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The WW Domain of Neural Protein FE65 Interacts with Proline-rich Motifs in Mena, the Mammalian Homolog of *Drosophila* Enabled*

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The neural protein FE65 contains two types of protein-protein interaction modules: one WW binding domain and two phosphotyrosine binding domains. The carboxyl-terminal phosphotyrosine binding domain of FE65 interacts *in vivo* with the β-amyloid precursor protein, which is implicated in Alzheimer disease. To understand the function of this adapter protein, we identified binding partners for the FE65 WW domain. Proline-rich sequences sharing a proline-proline-leucine-proline core motif were recovered by screening expression libraries for ligands of the FE65 WW domain. Five proteins of molecular masses 60, 75, 80, 140, and 200 kDa could be purified from mouse brain lysates by affinity to the FE65 WW domain. We identified two of these five proteins as the 80- and 140-kDa isoforms encoded by Mena, the mammalian homolog of the *Drosophila* Enabled gene. Using the SPOTs technique of peptide synthesis, we identified the sequences in Mena that interact with the FE65 WW domain and found that they contain the signature proline-proline-leucine-proline motif. Finally, we demonstrated that Mena binds to FE65 *in vivo* by coimmunoprecipitation assay from COS cell extracts. The specificity of the Mena-FE65 WW domain association was confirmed by competition assays. Further characterization of the FE65-Mena complex may identify a physiological role for these proteins in β-amyloid precursor protein biogenesis and may help in understanding the mechanism of molecular changes that underlie Alzheimer disease.

FE65 is a brain-enriched protein with the modular structure of a typical adapter containing two types of protein-protein interaction domains: the WW domain and two phosphotyrosine binding (PTB)[1] domains (Refs. 1 and 2; see Fig. 1). WW domains bind proline-rich proteins containing the PP*X*Y or PPLP core motifs (where *X* signifies any amino acid) (3–5). PTB domains bind proteins containing the consensus sequence NP*X*Y; in some instances, the tyrosine must be phosphorylated to mediate high-affinity binding (6–8). Importantly, the carboxyl-terminal PTB domain of FE65 binds *in vitro* and *in vivo* to the cytoplasmic portion of the β-amyloid precursor protein (βAPP), a large transmembrane protein implicated in Alzheimer disease (1, 9–11). βAPP is a precursor protein of the β-amyloid peptide, the major constituent of the extracellular senile plaques in the Alzheimer brain. In pathological conditions, the level of β-amyloid peptide production and/or accumulation is increased dramatically compared with the normal physiological state. The formation of amyloid plaques correlates well with the onset of Alzheimer disease (12–14). The biogenesis of βAPP is a complex process that involves specific proteolytic activities, as well as other steps, which include additional posttranslational modifications, trafficking, and secretion (15–19). Little is known about the partner molecules that interact with βAPP and control its processing. In addition to FE65, three other proteins have been shown recently to interact with the cytoplasmic domain of βAPP: the heterotrimeric G protein G_o (20–22), the neuron-specific X11 protein (23), and a 59-kDa βAPP-binding protein 1 (24).

The modular, adapter-like structures of FE65 and X11 proteins suggest that they may link the cytoplasmic portion of βAPP to cytosolic proteins in a manner similar to signal transduction cascades involving growth factor or hormone receptors and integrins (reviewed in Ref. 2). The molecular components and the significance of the putative βAPP signaling remain to be defined, but it is possible that defects in this pathway could be involved in the pathogenesis of Alzheimer disease (2, 25–27).

Biochemical and genetic analyses of proto-oncogenes have elucidated many facets of signaling processes that underlie neuronal function and development (reviewed in Ref. 28). The study of the *Drosophila* homolog of the *Abl* proto-oncogene implicated this gene in the processes of axonal outgrowth and fasciculation (reviewed in Ref. 29). Using genetic modifier screens, mutations in several new genes have been identified

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF020191 (clone 13), AF020311 (clone 7), AF020312 (clone 9-1), and AF020313 (clone 48).

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[1] The abbreviations used are: PTB, phosphotyrosine binding domain; GST, glutathione *S*-transferase; βAPP, β-amyloid precursor protein; Ena, *Drosophila* Enabled; Mena, mammalian Enabled; VASP, vasodilator-stimulated phosphoprotein; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; MAP, microtubule-associated protein; EVH, Ena-VASP homology; mbh1, *myc* basic motif homolog-1; RIPA buffer, 10 mM Tris HCl, pH 7.4, 5 mM EDTA, 300 mM NaCl, 0.1% SDS; Tris/Tween buffer, 50 mM Tris HCl, pH 7.5,100 mM NaCl, 1 mM EDTA, 0.1% Tween 20, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride; TBS-T buffer, 20 mM Tris HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20; HMK buffer, 20 mM Tris HCl, pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$; CMV, cytomegalovirus.

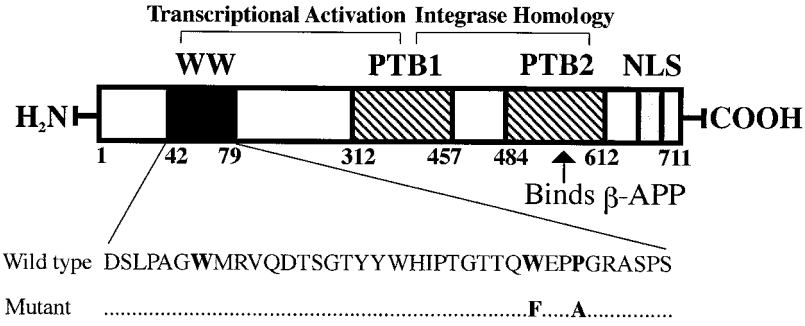

FIG. 1. **Schematic diagram of the domain structure of FE65.** The FE65 protein contains two distinct types of protein binding domains. The WW domain is located toward the amino-terminal sequence. PTB1 and PTB2 are located toward the carboxyl-terminal part of the FE65 protein, followed by the nuclear localization signal (NLS). The sequences of wild-type and mutant WW domains of FE65 are indicated at the bottom, distinguished by the mutations W69F and P72A. These two mutations render the WW of FE65 inactive, in terms of ligand binding, with minimal apparent effect on the structure of the domain. Signature tryptophans, the conserved carboxyl-terminal proline, and substituted amino acids in the mutant are shown in *boldface*. H_2N and *COOH* indicate the amino and carboxyl termini, respectively. Numbers indicate positions of amino acids.

that modify *Abl*-dependent phenotypes. These genes include *Disabled*, *Prospero*, *Failed Axon Connection*, and *Enabled* (*Ena*) (30, 31). *Ena* was identified in a specific screen for dominant mutations that alleviate the *Abl* phenotype (32). The Ena protein is axonally localized and is a substrate for the *Drosophila* Abl kinase and a ligand for the SH3 domains of Abl and Src (33). Recently, two vertebrate proteins closely related to Ena were identified. These two proteins, mammalian Enabled (Mena) and Ena-VASP-like are closely related to Ena (34) as well as to vasodilator-stimulated phosphoprotein (VASP) (35). Together, these molecules make up the Ena/VASP family of proteins and share three distinct regions of similarity: the amino-terminal 115 amino acids (the Ena-VASP homology 1 (EVH1) domain), a proline-rich core, and the carboxyl-terminal 226 amino acids (the EVH2 domain). The EVH1 domain mediates subcellular targeting of Ena/VASP family proteins by engaging in protein-protein interactions with a distinct proline-rich motif (34, 36). Ena/VASP family proteins are concentrated in focal adhesions and actin stress fibers and are found in areas of dynamic actin remodeling, such as lammellipodia and axonal growth cones. The subcellular distribution, interactions with profilin, and a small actin monomer-binding protein implicated in the regulation of actin dynamics suggest a role for Ena/VASP family proteins in the regulation of cell motility and morphology by modulating the actin-based cytoskeleton (Ref. 34; reviewed in Ref. 37). A specialized role for Mena in the nervous system is suggested by a 140-kDa neuron-specific isoform of Mena that is present in the developing and adult brain along with the broadly expressed 80-kDa form of Mena (34).

Here we report the identification of a cognate ligand for the WW domain of FE65. Using a combination of functional screens of mouse embryo cDNA expression libraries, the SPOTs technique of peptide synthesis, pull-down experiments from mouse brain lysates, and coimmunoprecipitation from mammalian cell extracts, we were able to characterize the potential ligands of the FE65 WW domain. We identified one of these ligands as Mena, and we demonstrate herein the interaction of the FE65 WW domain with Mena through specific proline-rich motifs that contain the PPLP core.

EXPERIMENTAL PROCEDURES

Construction, Purification, and Labeling of Fusion Proteins—The cDNA regions corresponding to the WW domains of rat FE65, mouse YAP (WW1), Nedd4 (WW2), Nedd4 (WW3), Ess1, and Msb1 were amplified using the polymerase chain reaction method. Primers designed with *Bam*HI site at the 5′ end and *Eco*RI site at the 3′ end were as follows: 5′-dACA GGA TCC GAT CTA CCG GCT GGA-3′ and 5′-dTGT GAA TTC CCC TGT GAT GGG GAG-3′ (FE65); 5′-dCTA TAC GGA TCC CAG TCC TCC TTT GAG ATC CCT-3′ and 5′-dTAC GAC GAA TTC GGC TGG CAG GCG CAG GAA CGT TCA-3′ (YAP WW1); 5′-dCTA TAC GGA TCC ACA CTT CCT GTG CTT TTG CCT-3′ and 5′-dTAC GAC GAA TTC CTC TCA GAT GAG CAG GGA TTT TCG-3′ (Nedd4 WW2); 5′-dCTA TAC GGA TCC AGA GGA AAG ACT GAC TCC AAT-3′ and 5′-dTAC GAC GAA TTC TGG AGT AGG GCA CTG CTG GTC CAG-3′ (Nedd4 WW3); 5′-dCTA TAC GGA TCC CCA TCT GAC GTA GCA TCG AGC-3′ and 5′-dAAG GAC GAA TTC GAT GGT CTC TTA AGT GCT TGT GTA-3′ (Ess1); 5′-dACG TAG GGA TCC CCC CCC TCT CCT CCC AAA CCA AAA ACC-3′ and 5′-dGCA AGG AAT TCC AGC TTC ATG CTC AAG GCT GGC ATC-3′ (Msb1); 5′-dCGT GGG ATC CGG TGC ATT AAA AGC AAA-3′ and 5′-dTCT GAA TTC TAC AGT AAC ACC ACC-3′ (Yes unique domain). After purification the amplified fragments were subcloned into the pGEX-2TK vector (Pharmacia Biotech Inc.) between the *Bam*HI and *Eco*RI sites. The nucleic acid sequences of the constructs were verified by sequence analysis (Sanger method; Ref. 38), and the expression level and the molecular weight of the glutathione *S*-transferase (GST) fusion proteins were verified by SDS-PAGE. The GST-13Pro, GST-7/9-1Pro, GST-48Pro, GST-*myc* basic motif homolog 1 (mbh1) fusion proteins were obtained using similar strategy. Considering that proline-rich regions of clones 7 and 9-1 are identical (Table I), only the polyproline motif of clone 7 was expressed in the pGEX-2TK vector. The inserts were generated by polymerase chain reaction amplification using the following primers: 5′-dCTA TAC GGA TCC CTG CAG CCA ACC TAC TGC TCA-3′ and 5′-dTAC GAC GAA TTC CTG TAT CGT AGG TCA GCG AGA AAG-3′ for GST-13Pro; 5′-dCTA TAC GGA TCC GCG CCG CCG CCG CCG CCG CCG CTG-3′ and 5′-dTAC GAC GAA TTC CTG GTG GCG GGG GCG GCG GCA-3′ for GST-7/9-1Pro; 5′-dCTA TAC GGA TCC CTA GAC CTT CTG CCT CCT CCT-3′ and 5′-dTAC GAC GAA TTC TAG AGA GAA GTT CAG GAG GAG-3′ for GST-48Pro; 5′-dCTA TAC GGA TCC GCC ACT GGA CAG ATG AAT CTG-3′ and 5′-dCAT TAC GGA TCC CAG GCA GCC CTC CAA GTG GCT-3′ for GST-mbh1 (see Table I). SURE cells (Stratagene) transformed with the pGEX-2TK-recombinant constructs were induced with 4 mM isopropyl-β-D-galactoside (Sigma) for 4 h at 37 °C and then sonicated in PBS (147 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4{\cdot}7H_2O$, 1.5 mM KH_2PO_4) with 1% Triton X-100. To purify the GST fusion proteins, bacterial lysates were incubated with glutathione agarose beads (Sepharose 4B, Pharmacia) for 2 h at 4 °C. The fusion proteins were eluted with a Tris buffer (50 mM, pH 8.0) containing 30 mM of reduced glutathione (Sigma) on poly-prep chromatography columns (Bio-Rad). The labeling reaction of GST, mutant GST-FE65 WW, or GST-FE65 WW was performed with 200 μg of fusion protein bound to 100 μl of glutathione beads in the presence of 50 μCi of [^{32}P]dATP, 120 units of bovine heart catalytic subunit of cAMP-dependent protein kinase A (Sigma), 50 μl of 1 × HMK buffer, and 1 mM dithiothreitol. The hemagglutinin (HA)-FE65 construct with CMV promoter was generated by cloning the FE65 cDNA coding region into the pcDNA-MHA vector (a kind gift from Francesca Fiore, Università degli Studi di Napoli "Federico II," Naples, Italy) in frame with the DNA sequence encoding an HA tag epitope.

Mutagenesis of FE65 WW Domain—Mutant GST-FE65 WW fusion protein was generated in which tryptophan (Fig. 1, W) at position 69 was substituted with phenylalanine (F) and proline (P) at position 72 was substituted with alanine (A). These mutations of the FE65 WW domain were designed based on NMR structural data for the human YAP WW domain and on empirical data from mutants of human YAP WW domain that did not bind proline-rich ligands, although the structure of the domain was maintained (39, 40). The pGEX-2TK-FE65WW construct was used as a template. The mutagenesis was performed

TABLE I

cDNA clones isolated from mouse embryo expression libraries screened with radioactively labeled GST-FE65 WW fusion protein

a, *b*, *c*, *d*, and *e* represent minimal sequences of isolated clones sufficient for binding to GST-FE65 WW fusion protein in *in vitro* binding assay (see text).

Clone number	Times isolated	Length of insert	Source
		bp	
9–1[a]	12	684	12d, 14d, 16d cDNA library
7[b]	4	487	12d, 16d cDNA library
13[c]	1	1384	12d cDNA library
48[d]	1	2233	14d cDNA library
mbh1[e]	2	450	12d, 16d cDNA library

[a] PPPPPPLPPPPPP.

[b] PPPPPPLPPPPPP.

[c] PPPPPPPPPPPPLPAPPQPQQPHFLPPGYLSALHFLPPPPPPPS-PP.

[d] LDLLPPPPPPPGLLS.

[e] SERQGKAQVEIITDGEEPAEMIQVLGPKPALKEGNPEEDITAD-QTNAQAAALYKVSDATGQMNLTKVADSSPFASELLIPDDCFVLDN-GLCGKIYIWKGRKANEKERQAALQVADGFISRMRYSPNTQVEILR-QGRESPIFKQFFKNWK.

using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene). PAGE-purified mutagenic primer used for the mutagenesis was as follows: 5′-pGGA GGC CCG GCC GGC GGG TTC GAA CTG GGT GGT CCC-3′.

Molecular Cloning of FE65 WW Domain Ligands—Mouse embryo cDNA expression libraries made in pEXlox vectors from 12, 14, and 16 day mRNA (Novagen) were screened with ^{32}P-labeled GST-FE65 WW mutant and wild-type fusion proteins by standard methods (41, 42). Three to four rounds of enrichment were performed until pure and uniformly positive phages were isolated. Plasmids containing recovered cDNA inserts of positive clones were generated by *cre*-mediated excision. Both strands of the cDNA clones were sequenced by the method of Sanger *et al.* (38).

Analysis of Binding Motif by the SPOTs Method—The SPOTs technique of peptide synthesis on derivatized cellulose membrane was performed as described (43, 44). All reagents and equipment, including amino acids, derivatized membranes, instruction manual, and software (SPOTs, Release 1.0′), were purchased from Cambridge Research Biochemicals and Genosys Biotechnologies, Inc. The Mena SPOTs membrane was a kind gift from Juergen Wehland and Ronald Frank (Gesellschaft für Biotechnologische Forschung mbH, Braunschweig, Germany). The membranes were blocked with the blocking buffer supplied by the manufacturer and thereafter probed with the ^{32}P-labeled GST, GST-FE65 WW, or GST-mYAP WW1 fusion protein.

Antibodies—The following antibodies were used for Western blot analysis: affinity-purified antisera raised against 19-mer peptide from carboxyl-terminal portion of Mena (34); antisera against mbh1 (a kind gift from Edward Ziff, New York University, New York, NY); monoclonal antibodies against gelsolin (Transduction Laboratories); monoclonal anti-MAP antibodies (Sigma; a kind gift from Gianni Piperno, Mount Sinai Medical Center, New York, NY); antisera against myosin binding protein H (a kind gift from Donald Fischman, Cornell University Medical School, New York, NY).

Pull-down and Western Blotting Experiments—Mouse brains were homogenized in RIPA buffer. Clarified lysates were diluted 10-fold with Tris/Tween buffer to the final protein concentration of 1 mg/ml and incubated with various GST fusion proteins bound to glutathione agarose beads (200 μg of protein/precipitation reaction) for 12 h at 4 °C. The beads were then washed twice in 30 volumes of PBS with 0.5% Triton X-100 and twice in PBS only. Protein complexes separated by 7.5% SDS-PAGE were subsequently transferred to nitrocellulose membrane (Bio-Rad). The blots were probed either with the ^{32}P-labeled GST fusion proteins or anti-Mena antibodies.

Coimmunoprecipitation—COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin mixture in 5% CO_2 atmosphere at 37 °C. 3×10^6 cells were transfected by electroporation at 250 microfarads and 220 V with 20 μg of CMV-HA-FE65 plasmid. 62 h after the transfection, the cells were harvested in ice-cold PBS and centrifuged at 2000 rpm at 4 °C, and the pellet was dissolved in lysis buffer (10 mM Tris HCl, pH 7.5; 150 mM NaCl; 0.1 mM sodium vanadate; 50 mM NaF; 0.5% Nonidet P-40; 1 mM phenylmethylsulfonyl fluoride; 10 μg/ml aprotinin, leupeptin, and pepstatin). The extracts were clarified by centrifugation at 16,000 $\times$ *g* at 4 °C, and 4 mg of supernatant were incubated for 1 h at 4 °C with an anti-HA monoclonal antibody (a kind gift from Francesca Fiore) or with an unrelated monoclonal antibody (a kind gift from Caterina Turco, Università degli Studi di Napoli "Federico II"). Thereafter, 30 μl of protein A-Sepharose resin (Pharmacia) were added to each sample of the extract-antibody mixture, and the immunocomplexes were eluted with 50 mM Tris HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM dithiothreitol, 0.01% bromphenol blue. The proteins were resolved by 7.5% SDS-PAGE and transferred to Immobilon-P membranes (Millipore). The filter was blocked in 5% nonfat dry milk in TBS-T buffer and incubated with anti-Mena antibodies at 1:1000 dilution for 1 h at room temperature. After being washed in TBS-T, the filter was exposed to horseradish peroxidase-conjugated protein A (Amersham Corp.) at a dilution of 1:5000 for 30 min at room temperature. The signals were detected by chemiluminescence using the ECL system (Amersham Corp.).

Competition Experiments—The precipitation from mouse brain lysates was performed as described above. During probing with 2.5 μg/ml radioactively labeled GST-FE65 WW, the GST-13Pro fusion protein containing polyproline region of clone 13 was added at two arbitrarily chosen concentrations, 15 μg/ml and 40 μg/ml. Blots were then washed three times for 15 min and exposed.

Northern Blot Analysis—Multiple tissue Northern blot (CLONTECH Laboratories, Inc.) of poly(A$^+$) RNA from mouse heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis was hybridized with ^{32}P-labeled cDNA of clones 13, 48, and 9-1 under standard conditions (45).

RESULTS

FE65 WW Domain Interacts with Several Proteins Present in Mouse Brain Lysates—To demonstrate that the putative WW domain of FE65 indeed interacts with the protein(s) present in the mammalian nervous system, we performed pull-down experiments by incubating wild-type GST-FE65 WW fusion protein with mouse brain lysates. GST and mutant GST-FE65 WW domain proteins were used in control experiments. Bound proteins from mouse brain lysates were analyzed by Western ligand blots probed with either radioactively labeled GST, mutant GST-FE65 WW, or GST-FE65 WW fusion proteins (Fig. 2). Precipitation with GST and mutant GST-FE65 WW did not show any specific interaction (Fig. 2, *A* and *B*), whereas precipitation with the wild-type GST-FE65 WW revealed at least five proteins, with molecular masses of 60, 75, 80, 140, and 200 kDa (Fig. 2*C*).

Identification of Specific Amino Acid Sequences Interacting with the WW Domain of FE65—As previously demonstrated (3–5), WW domains interact with proteins containing the PP*X*Y or PPLP core motifs. The first step toward the identification of proteins interacting with the FE65 WW domain was the elucidation of the core motif required for this binding. Thus, we identified potential ligands by screening phage expression libraries and analyzed these proteins by the SPOTs technique of peptide synthesis. We screened 12-, 14-, and 16-day mouse embryo cDNA expression libraries with radioactively labeled GST-FE65 WW fusion protein. Twenty positive clones were isolated that did not bind radioactively labeled GST or mutant GST-FE65 WW. They were grouped into five independent clones (Table I). Two clones, 7 and 9-1, encoded an identical polyproline sequence with the PPLP motif, suggesting that the WW domain of FE65 may belong to the class of WW domains that interact with PPLP rather than PP*X*Y core motifs. By sequence and Northern blot analyses, clones 7 and 9-1 appeared to contain binding sites within cryptic open reading frames present in untranslated regions of the cDNAs (data not shown). Clone 13 represents a novel cDNA with a long open reading frame that also contains the PPLP motif in the coding frame. Northern blot analysis revealed that it is testes-specific (data not shown), making it unlikely to be a ligand of the brain-enriched form of FE65. Clone 13 may represent a ligand of the WW domain of the related FE65L2 protein, which is

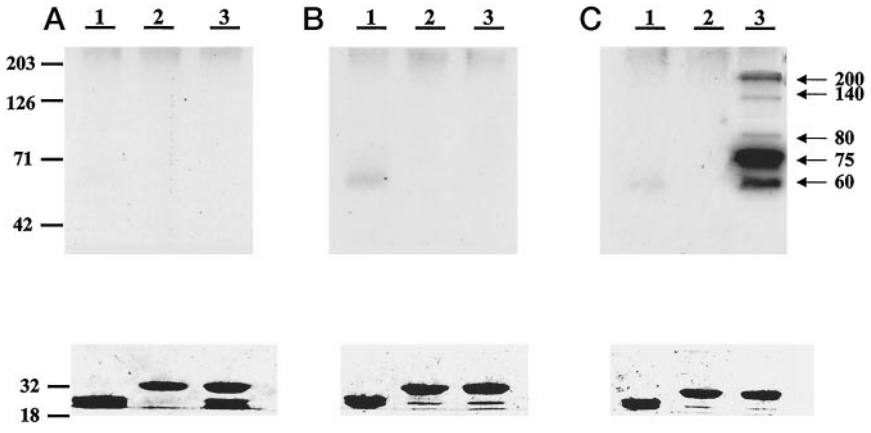

FIG. 2. **Precipitation of ligand proteins for the GST-FE65 WW domain fusion protein in pull-down experiments with mouse brain lysate.** *A–C*, *lane 1,* precipitation with GST; *lane 2,* precipitation with mutant GST-FE65 WW fusion protein; *lane 3,* precipitation with wild-type GST-FE65 WW fusion protein. Blots were probed with ^{32}P-labeled GST (*A*), mutant GST-FE65 WW (*B*), and GST-FE65 WW (*C*). Five bands of 60, 75, 80, 140, and 200 kDa were detected with the wild-type FE65 WW domain as indicated by *arrows* and numbers at the *right*. *Bottom panels* show that amount of protein loaded on gels was normalized and did not vary significantly (Coomassie stain). Numbers at *left* indicate molecular weight markers in kDa.

present in both brain and testes.[2] Clone 48 corresponds to a ubiquitously expressed mRNA that does not encode a perfect PPLP motif but instead encodes several proline-rich regions that bind to WW domain of FE65 with relatively weaker affinity. Finally, one of the isolated clones encodes the carboxyl-terminal portion of mbh1, also known as macrophage capping protein 39 (46, 47). mbh1 is a 45-kDa actin-binding protein phosphorylated at multiple serines and threonines that belongs to the gesolin/severin family (48–50). The molecular function of this protein is unknown.

To confirm binding of cloned proteins, GST fusion proteins that represented full and deleted portions of the isolated clones were generated. Radioactively labeled GST-FE65 WW fusion protein, which showed specific binding to the GST-ligand fusion proteins, was used as a probe for Western ligand blot analysis of the deletion series. GST alone or mutant GST-FE65 WW were used as controls (data not shown). The results of the deletion analysis determined the minimal sequences within positive clones sufficient and necessary for binding (Table I). Interestingly, but not surprisingly, the GST-mbh1 fusion protein of the full cloned mbh1 segment (450 base pairs) was required to bind GST-FE65 WW. This binding event might be explained by helix-loop-helix structure of mbh1 in this region (46). Distant amino acids, including prolines, might be juxtaposed by folding to form a binding motif sufficient to interact *in vitro*. The pull-down experiments reported in Fig. 2 do not show any band with an apparent molecular weight compatible with that of mbh1. Furthermore, we were not able to demonstrate the complex between mbh1 and FE65 *in vivo* by coimmunoprecipitation from PC12 cells or mouse brain lysates with anti-mbh1 antibodies. Therefore, it seems likely that the mbh1-FE65 WW domain interaction we observed *in vitro* is spurious.

To gain more insight into the polyproline sequence identified in the positive clones, we performed a "valine scan" of a proline-rich sequence of clone 13 by the SPOTs technique to determine which residues are required for binding to the WW domain of FE65. Valine was used for substitutions instead of the typical alanine replacement because the fragment of clone 13 contained alanine. As shown in Fig. 3, six consecutive prolines are necessary for significant binding *in vitro* between the WW domain and peptide ligands (spots 4–7). Substitutions of leucine at position 8 of the target peptide with glutamic and aspartic acids (spots 19 and 20) result in a weaker binding to the WW domain of FE65. In contrast, replacements with basic amino acids, such as lysine and arginine (spots 25 and 31), lead to a relative increase in interaction between the FE65 WW domain and its ligand peptides. Spot 37 represents a binding motif found in clones 7 and 9-1 that binds strongly to GST-FE65 WW fusion protein. Peptides 38–40 are proline-rich segments of sequence from clone 48. Only peptide 38 (VEPMLDLLPPPPPPPP) showed interaction on the filter assay. Spot 41 represents the proline-rich sequence found in formin and containing the PPLP motif. Leder and colleagues (4) have reported that this sequence interacts *in vitro* with certain SH3 as well as WW domains, and we observed relatively strong binding to WW domain of FE65 (Fig. 2, spot 41). Finally, a 15-mer peptide containing exclusively prolines (spot 42) interacts robustly with the FE65 WW domain, confirming our prediction that the presence of six or more consecutive prolines is sufficient for an oligopeptide to bind to the WW domain of FE65. Together, these experiments indicate that the consensus sequence for a ligand of FE65 WW domain may include a minimal sequence of six consecutive prolines or a longer sequence of polyprolines that could be interrupted by specific amino acids, such as leucine, lysine, or arginine.

Identification of 80- and 140-kDa Bands as Mena—To identify candidate FE65 WW ligands, we searched the GenBank™ and EMBL data bases for proteins that contain at least six consecutive prolines with probable core sequences of PPLP or PP(K/R)P, that are expressed in the brain, and of which the estimated molecular weights are similar to those identified by pull-down experiments with the FE65 WW domain (see Fig. 2). Several potential candidates were identified, including MAPs (MAP1, MAP2, MAP5, Tau, β-tubuline, and tyrosine-tubuline), gelsolin, myosin binding protein H, and Mena. Western blot analysis with specific antibodies against these molecules identified Mena and none of the other proteins among the FE65 WW pulled down proteins. FE65 WW-bound protein complexes were separated by SDS-PAGE and transferred onto nitrocellulose membrane, and the blot was probed with radioactively labeled GST-FE65 WW (Fig. 4*A*) and thereafter with anti-Mena antibodies (Fig. 4*B*). Two bands of 80 and 140 kDa observed in both cases appeared to be identical in terms of relative migration and intensity. To confirm that these signals actually represent Mena, we compared lysates made from normal mouse brains to lysates from Mena null mutant mice that were engineered by homologous recombination of the Mena gene (34).[3] The FE65 WW domain pull-downs from Mena knockout mouse brain lysates lacked the 80- and 140-kDa bands. The third protein, represented by lower band of 60 kDa, which is stained with antibodies against Mena, represents a nonspecific signal because other Mena antisera fail to detect this signal in total brain lysates.[3]

FE65 and Mena Interact in Vivo—Currently available anti-FE65 antibodies are directed against the WW domain of the protein and thus may be inhibitory in coimmunoprecipitation

[2] T. Russo, unpublished data.

[3] F. Gertler, unpublished data.

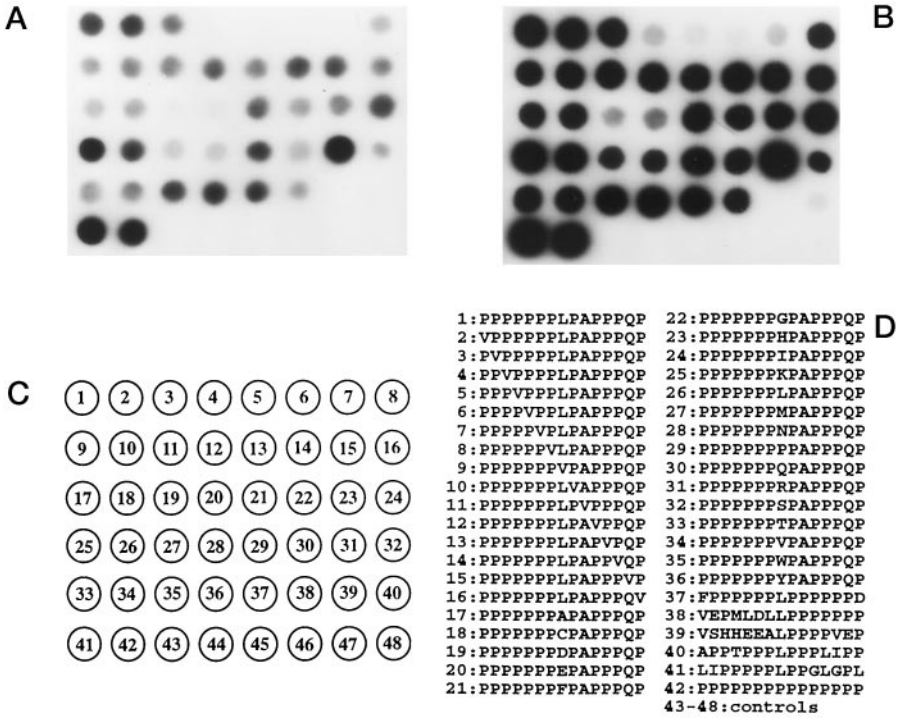

FIG. 3. **Mutational analysis of the $P_1P_2P_3P_4P_5P_6P_7L_8P_9A_{10}P_{11}P_{12}P_{13}Q_{14}P_{15}$ target peptide that binds to the WW domain of FE65.** The SPOTs technique was used to synthesize a repertoire of mutated peptides. Spot 1 represents the wild-type sequence of the target peptide as isolated from clone 13 in screening of cDNA expression libraries. Spots 2–16 represent a valine scan of the target peptide; each amino acid of the target peptide sequence was sequentially substituted with valine. A sequential substitution of L_8 with all remaining amino acids corresponds to spots 17–36. Spots 37–42 represent spots with potential binding affinities to the WW domain of FE65 (see "Results"). The control spots, 43–48, indicate derivatized spots onto which no amino acids were applied. For blotting, the ^{32}P-labeled GST-WW domain of FE65 was used. *A*, autoradiogram of the membrane exposed for 5 min; *B*, autoradiogram of the membrane exposed for 15 min; *C*, orientation of the derivatized spots on which peptides were synthesized; *D*, individual sequences of the peptides corresponding to numbered spots.

experiments due to their ability to compete with any protein for the binding to the WW domain. Therefore, we generated an expression vector in which the CMV promoter drives the expression of a HA-tagged FE65 protein. This construct was transfected transiently in COS-7 cells. Protein extracts from these cells were incubated with anti-HA antibodies, and immunocomplexes were analyzed by Western blotting using anti-Mena antibodies. As shown in Fig. 5, two bands of the 80-kDa isoform of Mena were detected in total cell lysate and in protein complexes immunoprecipitated by anti-HA antibodies but they were not detected by the unrelated control antibodies. It has been shown previously that the 80-kDa form of Mena migrates as a doublet (34). We noted an apparent difference in the pattern of the 80-kDa form of Mena precipitated with GST-FE65 WW domain from brain lysates (*e.g.* Fig. 4, *lane 9*: single band) and with HA-tagged FE65 from COS cells (Fig. 5, *lane 4*: doublet). This may be due to the presence of an additional form of Mena in COS-7 cells (Fig. 5, *lanes 1* and *4*).

Specificity of Mena-WW Domain Interaction—GST fusion proteins of the WW domains of FE65, mouse YAP (WW1), Nedd4 (WW2 and WW3), Ess1, Msb1, the unique domain of Yes (irrelevant sequence control), and GST alone were used in pull-down experiments with mouse brain lysates. A Western blot of the precipitated proteins was probed with anti-Mena antibodies (Fig. 6*A*). The WW domains of FE65 and Msb1 precipitated both the 80- and 140-kDa forms of Mena efficiently. The WW domains of YAP and Nedd4 (WW2) bound well only to the 140-kDa isoform, whereas GST alone, Nedd4 (WW3), Ess1, and the unique domain of Yes showed no interaction. The FE65 and Msb1 WW domains share a high degree of similarity and belong to one subclass of WW domains containing three consecutive aromatic residues in the middle of the WW domain sequence, for which the core of binding motif is PPLP (2). In contrast, YAP and Nedd4 WW domains bind to ligands containing the PP*X*Y motif (2, 5, 39, 51). Interestingly, the neural isoform of Mena contains the PPSY motif, which might be responsible for binding to YAP and Nedd4 WW domains. This suggestion is supported by observation that these WW domains bind much more strongly to the 140-kDa isoform of Mena than to the 80-kDa form (Fig. 6*A*), which lacks the PPSY sequence. Although the 80-kDa isoform was detected in pull-downs with mYAP WW1 and Nedd4 WW2, it was present at a very low level, which probably could be explained by the presence of heteromultimeric or homomultimeric Mena complexes.

FE65 WW Domain Interacts with Specific Polyproline Regions of Mena—To identify the binding motif through which Mena interacts with FE65, we probed a SPOTs filter containing overlapping 15-mer peptides, which represent the entire sequence of the neural isoform of Mena, with radioactively labeled GST-FE65 WW fusion protein (Fig. 7). As expected, spots 142 and 143, representing stretches of polyprolines interrupted with a single leucine, demonstrated the strongest binding. In addition, other proline-rich regions of Mena (spots 174–176 and 180–193) showed binding to FE65 WW domain, although with weaker affinity than spots 141–145 of Mena. Remarkably, all target peptides that bound to the WW domain of FE65 in this experiment contain the PPLP sequence (Fig. 7). Also, when a membrane supporting Mena peptides was probed with radioactively labeled GST-mYAP WW1 fusion protein, binding was detected only on spots 85–88, which contain the PPSY motif (data not shown). These results confirm that the binding of the 140-kDa isoform of Mena to the WW domains of mYAP and Nedd4, shown in a previous experiment, occurs through a sequence containing a PPSY core that is present in the alternatively included portion of the neuron-specific Mena isoform.

GST Fusion Protein Containing the Polyproline Region of Clone 13 (GST-13Pro) Competes with Mena for Binding to FE65 WW Domain—To confirm that binding between Mena and the FE65 WW domain does indeed occur through the proline-rich region of Mena with the PPLP core motif, we performed a competition assay in which GST-13Pro fusion protein was used (see Table I). Blots with proteins precipitated from mouse brain lysates with GST, mutant GST-FE65 WW, and wild-type GST-FE65 WW were incubated with radioactively labeled GST-FE65 WW alone (Fig. 8*D*) or with the addition of GST-13Pro fusion protein (Fig. 8, *A* and *C*). When increasing concentrations of competing protein were used, the interaction between Mena and the GST-FE65 WW fusion protein was proportionally decreased (Fig. 8, *A* and *C*). In addition

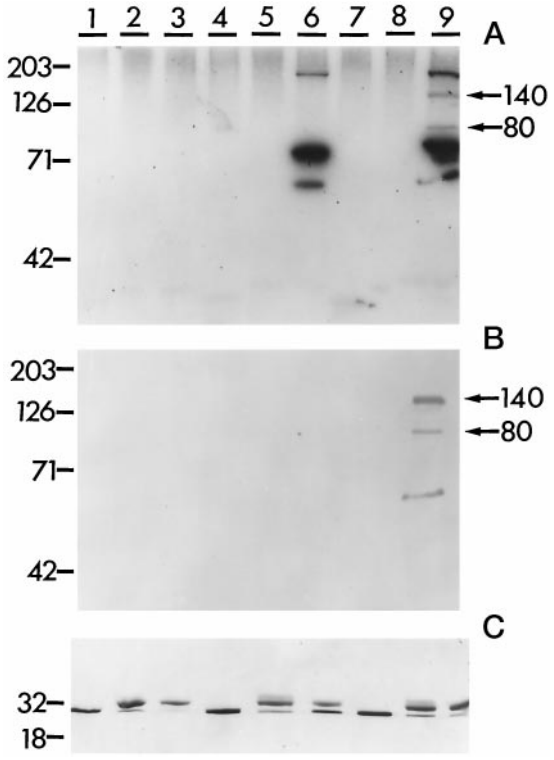

FIG. 4. **Identification of FE65 WW domain ligand as Mena by Western and far-Western blotting.** *A-C*, *lane 1*, GST; *lane 2*, mutant GST-FE65 WW fusion protein; *lane 3*, wild-type GST-FE65 WW fusion protein; *lane 4*, precipitation with GST from Mena knockout mouse brain lysate; *lane 5*, precipitation with mutant GST-FE65 WW from Mena knockout mouse brain lysate; *lane 6*, precipitation with wild-type GST-FE65 WW from Mena knockout mouse brain lysate; *lane 7*, precipitation with GST from control mouse brain lysate; *lane 8*, precipitation with mutant GST-FE65 WW from control mouse brain lysate; *lane 9*, precipitation with wild-type GST-FE65 WW from control mouse brain lysate. Nitrocellulose blot of precipitated proteins was probed with ^{32}P-labeled GST-FE65 WW fusion protein (*A*) and anti-Mena antibodies (*B*). The normalized amount of protein in each lane was confirmed by Coomassie stain (*C*). Two isoforms of Mena protein are indicated by *arrows* and numbers at the *right*. Numbers at *left* indicate molecular mass markers in kDa.

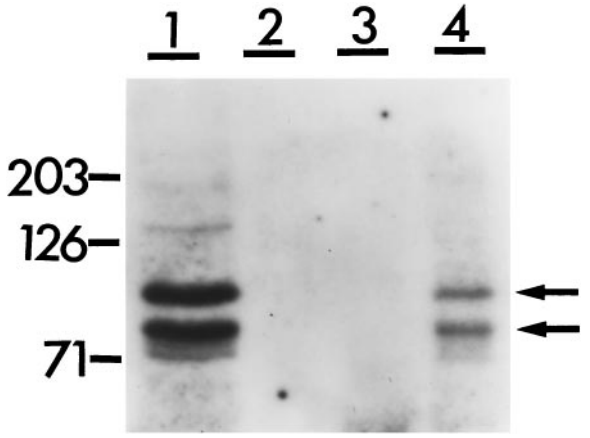

FIG. 5. **FE65 interacts with Mena in cell culture.** COS-7 cells transfected with CMV/HA-FE65 were used for coimmunoprecipitation. *Lane 1*, total cell lysate (20 μg of proteins); *lane 2*, molecular weight markers; *lane 3*, coimmunoprecipitation with control anti-mouse IgG (5 mg of proteins); *lane 4*, coimmunoprecipitation with anti-HA antibodies (5 mg of proteins). The blot was probed with anti-Mena antibodies. The precipitated Mena doublet of 80 kDa is indicated by *arrows*. Numbers at *left* indicate molecular mass markers in kDa.

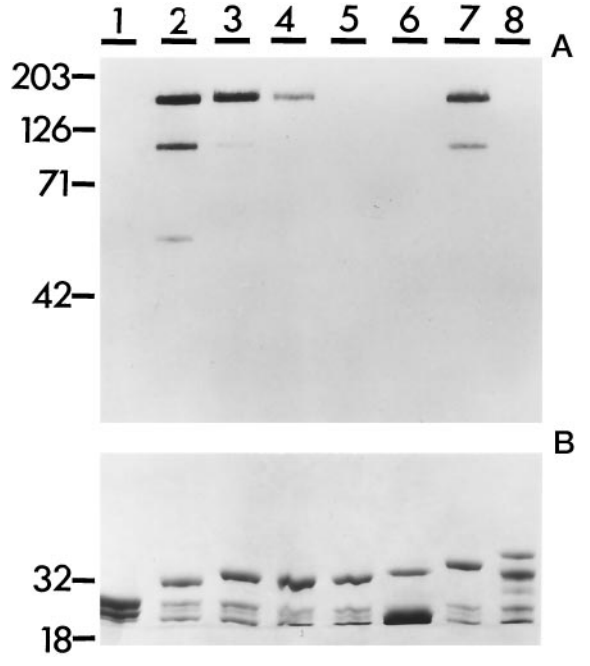

FIG. 6. **Specificity of interaction between Mena and different WW domains.** *A* and *B*, precipitation from mouse brain lysate with GST fusion proteins; *lane 1*, GST; *lane 2*, GST-FE65 WW; *lane 3*, GST-mYAP WW1; *lane 4*, GST-Nedd4 WW2; *lane 5*, GST-Nedd4 WW3; *lane 6*, GST- Ess1 WW; *lane 7*, GST-Msb1 WW; *lane 8*, GST-Yes unique domain. Blot was probed with anti-Mena antibodies (*A*). The normalized amount of protein in each lane was confirmed by Coomassie stain (*B*). Numbers at *left* indicate molecular mass markers in kDa.

to Mena, the 200-kDa protein was also competed (Fig. 8*A*). This result suggests that other ligands of FE65 WW domain may interact via a similar binding motif containing the PPLP sequence, although binding of the FE65 WW domain to 60- and 75-kDa proteins was not significantly affected by competition with GST-13Pro (Fig. 8, *A* and *C*).

DISCUSSION

We identified the Mena protein as one of the cognate ligands for the WW domain of FE65 adapter protein and mapped the sites of interaction on Mena to polyproline-rich regions containing the signature PPLP motif. More importantly, we documented the binding between Mena and the FE65 WW domain *in vitro* and *in vivo* by pull-down experiments from mouse brain lysates and by coimmunoprecipitations of the complex from COS cells overexpressing the FE65 gene product.

The following aspects of the work deserve brief comment: (*a*) specificity of the interaction between FE65 and Mena; (*b*) biological role of the FE65-Mena complex; and (*c*) functional implications of the FE65-Mena complex for the biogenesis of the βAPP.

Given the numerous examples of specificity and degeneracy in the protein-protein interactions mediated by SH2, SH3, and known WW domains (3, 4, 52, 53), we consider the FE65-Mena complex a cognate pair. The major criterion for this conclusion is the expression of these two proteins in the same cellular compartment (34, 54) and tissues (*i.e.* cytoplasm and neural tissues) and formation of the complex *in vivo* as revealed by its coimmunoprecipitation. Two other observations support the specificity of binding. First, the WW domain of FE65 belongs to the subset of WW domains that contain three aromatic positions in the middle of the linear sequence of the domain. This feature seems to correlate with the preference of the domain for polyproline ligands containing PPLP cores. In contrast, the WW domains with two consecutive aromatic amino acids in the middle bind ligands with PP*X*Y cores (2–4). Our results are

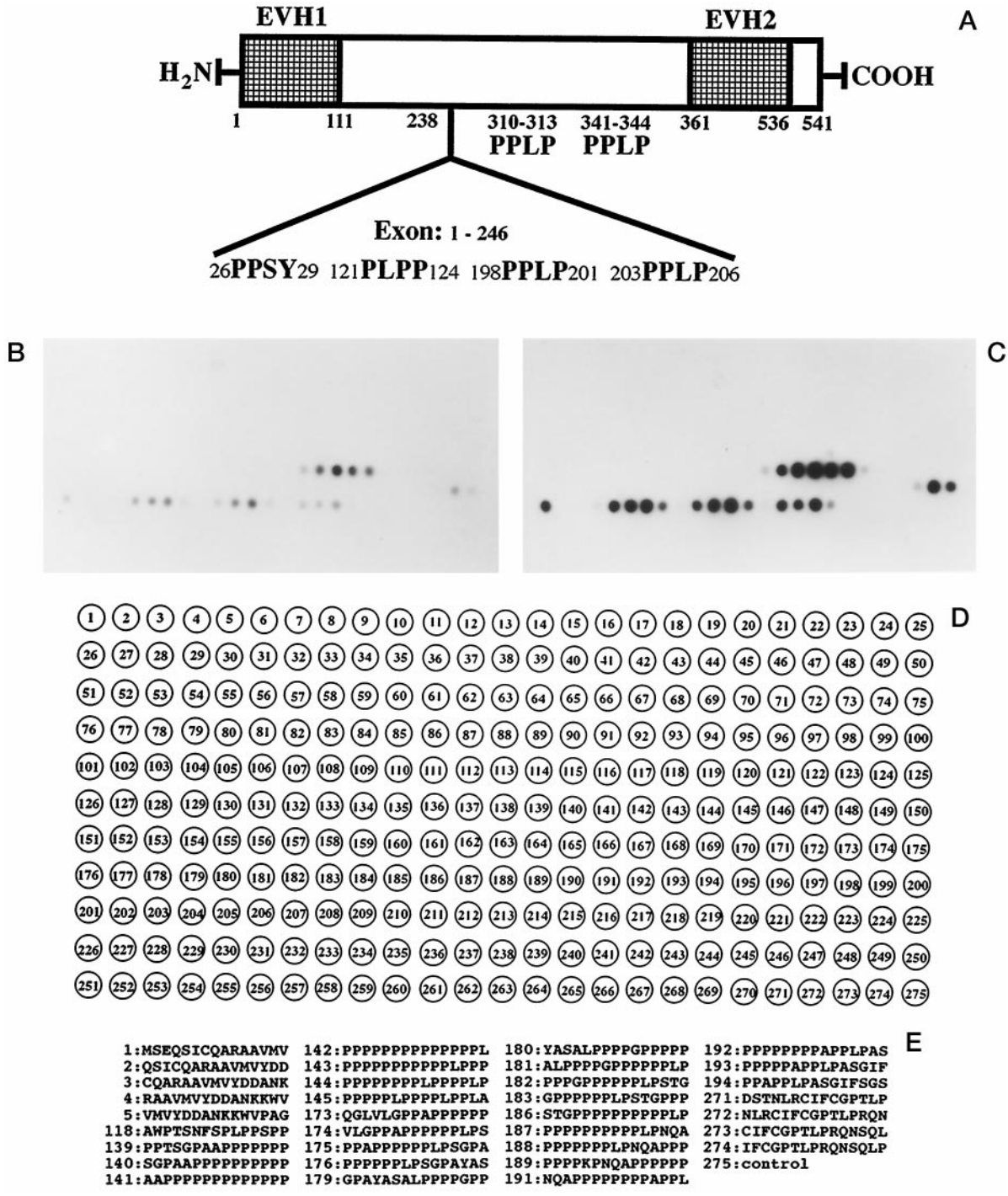

FIG. 7. **Screening of Mena with ^{32}P-labeled GST-FE65 WW fusion protein on SPOTs membrane.** Using the SPOTs technique, the 140-kDa isoform of Mena was synthesized on a derivatized membrane as series of consecutive 15-mer peptides with the offset of three amino acids. The control spot (*275*) (Mena) indicates a derivatized spot onto which no amino acids were applied. The membrane was probed with ^{32}P-labeled GST-WW domain of FE65. *A*, schematic representation of Mena. Numbers *1* and *541* denote the amino (H_2N) and the carboxyl (*COOH*) termini, respectively (80-kDa isoform). The alternatively spliced exon (neuron-enriched 140-kDa isoform) is indicated with *solid lines* and numbers (*1–246* (the length of the exon in amino acids)) in the lower part *panel A*. The PPLP and PPSY sequences are magnified and shown in *boldface*. The EVH1 and EVH2 domains are shown as *patterned boxes*. *B*, autoradiogram of the membrane exposed for 5 min; *C*, autoradiogram of the membrane exposed for 15 min; *D*, orientation of the derivatized spots on which peptides were synthesized; *E*, selected sequences of the peptides corresponding to numbered spots; the first five (1–5) and the last five (271–275) consecutive peptides are shown in addition to the sequences that scored positively in the binding assay.

consistent with this observation in that the strongest relative binding of the FE65 WW is to Mena peptides containing PPLP cores. Interestingly, the PPSY motif that is present in the neural isoform of Mena exhibited binding *in vitro* only to the WW domains of YAP and Nedd4. The unique stretches of homoprolines interrupted by leucines form binding sites to other modules, including the SH3 domains, EVH1 domains, and profilin. Although seemingly degenerate, these interactions may require specific core sequences within the polyproline regions as proposed in the "binary switch" hypothesis for overlapping core motifs for SH3 and WW domains (5, 51, 55). Because the proline-rich core of Mena binds to the profilin, WW, and SH3

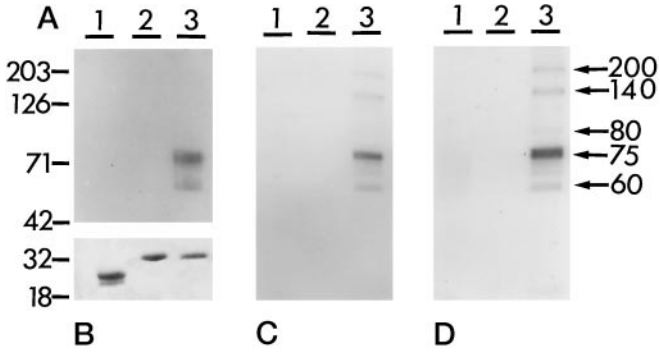

FIG. 8. **Competition of Mena and GST-13Pro for binding to FE65 WW domain.** The GST-13Pro fusion protein, which contains a proline-rich region from clone 13 (indicated in Table I) and binds to the FE65 WW domain in binding assay on filter, was used to compete interaction between Mena and radioactively labeled GST-FE65 WW domain fusion protein. *A-D*, precipitation from mouse brain lysate with GST fusion proteins; *lane 1*, GST; *lane 2*, mutant GST-FE65 WW; *lane 3*, GST-FE65 WW. Blots were incubated with 2.5 μg/ml ^{32}P-labeled GST-FE65 WW and 40 μg/ml GST-13Pro (*A*), with 2.5 μg/ml ^{32}P-labeled GST-FE65 WW and 15 μg/ml GST-13Pro (*C*), and with 2.5 μg/ml ^{32}P-GST-FE65 WW fusion protein alone (*D*). *B* shows that amount of protein loaded on gels was normalized and did not vary significantly (Coomassie stain). Positions of the 60-, 75-, 80-, 140-, and 200-kDa bands are indicated with *arrows* and numbers at the *right*. Numbers at *left* indicate molecular weight markers in kDa.

domains, it will be important to determine which of these interactions are compatible and which are competitive. Second, the competition experiment, in which PPLP-containing polypeptides were used to dissociate interactions between Mena and FE65, supports our conclusion on the specificity of binding, although the control, "scrambled" peptide was not used due to an intrinsic difficulty in permuting homoprolines and the significant affinity of the FE65 WW domain for hexamer or longer polyprolines.

Three other proteins present in mouse brain lysate bind to the WW domain of FE65, one of which, the 75-kDa protein, either is a strong binder or is present in relatively higher quantity in the lysates. The identification of these proteins and insight into their biological properties will further our understanding of FE65 function. Perhaps different ligands of the FE65 WW domain compete with each other and thus modulate the transduction of the signal from βAPP to the intracellular pathway (2).

The FE65 PTB2 domain interacts with carboxyl-terminal tail of βAPP *in vivo* (1, 8–10). Proteins that bind to PTB1 and WW domains of FE65 could be involved in biogenesis of βAPP. Specifically, cellular trafficking of βAPP and its derivatives is a complex process during which βAPP, as well as products of βAPP secretory cleavage, are transported to the endosomes/lysosomes in the clathrin-coated vesicles (56). The sequence NPTY, which is present in the cytodomain of βAPP and shown to be responsible for interaction with the FE65, was also found to be a specific targeting signal for internalization into the clathrin-coated vesicles of low-density lipoprotein receptor and other proteins (57). Therefore, FE65 may serve as an adapter protein that brings other partner molecules into the complex with βAPP, which would effect βAPP secretion, internalization, and/or trafficking. In light of this, Mena, being a cytoskeletal protein involved in microfilament assembly, is a good candidate to participate in cellular network of proteins interacting with βAPP in the cytoplasm. Interestingly, expression of the neuron-enriched isoform of Mena in fibroblasts induces the formation of actin-rich cellular outgrowths implicating Mena in the establishment of cytoskeletal microfilament connections (34). Similar to its relative, VASP, Mena has been shown to interact with the G-actin binding protein profilin. Furthermore, Mena, profilin, and vinculin (shown to bind to Mena *in vitro*) are concentrated in axonal growth cones (34). It is expected that the study of the Mena-FE65 complex may elucidate molecular events that affect or regulate βAPP biogenesis and may provide clues to the molecular changes that underlie Alzheimer disease.

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The WW Domain of Neural Protein FE65 Interacts with Proline-rich Motifs in Mena, the Mammalian Homolog of *Drosophila* Enabled

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