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

(Received for publication, August 8, 1997, and in revised form, September 23, 1997)

Kira S. Ermekova‡, Nicola Zambrano‡, Hillary Linn‡, Giuseppina Minopoli§, Frank Gertler¶, Tommaso Russo§, and Marius Sudol¶‡

From the ‡Department of Biochemistry, Mount Sinai School of Medicine, New York, New York 10029, the ¶Department of Biochemistry and Biotecnologie Mediche, Università degli Studi di Napoli “Federico II,” Via S. Pansini 5, I-80131 Napoli, Italy, and the †Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139-4307

The neural protein FE65 contains two types of protein-protein interaction modules: one WW binding domain and two phosphotyrosine binding domains. The carboxy-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) domains (Refs. 1 and 2; see Fig. 1). WW domains bind proline-rich proteins containing the PPXY or PPLP core motifs (where X signifies any amino acid) (3–5). PTB domains bind proteins containing the consensus sequence NPYX; in some instances, the tyrosine must be phosphorylated to mediate high-affinity binding (6–8). Importantly, the carboxy-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 Gα (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

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, 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 MgCl2; CMV, cytomegalovirus.
that modify Ab1-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 Ab1 phenotype (32). The Ena protein is axonally localized and is a substrate for the Drosophila Ab1 kinase and a ligand for the SH3 domains of Ab1 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 carboxy-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 lamellipodia and axonal growth cones. The subcellular distribution, interactions with profilin, and a small actin monomer-binding protein implicated in protein-protein interactions with a distinct proline-rich motif (34, 36).

The FE65 protein contains two distinct types of protein binding domains. The WW domain and COOH terminus indicate the amino and carboxy termini, respectively. Numbers indicate positions of amino acids.

**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 Mab1 were amplified using the polymerase chain reaction method. Primers designed with BamHI site at the 5' end and EcoRI site at the 3' end were as follows: 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC AGA GGA AAG ACT GAC TCC for GST-13Pro; 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC AGA GGA AAG ACT GAC TCC for GST-7/9-1Pro; 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC AGA GGA AAG ACT GAC TCC for GST-48Pro; 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC CCC GTG GTA-3 for GST-mbh1 (see Table I).

**Expression of Fusion Proteins**—Columns (Bio-Rad) were washed with HMK buffer, and 1 mM of d-thiogalactoside (Sigma) for 4 h at 37°C and then sonicated in PBS (147 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 7H2O, 1.5 mM KH2PO4) 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 5.0) containing 30 mM of reduced glutathione (Sigma) on poly-prep chromatography columns (Bio-Rad). The labeling reaction of GST, mutant GST-FE65 WW domains containing a specific screen for domains that modify Ab1-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 Ab1 phenotype (32). The Ena protein is axonally localized and is a substrate for the Drosophila Ab1 kinase and a ligand for the SH3 domains of Ab1 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 carboxy-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 lamellipodia and axonal growth cones. The subcellular distribution, interactions with profilin, and a small actin monomer-binding protein implicated in protein-protein interactions with a distinct proline-rich motif (34, 36).

The FE65 protein contains two distinct types of protein binding domains. The WW domain and COOH terminus indicate the amino and carboxy termini, respectively. Numbers indicate positions of amino acids.

**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 Mab1 were amplified using the polymerase chain reaction method. Primers designed with BamHI site at the 5' end and EcoRI site at the 3' end were as follows: 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC CCC GTG GTA-3 for GST-13Pro; 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC CCC GTG GTA-3 for GST-7/9-1Pro; 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC CCC GTG GTA-3 for GST-48Pro; 5'-dCTA TAC GGA TCC CCC GTG GTA-3 and 5'-dCTA TAC GGA TCC CCC GTG GTA-3 for GST-mbh1 (see Table I).

**Expression of Fusion Proteins**—Columns (Bio-Rad) were washed with HMK buffer, and 1 mM of d-thiogalactoside (Sigma) for 4 h at 37°C and then sonicated in PBS (147 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 7H2O, 1.5 mM KH2PO4) 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 5.0) containing 30 mM of reduced glutathione (Sigma) on poly-prep chromatography columns (Bio-Rad). The labeling reaction of GST, mutant GST-FE65 WW domains containing a specific screen for domains that modify Ab1-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 Ab1 phenotype (32). The Ena protein is axonally localized and is a substrate for the Drosophila Ab1 kinase and a ligand for the SH3 domains of Ab1 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 carboxy-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 lamellipodia and axonal growth cones. The subcellular distribution, interactions with profilin, and a small actin monomer-binding protein implicated in protein-protein interactions with a distinct proline-rich motif (34, 36).

The FE65 protein contains two distinct types of protein binding domains. The WW domain and COOH terminus indicate the amino and carboxy termini, respectively. Numbers indicate positions of amino acids.
cDNA inserts of positive clones were generated by uniformly positive phages were isolated. Plasmids containing recovered mutant and wild-type fusion proteins by standard methods (41, 42).

Three to four rounds of enrichment were performed until pure and

mRNA (Novagen) were screened with32P-labeled GST-FE65 WW fusion protein. Twenty positive clones were isolated that did not bind radioactively labeled GST or mutant GST-FE65 WW fusion protein. Twenty positive clones were isolated that did not bind radioactively labeled GST or mutant GST-FE65 WW fusion protein. 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 interacts with the protein(s) present in 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. 2C).

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 PPIX 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 interacts with the protein(s) present in 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. 2C).

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 PPIX 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 PPIX 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 tissue-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

Table I

| Clone number | Times isolated | Length of insert | Source |
|--------------|----------------|-----------------|--------|
| 9–1         | 12             | 684             | bp     |
| 7           | 4              | 487             | bp     |
| 13          | 1              | 1384            | bp     |
| 48          | 2              | 2233            | bp     |
| mbh1        | 2              | 450             | mbh1   |

a PPPPPPFLPPPPP.  
b PPPPPPFLPPPPP.  
c PPPPPPFLPPPAPPPQPPQHFLPPGLALHFLPPPPPP-P-P.  
d LLDDPPPPLGGLS.  

SERQGKAQVEIITDGEEPAEMIQVLGPKPALKEGNEPEDITADQITNAQAAALYKVDATGQMNLTVADSSFFASELILLPFDVCFLNDGCGKPYKTWGRKANHKEQKLQAIQAVDGFISRMLKNQTSQVEIHLGGESPIFQKFXNWK.

using the Chameleon double-stranded, site-directed mutagenesis kit (Stratagene). PAGE-purified mutagenic primer used for the mutagenesis was as follows: 5’-pGGA GCC CCG GCC GCG GGG TTC GAA CTG GGT GTG 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 PPIX-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 Genoys Biotechnologies, Inc. The Mena SPOTs membranes were 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 Tritis/Tween buffer to the final protein concentration of 1 mg/ml and incubated with various GST-FE65 WW 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 32P-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 proteins 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. 2C).
present in both brain and testes. 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 carboxy-terminal portion of mbh1, also known as macrophage capping protein (39). mbh1 is a 45-kDa actin-binding protein phosphorylated at multiple serines and threonines that belongs to the gelsolin/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 communoprecipitation from PC12 cells or mouse brain lysates with antimbh1 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 from 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 leucine, lysine, or arginine, 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 PP(L/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 domain 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. 4A) and thereafter with anti-Mena antibodies (Fig. 4B). 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). 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.

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 communoprecipitation.
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 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 alone (Fig. 8,A). The control spots, 43–48, indicate derivatized spots onto which no amino acids were applied. For blotting, the 32P-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.

Specificity of Mena-WW Domain Interaction—GST fusion proteins of the WW domains of FE65, mouse YAP (WW1), Neddd4 (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. 6A). The WW domains of FE65 and Msb1 precipitated both the 80- and 140-kDa forms of Mena efficiently. The WW domains of YAP and Neddd4 (WW2) bound well only to the 140-kDa isoform, whereas GST alone, Neddd4 (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 Neddd4 WW domains bind to ligands containing the PPXY motif (2, 5, 39, 51). Interestingly, the neural isoform of Mena contains the PPXY motif, which might be responsible for binding to YAP and Neddd4 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. 6A), which lacks the PPSY sequence. Although the 80-kDa isoform was detected in pull-downs with mYAP WW1 and Neddd4 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 neuronal 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 180–189) showed binding to FE65 WW domain, although with weaker affinity than spots 142–145 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 PPXY motif (data not shown). These results confirm that the binding of the 140-kDa isoform of Mena to the WW domains of mYAP and Neddd4, shown in a previous experiment, occurs through a sequence containing a PPXY 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 coprecipitation 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. 8D) 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
to Mena, the 200-kDa protein was also competed (Fig. 8A). 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 

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 PPX-Y cores (2–4). Our results are

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-Ees1 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 (C). Numbers at left indicate molecular mass markers in kDa.
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
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 homologous 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 will 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 FE65 WW and 40 μg/ml GST-13Pro (A), with 2.5 μg/ml 32P-labeled FE65 WW and 15 μg/ml GST-13Pro (C), and with 2.5 μg/ml 32P-labeled 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.

**REFERENCES**

1. Fiore, F., Zambrano, N., Minopoli, G., Denini, V., Duilio, A., and Russo, T. (1995) *J. Biol. Chem.* 270, 30853–30856
2. Ermeкова, K. S., Chang, A., Zambrano, N., de Candia, P., Russo, T., and Sudol, M. (1997) *Adv. Exp. Med. Biol.* in press
3. Chen, H. J., and Sudol, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 7819–7823
4. Chan, D. C., Bedford, M. T., and Leder, P. (1996) *EMBO J.* 15, 1045–1054
5. Sudol, M. (1996) *Prog. Biophys. Mol. Biol.* 65, 113–132
6. Bork, P., and Margolis, D. (1995) *Cell* 80, 693–694
7. Kavanaugh, W. M., Tureck, C. W., and Williams, L. T. (1995) *Science* 268, 1177–1179
8. van der Geer, P., and Pawson, T. (1995) *Trends Biochem. Sci.* 20, 277–280
9. Behn, J. Y., Chen, J., Jondro, P. D., and Tanzi, R. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 10823–10837
10. Zambrano, N., Buxbaum, J. D., Minopoli, G., Fiore, F., de Candia, P., De Renzis, S., Parnonio, R., Sals, G., Cheetham, J., Sudol, M., and Russo, T. (1997) *J. Biol. Chem.* 272, 6399–6405
11. McLaughlin, D. M., and Miller, C. C. J. (1996) *FEBS Lett.* 397, 197–200
12. Selkoe, D. J. (1997) *Science* 275, 630–631
13. Yanker, B. A. (1996) *Neuron* 15, 921–932
14. Kang, J., Lemaire, H., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) *Nature* 325, 733–736
15. Oltersdorff, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I., and Fritz, I. C. (1990) *J. Biol. Chem.* 265, 4492–4497
16. Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorff, T., McClure, D., and Ward, P. J. (1990) *Science* 242, 1122–1124
17. Shiyo, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., Cai, X., McKay, D. M., Timrner, R., Frangione, B., and Younkin, S. G. (1992) *Science* 256, 126–129
18. Keo, E. H., and Squazzo, S. L. (1994) *J. Biol. Chem.* 269, 17386–17389
19. Cai, X.-D., Golde, T. E., and Younkin, S. G. (1993) *Science* 259, 514–516
20. Nishimoto, I., Okamoto, T., Matsuura, Y., Takahashi, S., Okamoto, T., Murayama, Y., and Ogata, E. (1993) *Nature* 362, 75–79
21. Okamoto, T., Takeda, S., Murayama, Y., Ogata, E., and Nishimoto, I. (1995) *J. Biol. Chem.* 270, 4205–4208
22. Yamatsumi, T., Matsui, T., Okamoto, T., Komatsuzaki, K., Takeda, S., Fukumoto, H., Iwatsubo, T., Suzuki, N., Asami-Okada, A., Ireland, S., Kinane, B., Giambarella, U., and Nishimoto, I. (1996) *Science* 272, 1349–1352
23. Borg, J.-P., Oei, J., Levy, E., and Margolis, B. (1996) *Mol. Cell. Biol.* 16, 6229–6241
24. Chow, N., Korenberg, J. R., Chen, X.-N., and Neve, R. L. (1996) *J. Biol. Chem.* 271, 11339–11346
25. LaFerla, F. M., Tinkle, B. T., Biegerich, C. J., Haudenschild, C. C., and Jay, G. (1995) *Nat. Genet.* 9, 21–29
26. Oster-Granite, M. L., McPhie, D. L., Greenan, J., and Neve, R. L. (1993) *J. Neurosci.* 13, 6732–6741
27. Yamatsumi, T., Okamoto, T., Takeda, S., Murayama, Y., Tanaka, N., and Nishimoto, I. (1996) *EMBO J.* 15, 498–509
28. Sudol, M., Granit, S. G. N., and Maisonnier, P. C. (1993) *Neurochem. Int.* 22, 369–384
29. Hoffmann, M. F. (1991) *Trends Genet.* 7, 351–355
30. Gertler, F. B., Bennet, R. L., Clark, M. J., and Hoffmann, F. M. (1989) *Cell* 58, 105–110
31. Gertler, F. B., Hill, K. K., Clark, M. J., and Hoffmann, F. M. (1993) *Genes Dev.* 7, 441–453
32. Gertler, F. B., Doctor, J. S., and Hoffmann, F. M. (1990) *Science* 245, 857–860
33. Gertler, F. B., Comer, A. R., Jiang, J. L., Ahern, S. M., Clark, M. J., Liebl, E. C., and Hoffmann, F. M. (1995) *Genes Dev.* 9, 521–533
34. Gertler, F. B., Niebuhr, K., Reinhard, M., Wehland, J., and Soriano, P. (1996) *Cell* 87, 248–59
35. Niebuhr, K., Eisel, P., Reinhard, M., Domann, E., Carl, U. D., and Walter, U. (1995) *EMBO J.* 14, 19–27
36. Niebuhr, K., Eisel, P., Reinhard, M., Domann, E., Carl, U. D., and Walter, U. (1995) *EMBO J.* 14, 19–27
37. Pendergast, and George Ziff for the anti-mbh1 antibodies and constructive advice. We are grateful to Jurgen Wehland and Ron Frank for their generous gift of the Mena SPO'T filter.

**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 assays 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 32P-labeled GST-FE65 WW and 40 μg/ml GST-13Pro (A), with 2.5 μg/ml 32P-labeled GST-FE65 WW and 15 μg/ml GST-13Pro (C), and with 2.5 μg/ml 32P-labeled 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.
42. Young, R. A., and Davis, R. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1194–1198
43. Frank, R., and Doring, R. (1988) Tetrahedron 44, 6031–6040
44. Blankenmayer-Menge, Il., Nimtz, M., and Frank, R. (1990) Tetrahedron 31, 1701–1704
45. Sudol, M. (1994) Oncogene 9, 2145–2152
46. Prendergast, G. C., and Ziff, E. B. (1991) EMBO J. 10, 757–766
47. Dahiri, G. A., Young, C. L., Rosenblom, J., and Southwick, F. S. (1992) J. Biol. Chem. 267, 16545–16552
48. Bearer, E. L. (1991) J. Cell Biol. 115, 1629–1638
49. Johnston, P. A., Yu, F., Reynolds, G. A., Yin, H. L., Moomaw, C. R., Slaughter, C. A., and Sudhof, T. C. (1990) J. Biol. Chem. 265, 17946–17952
50. Onoda, K., Yu, F., and Yin, H. L. (1993) Cell Motil. Cytoskeleton 26, 227–238
51. Bedford, M. T., Chan, D. C., and Leder, P. (1997) EMBO J. 16, 2376–2383
52. Staub, O., Dho, S., Henry, P., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) EMBO J. 15, 1045–1054
53. Pawson, T. (1995) Nature 373, 573–580
54. Simeone, A., Dullro, A., Fiore, F., Accampa, R., De Felice, P., Pia, S., Cimino, F., and Russo, T. (1994) Dev. Neurosci. 16, 53–60
55. Sudol, M. (1996) Trends Biochem. Sci. Lett. 21, 161–163
56. Nordstedt, C., Caporaso, G. L., Thyberg, J., Gandy, S. E., and Greengard, P. (1993) J. Biol. Chem. 268, 608–612
57. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) Annu. Rev. Cell Biol. 9, 129–161