A Single Point Mutation in a Group I WW Domain Shifts Its Specificity to That of Group II WW Domains*

(Received for publication, January 28, 1999, and in revised form, March 26, 1999)

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WW domains can be divided into three groups based on their binding specificity. By random mutagenesis, we switched the specificity of the Yes-associated protein (YAP) WW1 domain, a Group I WW domain, to that of the FE65 WW domain, which belongs to Group II. We showed that a single mutation, leucine 190 (βB5) to tryptophan, is required to switch from Group I to Group II. Although this single substitution in YAP WW1 domain is sufficient to precipitate the two protein isoforms of Mena, an in vivo ligand of FE65, we showed that an additional substitution, histidine 192 (βB7) to glycine, significantly increased the ability of YAP to mimic FE65. This double mutant (L190W/H192G) precipitates eight of the nine proteins (FBPs) that interact with the same 10-mer motif found in FBP21 WW1 and WW2 domains, interact with the core sequence PPXY (17). Group II WW domains, such as the FE65 WW domain, bind to a long stretch of prolines often interrupted by a leucine (PPLP motif) (5, 6, 12). Group III WW domains, represented by the FBP21 WW1 and WW2 domains, interact with a repeated proline-rich region containing glycines and methionines, called the PGM motif (18). Recently, a phosphoserine/phosphothreonine-containing peptide has been shown to interact with some WW domains, such as the mitotic rotamase Pin 1, this finding might generate a fourth group (19).

YAP, which contains the first described WW domain, exists in two isoforms: the shorter possesses one WW domain, and the longer contains two WW domains. Both of these YAP isoforms also possess an SH3 binding motif that allows the connection to c-Yes kinase SH3 domain (20). Even though the precise function of YAP remains unclear, its overall organization suggests that it may function as an adaptor protein. YAP may mediate signaling between tyrosine and serine kinases (20). YAP WW1 domain, which is common for the two YAP isoforms, has a strong affinity for the PPXY motif (17, 21). The YAP WW1

* This work was supported by United States Public Health Service grants DK50795, CA46757, and CA10165 and by grant from the Muscular Dystrophy Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of fellowships from the Association pour la Recherche contre le Cancer and from the Phillippe Foundation.

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The abbreviations used are: YAP, Yes-associated protein; FBP, formin-binding protein; GST, glutathione S-transferase; SH, Src homology; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

Since the isolation of Src homology 2 (SH2) domain (1), the protein-protein interaction field has seen the emergence of several other protein modules, such as SH3, PTB, PDZ, and WW domains (reviewed in Refs. 2 and 3). Most of these domains have their own binding specificity; for example, SH3 domain ligands contain a PXXP motif, whereas SH2 domain ligands possess a phosphotyrosine residue followed by a hydrophobic amino acid at position +3 (4). Usually, the core consensus of the ligands is short, allowing different domains sometimes to recognize the same binding motif. This was notably described for the WW and SH3 domains of formin-binding proteins (FBPs) that interact with the same 10-mer motif found in formin isoforms, which are implicated in limb and kidney development (5, 6). In addition to the ligand core sequences, other “surrounding” amino acids are also necessary for a stable and specific interaction with protein modules. For example, the phosphotyrosine-hydrophilic-hydrophilic-hydrophobic motif binds to the Src SH2 domain, whereas the phosphotyrosine-hydrophobic-x-hydrophobic motif preferentially interacts with the phospholipase C-γ carboxyl-terminal SH2 domain (4).

Based on this observation, subconsensus sequences have emerged that lead to the subdivision of the domains into different classes according to their binding preferences (reviewed in Refs. 3 and 4). It seems that only a few amino acids within domains are responsible for these binding preferences, because a single substitution in SH2 or SH3 domains can switch their binding specificity (7–9).

The understanding of the mechanisms that dictate the specificity of domain-ligand interactions is important because several diseases were shown to be related to domain dysfunction. We and others have described that the loss of interaction between selected WW domains and their ligands could underlie such human diseases as Liddle’s syndrome, Duchenne and/or Becker muscular dystrophies, and Alzheimer’s disease (10–15).

WW domains are composed of 38 amino acids that place them among the smallest globular domains known to mediate protein-protein interactions. Their major features are (i) the conservation of two tryptophans separated by 20–22 amino acids, (ii) the presence of aromatic amino acids in the middle of the two tryptophans, (iii) and the presence of a proline at position +2 in relation to the second tryptophan (reviewed in Refs. 10 and 11). WW domains interact with polyproline-rich motifs through a small hydrophobic pocket formed by three antiparallel β-sheets (16). Similar to other protein modules, WW domains can also be subdivided thus far into three groups. Group I WW domains, like Yes-associated protein (YAP) WW1 and WW2 domains, interact with the core sequence PPXY (17). Group II WW domains, such as the FE65 WW domain, bind to a long stretch of prolines often interrupted by a leucine (PPLP motif) (5, 6, 12). Group III WW domains, represented by the FBP21 WW1 and WW2 domains, interact with a repeated polyproline-rich region containing glycines and methionines, called the PGM motif (18). Recently, a phosphoserine/phosphothreonine-containing peptide has been shown to interact with some WW domains, such as the mitotic rotamase Pin 1, this finding might generate a fourth group (19).
domain/cognate peptide NMR structure showed that leucine 190 (βB5) and histidine 192 (βB7) interact with the tyrosine of the ligand and that tryptophan 199 (βC5) binds to the two “central” prolines of the ligand (PPXY) through Van der Waals contacts. Glutamine 195 (βC1) may also establish a hydrogen bond with the tyrosine of the ligand (16).

FE65 is an adaptor protein that in addition to its WW domain possesses two PTB domains. The carboxyl-terminal PTB domain of FE65 binds to the β-amyloid precursor protein, which is a transmembrane protein implicated in Alzheimer’s disease. It is proposed that this interaction regulates β-amyloid precursor protein processing (reviewed in Ref. 22). FE65 also interacts through its WW domain with Mena (mammalian homologue of Drosophila enabled), which is implicated in the regulation of the cytoskeleton dynamics (12, 25). We have shown that the PPPPPPLPPPPP motif, which is present several times in Mena, is required for the efficient binding of the FE65 WW domain (12). In fact, a stretch of six or more prolines in a row can also bind to the FE65 WW domain in vitro. The three-dimensional structure of the FE65 WW domain is not yet known. However, the crystal structure of the Pin 1 WW domain displays the same overall structure as the YAP WW1 domain, including the three protruding amino acids in the pocket (Phe-25, His-27, and Trp-34) (24). Based on this evidence and on modeling programs, such as Modeller (25), which also predicts three β-sheets for the FE65 WW domain, one can suppose that the overall structure of the FE65 WW domain is similar to those of the YAP and Pin 1 WW domains.

Group III is the less characterized, because its description is very recent. Only the two WW domains of FBP21, which is a homologue of the precursor protein processing (reviewed in Ref. 22). FE65 also binds a proline-rich motif, where methionines and glycines are those of the YAP and Pin 1 WW domains.

To address the question of ligand selectivity of WW domains, we screened mutants of the YAP WW1 domain for those that would acquire ligand predilection of the FE65 WW domain. Our results show that the YAP Leu-190 substitution by Trp is necessary and sufficient for the switch. Moreover, this substitution is enough to precipitate the two isoforms of Mena. However, an additional substitution, His-192 to Gly, enhanced the ability of the mutated YAP WW1 domain to act like the FE65 WW domain. Our data also suggest that a block of three aromatic amino acids located in the second β-sheet of WW domains is required, but is not always sufficient, for a WW domain to belong to Group II.

EXPERIMENTAL PROCEDURES

Library and Constructions of Mutants—All inserts were cloned into pGEX-2TK vector (Amersham Pharmacia Biotech) at BamHI and EcoRI sites, after polymerase chain reaction amplification using the Deep Vent DNA polymerase from New England Biolabs. All constructs were verified by Sanger sequencing using either T7 Sequenase v2.0 kit (Amersham Pharmacia Biotech) or an automated DNA sequencer (ABI model 373). Primer nomenclature is as follows: underlined nucleotides indicate introduced mutation(s), whereas boldface nucleotides show the generated restriction site(s). For annealed primers, only the coding strands are shown.

GST-YAP corresponds to the human cDNA region (nucleotides 758–926; GenBank™ accession no. P46933) that corresponds to the WW domain (nucleotides 458–568; amino acids 43–79; see Fig. 1A) (12).

7- PPLP comes from clone 7, which was isolated from a mouse expression library screened with GST-FE65 (12). This clone codes for PPPPPPLPPPPP peptide in GST fusion.

P7V5 was obtained by polymerase chain reaction using XE-BamHI (5′-ATCTAGGATCCATCACAGCTCATGCATGACCT-3′) and EX-EcoRI (5′-ACATCGAATTCCGGCGGCGGATGCTACACGAC-3′) primers on human p53BP-2 cDNA (GenBank™ accession no. U58334). The amplified fragment codes for the PY motif (amino acids 729–768) under proper conditions. GST fusion proteins were induced by adding 1 mM IPTG to exponentially growing bacteria for 2 h at 30 °C. Bacterial protein extracts were obtained by sonication in phosphate-buffered saline + 1% Triton X-100. The extracts were then incubated, at 4 °C under vigorous shaking, for at least 2 h with glutathione-Sepharose beads (Amersham Pharmacia Biotech). The GST fusion proteins were purified by rapid centrifugations and phosphate-buffered saline washes.

About 50 μg of the GST fusion proteins bound to the beads were labeled with 150 units of protein kinase A from bovine heart (Sigma) in 40 μl of kinase buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 12 mM MgCl₂) with 1 mM dithiothreitol in the presence of 30 μCi of [γ-32P]ATP, for 30 min on ice. After several phosphate-buffered saline washes, GST fusion proteins were eluted from beads on a flow-through column, by 10 mM free glutathione in 50 mM Tris, pH 8.

Pull-down Experiments and Western Blots—Rat brains were lysed in radioligand precipitation buffer (10 mM Tris, pH 7.4, 300 mM NaCl, 0.01% Triton X-100, 5 mM EDTA, 1% sodium deoxycholate) in the presence of a protease inhibitor mixture (Complete™,Boehringer Mannheim). Protein extracts were clarified by two or three centrifugations (12,000 rpm for 10 min at 4 °C). Lysates were diluted 10 times in Tween buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Tween-20, 1% bovine serum albumin) and were incubated, at 4 °C for 14 h under agitation, with GST fusion proteins (50 μg) bound onto glutathione beads. Then GST fusion proteins/beads were washed three times with Tween buffer without bovine serum albumin. Pellets were resuspended volume to volume in loading buffer (50 mM glycerol, 125 mM Tris, pH 6.8, 10 μg/ml bromphenol blue, 5% β-mercaptoethanol, 2% SDS).

Samples were run on SDS-polyacrylamide gels (10.5%) and then electrotransferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in TBS-T buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.01% Triton X-100) with 5% low fat dried milk and incubated with the radiolabeled probe for 14 h at 4 °C under constant shaking. Then blots were washed four times with the same wash solution.

Library Screenings and Membrane Binding Assays—Bacteria were plated onto LB plates containing 50 μg/ml ampicillin and 0.5 mM IPTG. 14 h later, nitrocellulose membranes were applied onto colonies for 10 min. Membranes were lifted and placed inside a chloroform chamber for

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20 min. Then, membranes were incubated for 2–12 h in lysis buffer (50 mM Tris, pH 7.4, 400 μg/ml lysozyme, 5% low fat dried milk, 1 unit/ml DNase I, 1 mM MgCl2, 150 mM NaCl). Then, blots were transferred into Western wash solution (see above) with 5% low fat dried milk and incubated with the probe under constant shaking for 14 h at 4 °C. Washing conditions were described previously.

“SPOTs” Techniques—Peptides were synthesized on a derivatized cellulose membrane provided by Genosys Biotechnologies, Inc. as described by Frank and co-workers (26, 27). SPOTs membranes were blocked, probed, and washed with Western wash solution prescribed by the SPOTs technique; peptides were synthesized on a derivatized cellulose membrane (SPOTs technique; X means any amino acid) with the

RESULTS

The Single Substitution Leu-190 to Trp Is Sufficient to Switch the Specificity of the YAP WW1 Domain from Group I to That of Group II—Because the NMR structure of the YAP WW1 domain shows that Leu-190, His-192, and Gln-195 seem to directly interact with the Tyr of the ligand, we decided to randomly mutate these three residues to determine whether one of these mutants can acquire the specificity of the FE65 WW domain. To achieve this goal, we constructed two libraries in pGEX-2TK vector containing the YAP WW1 domain with random sequences at the position corresponding either to Leu-190 and His-192 together (YAFE-LH) or to Gln-195 (YAFE-Q) alone. Approximately 20,000 colonies/library were plated onto LB plates supplemented with IPTG to allow the expression of GST fusion proteins. The membranes were lifted from the plates and probed with the radiolabeled GST-7-PPLP (7-PPLP), a ligand of FE65 WW domain. This technique was sensitive and specific, because a single colony expressing the GST-FE65 WW domain (GST-FE65) could be readily detected with the radiolabeled 7-PPLP probe, whereas bacterial colonies expressing the GST-YAP WW1 domain (wild type; GST-YAP) were not detectable (Fig. 1A). The converse was also true, because GST-YAP was the only construct recognized by the GST-PY5 probe (PY5), which corresponds to the region of the p53BP-2 protein that interacts with YAP through its WW1 domain.2 In both cases, GST alone could not interact with these two probes (Fig. 1A).

From the YAFE-LH library, we obtained more than 200 positive clones, whereas no positive clones were isolated in the YAFE-Q library. Six YAFE-LH clones were fully analyzed, using two complementary techniques: a membrane binding assay and far Western blot. The membrane binding assay maintains the native conformation of GST fusion proteins but is not quantitative, whereas far Western blot is quantitative but requires one step of denaturation due to SDS treatment followed by one renaturation step. Renaturation in some cases can be difficult. For example, GST-FE65 does not fold well under far Western blot conditions, whereas GST-YAP seems to fold well, as judged by the intensity of their respective signals when probed with 7-PPLP or PY5 (Fig. 1D). As depicted in Fig. 1, C and D, these six clones can bind to the 7-PPLP probe but not to the PY5 probe in either the membrane binding assay or far Western blot. In fact, overexposure shows that the clone YAFE-LH3 can still interact weakly with PY5 motif.

The sequence analysis of these six mutants shows that Leu-190 is substituted each time by Trp, which is the amino acid in the FE65 WW domain (Trp-61) corresponding to Leu-190 of YAP. In addition, His-192 can be maintained or substituted by at least five different amino acids: Lys, Arg, Ser, Asp, or Gly (Table I), suggesting that position 192 is not critical for this switch.

The Amino Acid in Position 192 Also Interacts with Ligands—In order to determine whether position 192 is neutral, as the previous experiment suggested, we screened a PPPP repertoire because overexpression of the PY5 probe (PY5) was loaded in parallel on three 10.5% SDS-polyacrylamide gels. Two gels were electrotransferred and probed with either the 7-PPLP (left) or the PY5 (right) probes. The third gel was stained with Coomassie Blue to show the calibration. Molecular mass markers are indicated in kDa.

| Clone | Amino acid sequence |
|-------|---------------------|
| YAFE-LH1 | WENAXTSSGRYFPNKIDQTCTTW |
| YAFE-LH2 | WENAXTSSGRYFPNKIDQTCTTW |
| YAFE-LH3 | WENAXTSSGRYFPNKIDQTCTTW |
| YAFE-LH4 | WENAXTSSGRYFPNKIDQTCTTW |
| YAFE-LH5 | WENAXTSSGRYFPNKIDQTCTTW |
| YAFE-LH6 | WENAXTSSGRYFPNKIDQTCTTW |

The underlined amino acids correspond to the mutations that allow the switch of specificity.
showed a strong affinity for the PPPPPP peptide as the FE65 WW domain, only the YAFE-LH10 mutant displayed exactly the same pattern as that of the FE65 WW domain. This pattern showed, in addition to the strong binding to the PPPPPP peptide, a moderate binding to PPPPRP and a weak binding to PPPPPKP peptide. The YAFE-LH6 mutant can also bind to those two basic peptides but with an affinity the same as or stronger than that of the PPPPPP motif. Interestingly, the YAFE-LH3 mutant can interact with both PPPPPP and PPPPPYP peptides. This result is consistent with the weak interaction observed in Fig. 1, C and D, between YAFE-LH3 and the PY5 motif. Taken together, these data suggest that position 192 plays a role in the interaction with ligands.

The Single Point Mutation Leu-190 to Trp Is Sufficient to Precipitate FE65 WW Domain Ligands—To determine whether the YAFE-LH mutants can also interact with the other FE65 WW domain ligands, we performed pull-down experiments from rat brain lysates with YAFE-LH mutants. As negative controls, we used GST alone and a FE65 WW domain mutant (FE65 mut), where Trp-69 and Pro-72 were substituted by Phe and Ala, respectively. These two mutations render the FE65 WW domain inactive in terms of ligand binding (Fig. 3B) (12). As shown in Fig. 3A, almost all of the ligands precipitated by FE65 WW domain were also precipitated by YAFE-LH mutants, whereas none of the major ligands of YAP WW1 domain were precipitated by the mutants. Interestingly, as shown in the SPOT technique data (Fig. 2), the YAFE-LH10 mutant is most similar to the FE65 WW domain, because the precipitation patterns of YAFE-LH10 and FE65 are identical except for the faint 90-kDa protein band that can only be pulled down by the FE65 WW domain. It is worth noticing that the YAFE-LH3 mutant, although displaying a hybrid specificity on SPOT technique (PPPPPP and PPPPPYP; Fig. 2), cannot precipitate any YAP ligands, confirming that its affinity for PXXY is poor.

To show that the co-migrating protein bands observed in the mutants and FE65 pull-down experiments correspond to the same proteins, we probed them with an antibody against Mena, which interacts in vivo with FE65 through the WW domain (12). As shown in Fig. 3C, all of the mutants, like the FE65 WW domain, precipitated the two isoforms of Mena (80 and 140 kDa), whereas YAP can only precipitate the neuronal form of Mena (140 kDa) due to the presence of a PPSY motif (12). As expected, a third band (60 kDa), visualized only with FE65 and the mutants, can also be detected with this antibody, suggesting that another form of Mena or a related protein interacts with the FE65 WW domain. It is worth noticing that the Mena antibody also recognizes a band migrating above the Mena-related protein. This band is apparently none specific, because the signal is present in every lane, including the GST lane, when the film is exposed for a longer period (data not shown). These results confirm that all the co-migrating bands observed with the mutants and with FE65 correspond to the same proteins.

The Extra Length of YAFE Mutants Is Not Responsible for Their Failure to Precipitate the 90-kDa Band—A close observation shows that the YAP WW1 domain possesses 21 amino acids between the two conserved tryptophans, whereas in the FE65 WW domain there are only 20 (Fig. 1A). In order to address the importance of this extra amino acid for the switch and to examine whether the length is the factor that prevents the 90-kDa band precipitation, we deleted the Asn-191 in the YAFE-LH3 construct (YAFE-WH). The YAFE-WH mutant possesses the same second β-sheet end (YWHR-HD) as FE65 WW domain (YWHRHP). Fig. 4 shows that YAFE-WH can still fold properly because it can bind to the 7-PLLP probe. YAFE-WH can also precipitate Mena (Fig. 4C, right panel), but not the 90-kDa band. In addition, its precipitation pattern is not as close to FE65 as that of YAFE-LH10 (Fig. 4C, left panel). Interestingly SPOT analysis shows that YAFE-WH exclusively binds to the PPPPPP peptide and not to the PPPPPYP peptide as YAFE-LH3 does.

Three Aromatic Amino Acids in the Middle of YAP WW1

Fig. 2. Spot analyses of YAFE-LH mutants. Eight membranes with a repertoire of 20 peptides of the type PPPXPAAANA (where X indicates any amino acid), were probed with eight different radiolabeled probes, indicated on the left. Spot intensity cannot be compared from one membrane to another because labeling was not constant from one probe to another.

Fig. 3. Like FE65 WW domain, YAFE mutants can pull down the non-neuronal isoform of Mena. A, pull-down experiments. 800 μg of rat brain lysates were incubated with nine different GST fusion proteins. GST fusion proteins were precipitated with glutathione beads, extensively washed, and then loaded on 10.5% SDS-polyacrylamide gels. Proteins were electrophoresed onto a membrane that was cut into nine strips according to the nine loaded samples. Each of the strips was probed with the radiolabeled GST fusion protein used for the pull-down. The open arrow indicates the 90-kDa band. B, pull-down controls. We also performed a pull-down with a mutant of FE65 (FE65 mut), in which its WW domain was inactivated in terms of binding by two point mutations, W69F and P72A. We used either FE65 mut or FE65 as a probe onto FE65 mut and FE65 pull-down experiments to see whether any of the nine bands precipitated by FE65 are nonspecific. Lower panels correspond to the calibration of the loaded GST proteins. These two same blots were stripped and probed back with a rabbit polyclonal antibody against GST. C, Mena can be precipitated by the YAFE-LH mutants. Using the same pull-down experiments as in A, we ran and elecrotransferred them onto a membrane that we probed them with a rabbit polyclonal antibody against Mena, N-Mena (upper panel). Two solid arrows indicate the two Mena isoforms, and the open arrow indicates the Mena-related protein. The lower panel is the calibration of the previous blot with a GST antibody.

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**Fig. 4.** The short size of the FE65 WW domain is not responsible for the 90-kDa band precipitation. A, membrane binding assay. Upper panels, bacterial colonies containing GST, YAP, YAFE-WH, or FE65 were induced by IPTG on LB plates. Lower panels, bacterial colonies expressing GST, YAP, YAFE-WH, or A, pull-down experiments with 7-PPLP or PY5. B, far Western blots. Upper panels, filters, with GST fusion proteins electrotransferred onto them, were probed with the radiolabeled 7-PPLP or PY5 proteins. Lower panel, Coomassie Blue calibration of the GST fusion proteins analyzed in far Western blots. Molecular mass markers are in kDa. C, pull-down experiments with YAFE-WH. Conditions were the same as in Fig. 3A, D, pull-down experiments probed with an anti-Mena antibody, N-Mena. Conditions were the same as in Fig. 3C, E, spot analysis. Spot membrane, with the PPXXPAAAA peptide repertoire, was probed with the radiolabeled YAFE-WH probe.

**Domain Are Necessary but Not Always Sufficient to Allow the Switch**—A rapid comparison between Group I and II WW domains showed that Group I WW domains possess only two consecutive aromatic amino acids in the middle, whereas Group II WW domains have three aromatics (Table II). Because the YAFE-LH mutants are consistent with this observation, we investigated whether three aromatics in the YAP WW1 domain are sufficient, when we changed the composition and the position of the aromatic block.

To address the issue of the composition, we replaced Leu-190 with a different aromatic amino acid, such as Tyr (YAFE-LY), in case we had missed it in our screening of the YAFE-LH library. With this YAFE-LY mutant, we observed a weak and probably insignificant signal with either the 7-PPLP or PY5 probes (compare Fig. 5C and Fig. 1D). This conclusion is supported by the observations that the YAFE-LY mutant cannot pull down Mena from rat brain lysate and that SPOT analysis revealed a faint interaction only with the PPXXPAAAA peptide repertoire (data not shown).

To address the importance of the position of the aromatic amino acids, we substituted Arg-187 with Tyr (YAFE-RY). As shown in Fig. 5, the YAFE-RY mutant acts as the YAP wild type. We confirmed this result using the SPOT technique. In this experiment, both mutant and wild type presented the same pattern (data not shown).

Taken together, these data show that the position and the composition of the aromatic amino acids within the second β-sheet of the YAP WW1 domain are important for the switch of the specificity.

**DISCUSSION**

Based on their binding specificity, WW domains can currently be divided into three groups. In this report, we focused our study on the specificity that drives Group I and Group II WW domains to interact with PPXY core or a stretch of prolines containing the PPLP motif.

**TABLE II**

Partial listing of Group I and Group II WW domains for which ligands have been characterized

| Domain* | Sequence | Ref. |
|---------|----------|------|
| Group I | hYAP WW1 | WEMAKTSQGRFYLHIDQTTTW | 17 |
| Group I | hYAP WW2 | WEOAMQDGEIYYINHNNKSTSW | 5,6 |
| Group I | hNEDD4-WW1 | WEREQDILGRTYYVHBRRTQW | 28 |
| Group I | hNEDD4-WW2 | WEREQDILGRTYYVDHNRRTTW | 28 |
| Group I | hNEDD4-WW3 | WERURHAPKFGPRRFDITHQQTMTTW | 28 |
| Group I | hNEDD4-WW4 | WERURHAPKFGPRRFDITHQQTMTTW | 28 |
| Group I | hDystrophin WW | WERASIPKVRYYNHETFQCQW | 15, |
| Group I | hWWP1-WW1 | WERQKDPRGRTYYVHNRRTTW | 30 |
| Group I | hWWP1-WW2 | WERQKDPRGRTYYVHNRRTTW | 30 |
| Group I | hWWP1-WW3 | WERQKDPRGRTYYVHNRRTTW | 30 |
| Group I | hWWP2-WW1 | WERQELPGRRTYYVHNRRTTW | 30 |
| Group I | hWWP2-WW3 | WERQELPGRRTYYVHNRRTTW | 30 |
| Group I | hWWP3 | WERASIPKVRYYNHETFQCQW | 15, |
| Group II | rFE65 | WNRVQDTIG-RYYVHILGTQW | 12 |
| Group II | hMsb1 WW | WKTARDPREGKYYVHRTRQTQW | — |
| Group II | mFBP11 WW1 | WERHRSDRTYRYYHTRRTQW | 5,6 |
| Group II | mFBP11 WW2 | WERHRSDRTYRYYHTRRTQW | 5,6 |
| Group II | mFBP29 | WTEKTADKGRTYYVHRTRRTQW | 5,6 |

* h, human; m, mouse; r, rat.

**Fig. 5.** Three aromatics are not enough to allow the switch to the 7-PPLP ligand. A, membrane binding assay. Upper panels, bacterial colonies expressing GST, YAP, YAFE-RY, YAFE-LY, or FE65. Lower panels, filters from the upper single colonies were hybridized with either the 7-PPLP or PY5 probes. B, far Western blots. Conditions were the same as in Fig. 1D.
action with the 7-PPLL ligand. Although all the YAFE-LH mutants can precipitate the two Mena isoforms with the same strength as FE65, position 192 also imparts domain specificity, because analysis by SPOT and pull-down experiments revealed that these mutants can mimic the behavior of the FE65 WW domain to different degrees (Figs. 2 and 3). This observation is confirmed by the fact that a single mutation in YAP that changes this His-192 to a Phe switches the specificity from PPPPY to PPPFP, again suggesting that position 192 is also involved in imparting binding specificity.

According to SPOT and pull-down data, only two point mutations in the YAP WW1 domain, L190W and H192G (YAFE-LH10), generate a WW domain that acts similar to FE65. The only difference is a faint 90-kDa protein band that is precipitated by FE65 and not by YAFE-LH10 (Figs. 2 and 3). To ask whether the 90-kDa band precipitation is due to the size of FE65 WW domain (one amino acid less compared with the YAP WW1 domain), we generated the YAFE-WH mutant, which possesses the substitution Leu-190 to Trp and also an Asn-191 deletion. We chose the Asn-191 because its deletion results in a sequence similar to that of FE65. Interestingly, this YAFE-WH mutant can still fold properly because it can interact with the 7-PPLL probe and precipitate Mena. However, this mutant, like the other, cannot pull down the 90-kDa band, suggesting that the interaction with this band is not due to the length of FE65 WW domain. The 90-kDa band might correspond to a protein that, in addition to its interaction with the binding pocket of the FE65 WW domain, requires additional contacts with surrounding amino acids of the WW domain to stabilize its binding. This protein may also bind to FE65 via a nonlinear motif, as it has been previously described with Mbh I, an in vitro FE65 ligand that does not contain any stretches of prolines (12). This latter option is possible because Mbh I, expressed as a GST fusion protein, cannot interact with the YAFE-LH mutants or with YAFE-WH (data not shown).

A rapid comparison between Group I and Group II WW domains shows that Group I possesses two consecutive aromatic amino acids on their second β-sheet, whereas Group II has three (Table II). Although YAFE-LH mutants strengthen this observation, data from YAFE-RY and YAFE-LY mutants show that the position and the composition of the central aromatic amino acids are also important for imparting binding specificity. To summarize, three aromatic amino acids in the second β-sheet seem to be required for a WW domain to belong to Group II, but probably not all WW domains with three aromatics belong to Group II. Interestingly, the human and mouse FBP21-WW1 and -WW2 (Group III) have four aromatics, Y/E/V/Y/Y, except the mouse FBP21-WW1, which has three aromatics plus a His, a basic amino acid with an imidazole ring, HCYYY. More studies are needed to conclude that group III specificity is dictated by its four central aromatics or “rings.”

In order to confirm our hypothesis concerning the importance of the number of aromatic amino acids in the second β-sheet, we are trying to generate the reverse switch, by creating a modified FE65 WW domain that binds to the PY5 probe. Thus far, we have changed Trp-61 to a Leu (FEYA) with or without the addition of the extra amino acid, Asn. As expected, these FEYA mutants can no longer bind to 7-PPLL probe, but they also cannot bind to the PY5 probe (data not shown). Because these mutants can neither bind to any PPPYP peptides nor precipitate ligands from rat brain lysates (data not shown), we think that they cannot fold properly. This result suggests that to switch the FE65 WW domain binding affinity to the PY5 probe, we probably need to introduce, in addition to Leu, other changes to stabilize the structure and/or increase the affinity to the PY5 probe.

In this study, we have shown that a double or even a single mutation can switch the YAP WW1 domain specificity to another specificity. Now it would be interesting to generate modified WW domains that can restore the disrupted binding due to a point mutation in the proline-rich sequence. Such mutations have been described in the PPXY motif of a sodium channel subunit, β EcnA. These mutations prevent sodium channel degradation by Nedd4, a ubiquitin ligase possessing WW domains, and lead to Liddle’s syndrome, a form of severe hypertension (31–33).

Acknowledgments—We thank Hillary Linn, Kira Ermekova, and Frank Gertler (Massachusetts Institute of Technology, Boston, MA) for the SPOT peptide library and FE65 protein and for their valuable comments on the manuscript and to Paul Klotman for support.

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