 plasmid was constructed by cloning a PCR fragment corresponding to the intracellular domain (residues 648-695 of APP$_{695}$) of the Alzheimer's β-amyloid precursor protein (17) in the pET16 vector (Novagen). This construct was used to program the TnT-coupled transcription/translation reticulocyte lysate system (Promega) for the production of $[^{35}S]$methionine-labeled APP intracellular domain.

For co-precipitation assays glutatohione-Sepharose beads (10$^7$ μl) were saturated either with wild-type GST protein or with GST-Fe65 recombinant proteins, washed, and incubated with cellular extracts or with the $[^{35}S]$methionine-labeled APP intracellular domain for 2 h at 4°C in lysis buffer; unbound proteins were removed by three washes with lysis buffer. The bound proteins were eluted from the resin by boiling the samples in SDS-polyacrylamide gel electrophoresis gel loading buffer and resolved on the appropriate polyacrylamide gel. After the electrophoretic separation gels were subjected to fluorographic treatment (Amplify, Amersham Corp), dried, and exposed to x-ray films (Fuji).

Western blot analysis of the precipitated proteins with the anti-amyloid precursor protein 643-695 antibody (Boehringer Mannheim) was performed by the ECL chemiluminescence system (Amersham) according to the instructions of the manufacturers.

**Two-hybrid System-based Screening**—The screening to find the Fe65 ligand was based on the two-hybrid system in yeast (18) and performed as described in Ref. 19. The amino acid region chosen as a bait was from residue 312 to residue 612 of Fe65 (see Fig. 1); therefore, a PCR fragment from nucleotide 1034 to nucleotide 1912 of the rat Fe65 cDNA (15, 16) coding region was subcloned, using standard techniques, into pGBT10, an yeast expression vector carrying the Trp selective marker. The obtained plasmid was used to transform the H7c7 yeast strain (20), generating a clone that constitutively expresses the above mentioned Fe65 amino acid region fused to the DNA binding domain of the yeast GAL4 transcription factor. The H7c7 strain contains as reporter genes the HIS3 gene and the leu2 gene under the control of the GAL4 transcription factor cis-element (21). The human brain cDNA library cloned in the pGBT10 vector (Clontech), which carries the yeast GAL4 transcription factor, was used to transform the H7c7 Fe65-expressing strain. 6 × 10$^5$ transformants were obtained on Ura-Trp-Leu$^+$ plates, harvested in 20 ml of 65% glycerol, 10 μw Tris/HCl, pH 7.5, 10 μg MgCl$_2$ and stored in 1-ml aliquots at ~80°C. The transformants were then plated on Ura-Trp-Leu-His$^+$ plates, previously covered with nitrocellulose filters. After 3 days, the His$^+$ colonies were isolated. On these colonies the β-galactosidase assay was also performed as described in Ref. 19. The plasmid DNAs from positive clones were then rescued and introduced by electroporation into E. coli H8101. The cDNA inserts of the library plasmids were analyzed by digestion with EcoRI and by nucleotide sequence (22).

**RESULTS**

The Fe65 PID/PTB Domains Bind to Several Proteins—In order to evaluate if Fe65 PID/PTB domains interact with cellular proteins, we expressed and purified from E. coli a GST-Fe65 fusion protein in which GST was fused to the Fe65 region from amino acid 312 to 612, which contains two elements that can be aligned with the PID/PTB domain present in Shc (see "Materials and Methods" and Fig. 1). This protein associates with several $[^{35}S]$-labeled proteins from PC12 cell total extract, which are not precipitated in the control experiment with wild-type GST protein. As reported in Fig. 2 (panel A), the most intense bands are at 135, 115, 105, and 51 kDa.

A different isoform of the Fe65 protein (SF65) is encoded by the Fe65 gene, as a consequence of an alternative splicing affecting a very short exon of only 6 nucleotides, encoding 2 amino acids present in the NH$_2$-terminal PID domain. This results in the existence of a short transcript, which is barely detectable in all the tissues and cell lines tested, whereas the complete Fe65 transcript is abundant and restricted to neurons (15). Therefore we tested the ability of the SF65-PID domains to interact with PC12 protein extracts, by using a GST-SF65-PID fusion protein. Fig. 2 (panel B) shows that this protein associates with the same $[^{35}S]$-labeled proteins from PC12 cell total extract as the complete GST-Fe65-PID, and specifically with another protein of about 180 kDa, not associated with the complete GST-Fe65-PID.

Considering that the Fe65 transcript is neuron-specific, we tested if the PC12 proteins interacting with Fe65 are present in non-neuronal cells. Fig. 2 (panel C) shows that the pattern of $[^{35}S]$-labeled proteins from RAT2 fibroblasts co-precipitated with the GST-SF65-PID fusion protein is different to that of PC12 cells for the lower levels of the 135-kDa band. Furthermore, the GST-SF65-PID fusion protein precipitates the same proteins from $[^{35}S]$-labeled human cell extracts as those from rat cell extracts (data not shown).

The Intracellular Domain of the β-Amyloid Precursor Protein (APP) Binds to the Fe65 PID/PTB Domains—Using the two-hybrid system we screened a human brain cDNA library as described under "Materials and Methods," by using as a bait the Fe65 PID/PTB domains. 127 positive clones were isolated, which were His$^+$ and β-galactosidase-positive. They were analyzed by determining the restriction map of the cDNA inserts and by sequence analysis and resulted to be all identical to three different clones; the first one contains a fragment of the human APP cDNA (17), encoding the amino acids from 664 to 695, the second one contains a cloning artifact in which a segment of a repeated sequence (LINE) is fused to a fragment of APP cDNA overlapping to that present in the first clone. The nucleotide sequence of this clone demonstrated that an open reading frame is present, which allows the translation of an APP fragment from amino acid 657 to 695. The third clone contains a cDNA sequence, which is highly homologous to an APP-like cDNA (23). Fig. 3 shows the alignment between the deduced amino acid sequences of the three clones and the comparison with the known APP sequence. All the clones cover the COOH-terminal part of the APP, which represents its intracellular domain.

In order to confirm the interaction between Fe65 and APP, a human APP cDNA fragment encoding the intracellular domain of the protein (residues 648-695 of the APP$_{695}$) was in vitro transcribed and translated in reticulocyte lysate in the presence of $[^{35}S]$methionine. The labeled proteins were incubated with either complete GST-Fe65-PID or the GST-SF65-PID fusion proteins or with wild-type GST. Fig. 4 (panel A) shows that both GST-Fe65 fusion proteins associate with the in vitro translated APP fragment. Protein extracts from unlabeled PC12 cells were co-precipitated with GST-Fe65 fusion proteins and analyzed by Western blot using an anti-APP antibody. The major band evidenced by this antibody in PC12 cell total extracts also co-precipitated with both GST-Fe65 and GST-SF65 fusion proteins and not with the wild-type GST protein (see Fig. 4, panel B). Similarly, the ability of the SF65 form to interact with PC12 proteins was tested and found to be indistinguishable from that of Fe65.

**FIG. 1.** Domain structure of the Fe65 protein. The WW domain and the two PID/PTB domains are indicated by dashed boxes. The bar indicates the region (from amino acid 312 to 612) used to build the GST fusion proteins and the bait for the double hybrid screening.
The interaction with APP was demonstrated by the two-hybrid system in yeast. In fact, the region of SF65 containing the two PID/PTB domains can replace the SF65 sequence in the GAL4-SF65 construct, without changing the efficiency of the binding to the APP-GAL4 constructs.

**DISCUSSION**

SF65 PID/PTB domains interact with several proteins; one of these was identified to be APP. This is an integral membrane protein showing a large extracellular glycosylated domain and a short intracellular domain (17); a small fragment of this protein (amyloid-\(\beta\)-protein) accumulates in the brain of the patients suffering from Alzheimer's disease (AD) forming a great number of extracellular deposits (24). Very little is known about the functions of APP and about the proteins interacting with it, both extracellularly and in the cytoplasm.

Similarly, the functions of SF65 are still unknown. Besides the PID/PTB domains, SF65 amino acid sequence contains regions that can be aligned with other groups of proteins. One of these regions is significantly similar to various retroviral integrases (15). Another one contains the consensus sequence of a domain, identified in the Yes-associated protein YAP (25) and named WW domain (see Fig. 1). This domain is shared with several other proteins, such as dystrophin, Nedd-4, Rsp5, etc., and is known to interact with proline-rich ligands that have the XPPXY consensus (25, 26). The presence of the PID/PTB and the WW domains in SF65 suggests that it can act as an adaptor molecule.

The size of the shortest APP cDNA clone isolated (see Fig. 3) contributes to restrict the APP region that binds to SF65 to the last 32 residues at the COOH end of the intracellular domain of this protein. SF65 PID/PTB domains also bind to the human homolog of a mouse \(\beta\)-amyloid-like precursor (23). The alignment of this protein with the amino acid sequence of the APP cDNA clones indicates that the only significant similarity among these three regions is restricted to 11 amino acids (see Fig. 3); therefore, it can be deduced that the core sequence interacting with SF65 is represented by these 11 residues. An interesting feature of this amino acid stretch is represented by Tyr-687, which is included in the sequence Asn-Pro-Thr-Tyr, belonging to the family of endocytic codes (Gly/Asn)-Pro-Xaa-Tyr (27). This consensus, present in various growth factor receptors, is necessary for internalization, and the Tyr is not dispensable (28). Receptor autophosphorylation is necessary for internalization, because it causes a conformational change that results in the exposure of the Tyr-containing tight turn. A protein named Enigma was identified that binds the (Gly/Asn)-Pro-Xaa-Tyr endocytic codes present in the intracellular domain of the insulin receptor (29). This protein interacts with the receptor through a region that can be aligned to the so-called LIM domain. Computer-assisted analysis of the region of SF65 containing the two PID/PTB domains showed no similarity with the proteins containing a LIM domain. The observation that also Shc can interact with the Asn-Pro-Xaa-Tyr motif present in the intracellular domain of growth factor receptors (9), stimulates further experiments to test the possibility that the competition of endocytic codes for different cytoplasmic factors can contribute to define the fate of a membrane protein.

The PID/PTB domain of Shc interacts with the Asn-Pro-Xaa-Tyr motif present in several growth factor receptors and it is dependent on the phosphorylation of the Tyr present in the motif. In fact, mutant epidermal growth factor receptors, lack—

**FIG. 2.** Association of GST-SF65-PID/PTB domains with PC12 cell proteins. Panel A, glutathione-Sepharose beads were saturated either with wild-type GST protein (GST) or with GST-SF65-PID recombinant protein (SF65-PID) and incubated with PC12 cell extracts labeled with \(^{35}\)S-methionine as described under “Materials and Methods”; the bound \(^{35}\)S-labeled proteins were eluted from the resin and resolved on 10% polyacrylamide gels. Panel B, \(^{35}\)S-labeled proteins from PC12 cell extracts were incubated with either wild-type GST protein (GST) or with GST-SF65-PID or with GST-SF65-PID recombinant proteins (SF65-PID and SF65-PID). Panel C, \(^{35}\)S-labeled proteins from PC12 or Rat2 cell extracts were incubated with either wild-type GST protein (GST) or with GST-SF65-PID recombinant protein (SF65-PID).

**FIG. 3.** Alignment of the amino acid sequences deduced from those of the cDNAs isolated by screening the human brain cDNA library in yeast. On the top the COOH terminus sequence of the human \(\beta\)-amyloid-precursor protein (APP) is reported (17). The three cDNA clones (L.1, L.2, and L.3) are aligned to this sequence. Sequence in lowercase letters refers to the translation of the LINE repeated sequence found upstream (and in frame with) the APP cDNA fragment. The NPXY element is underlined.
Fe65 with APP and possibly of the functional interplay with other factors involved in the APP pathway is of interest to add tessereau to the puzzling scenario of AD.

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Fig. 4. Fe65 protein interacts with APP. Panel A, the [125I]methionine-labeled APP intracellular domain (residues 468–695 of APP$_{695}$) was synthesized in vitro by using the TNF-coupled transcription/translation reticulocyte lysate system, programmed with APP cDNA (APP-PRL), and incubated with wild-type GST protein or with GST-Fe65 recombinant proteins (Fe65-PID and SF65-PID), which saturate glutathione Sepharose beads (see “Materials and Methods”). The bound proteins eluted from the resin were resolved on 20% polyacrylamide gels. Panel B, total protein extracts from PC12 cell cultures were incubated in vitro with wild-type GST protein or with GST-Fe65 recombinant proteins (Fe65-PID and SF65-PID) bound to glutathione-Sepharose beads (see “Materials and Methods”). The bound proteins eluted from the resin were resolved on 10% polyacrylamide gels and analyzed by Western blot using an anti-amyloid precursor protein antibody.
The Regions of the Fe65 Protein Homologous to the Phosphotyrosine Interaction/Phosphotyrosine Binding Domain of Shc Bind the Intracellular Domain of the Alzheimer's Amyloid Precursor Protein

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