

## Research Paper

# Characterizing Class I WW domains defines key specificity determinants and generates mutant domains with novel specificities

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**Abstract**

**Introduction:** WW domains are small protein interaction modules found in a wide range of eukaryotic signaling and structural proteins. Five classes of WW domains have been annotated to date, where each class is largely defined by the type of peptide ligand selected, rather than by similarities within WW domains. Class I WW domains bind Pro–Pro–Xxx–Tyr containing ligands, and it would be of interest to determine residues within the domains that determine this specificity.

**Results:** Fourteen WW domains selected Leu/Pro–Pro–Xxx–Tyr containing peptide ligands via phage display and were thus designated as Class I WW domains. These domains include those present in human YAP (hYAP) and WWP3, as well as those found in ubiquitin protein ligases of the Nedd4 family, including mouse Nedd4 (mNedd4), WWP1, WWP2 and Rsp5. Comparing the primary structures of these WW domains highlighted a set of

highly conserved residues, in addition to those originally noted to occur within WW domains. Substitutions at two of these conserved positions completely inhibited ligand binding, whereas substitution at a non-conserved position did not. Moreover, mutant WW domains containing substitutions at conserved positions bound novel peptide ligands.

**Conclusions:** Class I WW domains contain a highly conserved set of residues that are important in selecting Pro–Xxx–Tyr containing peptide ligands. The presence of these residues within an uncharacterized WW domain can be used to predict its ability to bind Pro–Xxx–Tyr containing peptide ligands. © 2001 Elsevier Science Ltd. All rights reserved.

**Keywords:** Combinatorial peptide; Phage-display; Protein domain; Protein interaction module

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**1. Introduction**

The WW domain, first described by Bork and Sudol [1], also referred to as the WW domain containing protein

(WWP) domain [2] or the extragenic suppressor of spt3 (Rsp5) repeat [3], is a recently described protein interaction module [4,5]. The domain is approximately 40 amino acids in length and 340 WW domains have been discovered in 209 non-redundant proteins so far [6]. WW domains often occur multiple times in a single protein, ranging from one copy in the human Yes-associated protein (hYAP) to four in the human neuronally expressed, developmentally down-regulated protein 4 (Nedd4).

WW domains function as protein interaction modules that bind proline-rich peptide sequences. These domains fold into a compact structure containing three-antiparallel  $\beta$ -sheets, with the ligand binding face and N- and C-termini found on opposite sides of the domain [7]. Biochemical characterization of two proteins, WBP1 and WBP2, found to interact with the WW domain of YAP [4], revealed a common PPPPY peptide sequence and subsequent experiments with synthetic peptides revealed that the minimal binding motif is PPxY, where x is any amino

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*Abbreviations:* WW, refers to two highly conserved tryptophan residues within the WW domain; COLT, cloning of ligand targets; ENaC, epithelial amiloride-sensitive sodium channel; GST, glutathione *S*-transferase; HECT, homologous to the E6-AP C-terminus; PBS, phosphate-buffered saline; PTB, phosphotyrosine binding domain; SH2, Src homology 2; SH3, Src homology 3; WWP, WW domain containing protein; YAP, Yes-associated protein; Nedd4, neuronally expressed, developmentally down-regulated protein 4; PDZ, PSD-95, Dlg, ZO-1 homology domain; Rsp5, extragenic suppressor of spt3

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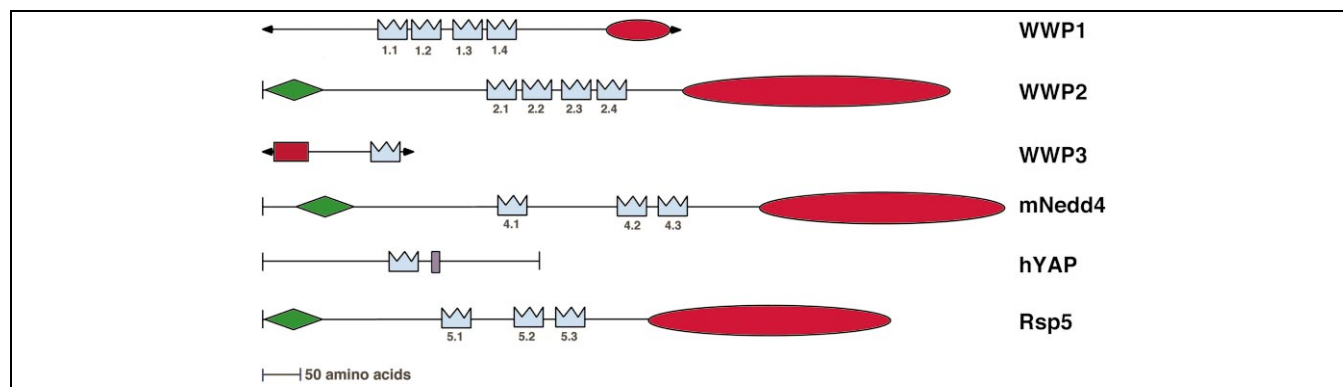


Fig. 1. Schematic diagram of some WWs. WWP1, WWP2 and WWP3 are human proteins previously isolated from human brain and skeletal muscle cDNA expression libraries. mNedd4 is the mouse isoform of the human Nedd4 while Rsp5 is a similar protein found in the yeast *S. cerevisiae*. YAP has a single WW domain. Of the 16 WW domains shown, only mNedd4.1 was not studied further after repeated attempts to screen the phage display library with this domain failed. Phage display-derived peptide ligands selected by the mNedd4.3 WW domain are published elsewhere [7]. The SH3 binding domain in hYAP contains a PxxP motif that was found to interact with the SH3 domain of Yes. A HECT domain is a region which encodes type E3 ubiquitin ligase activity. C2, refers to a  $\text{Ca}^{2+}$ -dependent lipid binding domain. In the diagram, = WW domain, = HECT domain, = guanylate kinase-like domain, = SH3 binding domain and = C2 domain. Arrows denote incomplete sequence data while vertical lines denote a complete coding sequence.

acid [4,8,9]. Based on this motif, a variety of proteins have been proposed to interact with WWs [8,10]. In one well characterized example, the WW domains of Nedd4 have been demonstrated to bind to a PPxY motif present in the cytosolic tails of the three subunits of the epithelial amiloride-sensitive sodium channel (ENaC; [11,12]). Furthermore, mutations in the PPxY sequences destroy the interaction between the ENaC and Nedd4, giving rise to a genetic form of hypertension termed Liddle's syndrome [11].

Combinatorial peptide libraries have recently provided a wealth of information on the ligand binding preferences of protein interaction modules. Peptide libraries have been utilized to delineate the ligand specificity of Src homology 2 (SH2) domains [13], phosphotyrosine binding domains (PTB) [14,15], Src homology 3 (SH3) domains [16–20] and PSD-95, Dlg, ZO-1 homology (PDZ) domains [21,22]. This information has been useful in suggesting what natural proteins may interact with these domains and provide peptide antagonists for *in vitro* and *in vivo* experiments and is discussed in recent reviews [23,24].

In this report, we have screened two bacteriophage M13 libraries displaying combinatorial peptides with WW domains from a total of five different proteins. These 14 WW domains include four from both WWP1 and WWP2 as well as one from WWP3, three proteins isolated from brain and skeletal muscle cDNA expression libraries using cloning of ligand targets (COLT; [8]), one from human YAP (hYAP, [25]), the central WW domain of mouse Nedd4 (mNedd4, [26]) and the three WW domains found in Rsp5, a ubiquitin ligase similar to mNedd4, found in *Saccharomyces cerevisiae* [27]. Concordant with previously published data, each WW domain was shown to prefer peptide ligands containing the core PxY motif. In comparing the primary structure of the WW domains that prefer this type 1 ligand, we noted that they all shared certain

residues, in addition to those originally noted to occur in WW domains [1–3]. Mutational analysis demonstrated that two of these conserved residues contribute to ligand specificity and binding. We propose this group of residues within Class I WW domains occupy positions important in determining specificity for PxY containing ligands.

## 2. Results

### 2.1. Affinity selection of peptide ligands for various WW domains

A schematic diagram of the proteins containing WW domains analyzed is shown in Fig. 1. To define the peptide ligand preference of each WW domain, we screened two combinatorial peptide libraries (i.e.,  $x_{12}$  or  $x_6\text{PPx}_6$ ) displayed at the N-terminus of mature protein III of bacteriophage M13. Phage isolated after three rounds of affinity selection were confirmed to bind to glutathione *S*-transferase (GST) fusions to WW domains, but not to GST alone, and sequenced. Of the 16 WW domains shown in Fig. 1, 14 were used as targets in this study. Peptides selected by mNedd4.3 are published elsewhere [23]. Only Nedd4.1 failed to select phage from the libraries and did not bind to any of the phage isolated by the other WW domains, even though other constructs of this WW domain have been reported elsewhere to bind proteins [11].

Fig. 2A lists the peptide sequences of phage successfully isolated by 14 WW domains. In all but two of the 76 different sequences isolated, a core PxY motif is present, flanked by either a leucine or proline residue just N-terminal of the core, yielding the motif (L/P)PxY. The two ligands that lack the PxY core, AWSLQGPSFYQS and CGYPNRPCEWM, were selected by WWP2.1 and WWP2.3, respectively. However, unlike other PxY pep-

A

WWP1.1		WWP2.2		Nedd4.2	
LKLDPYWESSAS	4 <sup>a</sup>	WSIWEGEPPPPY	4	LKLDPYWESSAS	3 <sup>a</sup>
GLPPPYDLTWVN	1	HWKDLDPPPY	3 <sup>d</sup>	LKLPEYWESSAS	2 <sup>b</sup>
GDVRFWGAPPPY	1	GWOLFEEPPPYE	2 <sup>c</sup>	WEDWGPPPYDYSD	1
LKLPEYWESSAS	1 <sup>b</sup>	GELPPSYQELFG	1 <sup>e</sup>	WVERLEPPPYEEAV	1
		TRWWYEDPPPYA	1	MRELGDPPSYLEAI	1
<u>ΨXΔPKY</u>		<u>WXX-XPPPY</u>		<u>ΨCΔPKYXE</u>	
WWP1.2		WWP2.3		Rsp5.1	
GWOLFEEPPPYE	4 <sup>c</sup>	PPAYGSWELFSP	11 <sup>e</sup>	LTEYLMPPSYKEAM	1
HWKDLDPPPY	3 <sup>d</sup>	CGYPNRPCEWM	3 <sup>#</sup>	WEWWGSPPAYERFG	1
WWWERPPPYREL	1	GELPPSYQELFG	1 <sup>e</sup>	WDVWFPPPLYGQV	1
		PSGPPPPYEAW	1	RFSWLLPPTYTDST	1
<u>WWXX-XPPPY</u>		HWKDLDPPPY	1 <sup>d</sup>	IPWWLRPPAYMEVD	1
		PLPPSYWAVVSQ	1 <sup>h</sup>	WKDYWAPPSYESAN	1
				WTEWLRPPAYGYDA	1
WWP1.3		<u>ΔPKY</u>		<u>WXXΔLXPPXY</u>	
GELPPSYQELFG	6 <sup>e</sup>				
PPAYGSWELFSP	2 <sup>a</sup>				
PPMYGSWWSTVN	1				
HWKDLDPPPY	1 <sup>d</sup>				
LFGPLPPYEVRT	1				
PLPPSYWAVVSQ	1 <sup>h</sup>				
<u>ΔPKY</u>					
WWP1.4		WWP2.4		Rsp5.2	
LLPNYGEWWRGG	3	PGMLLPPTYEEESI	3	AGTGDEPPPYSFWW	1
SLLPTYGHELFW	2	GMLPSYEAVMA	2	QYAGTPPPYWSVVQ	1
MLPEYTEYGFMS	1	PGMLLPPTYEEESI	2	SALKAPPPYWEIFP	1
TLLPGYLSDEYW	1	LKLDPYWESSAS	1 <sup>a</sup>	VKLRTPPPYAEQNW	1
LKLDPYWESSAS	1 <sup>a</sup>	LLPSYHIQGPWL	1	GKPLVRPPPYGSWL	1
SLLPEYNMPLYH	1	GMLPSYEEAVMA	1 <sup>g</sup>	WVQGRLPPLYLSDP	1
LMLPAYNEAVTW	1			MGTADSPPDYWSIV	1
LMLPHYGDMQFA	1			PARWYPPPAYTDVG	1
LLPMYGEAEAWF	1			WGSAYPPPYERVKL	1
		<u>LLPXYYE</u>		<u>ΨXXPPPY</u>	
		WWP3		Rsp5.3	
		GMLPSYEEAVMA	5 <sup>g</sup>	PVRLLPPTYQRGDS	1
		PIAPPTYEWAL	5	SRIWGDPPPYSPYW	1
		LPTYNEYLTRAA	2	AISSERPPPYELGL	1
		RLPAYKEPAATF	1	RISRDPPPLYEPAE	1
		LPSYSEWVAETR	1	MRPGIPPPPYQSGW	1
		PAVRVNLPPYPG	1	TPSWGDPPHYMHKV	1
		<u>LPXYXEΨ</u>		<u>PPPYXXΨ</u>	
WWP2.1		hYAP			
AWSLQGPSFYQS	4	LTTLPSYWEVMR	8		
GWELKPPEYREF	3	VWRSERLPPTYWE	1		
GKILPPPSYDSI	1	LVTLP SYAEAMQ	1		
QGGWSLMPPEYH	1	HSPPSYEEWKSK	1		
AWELPPPMYGRE	1	RPDPPSYSAVMR	1		
DRLPTYQEVAIG	1	GLRPPTYREWLA	1		
GDVLQPPQYSLV	1	CAPLPSYSEWLM	1		
		LEFLPSYSEWMQ	1		
<u>GXXLXPPXY</u>					
		<u>ΔPSYXEWΨ</u>			

Fig. 2.

<b>B</b>	
<u>Class I</u>	
WWP1.1	ΨXΔPXY
WWP2.1	GXXLXPPXY
RSP5.1	WXXΘLXPPXY
WWP1.2	WWXX-XPPPY
WWP2.2	WXX-XPPPY
RSP5.2	ΨXXPPPY
 <u>Class II</u>	
WWP1.3	ΔPPXY
WWP2.3	ΔPPXY
WWP1.4	LLPXYEXXΘ
WWP2.4	LLPXYEE
WWP3	LPXYXEΘΨ
hYAP	ΔPSYXEWΨ
Nedd4.2	LKLP-YWESSAS
Nedd4.3	PPXYEXΨΘ
Rsp5.3	PPPYXXΨ

Fig. 2. A: Sequences of peptides selected from two combinatorial peptide libraries for 14 different WW domains. Two libraries of bacteriophage M13, displaying either  $x_{12}$  or  $x_6PPx_6$  combinatorial peptide libraries at the N-terminus of the mature coat protein III, were affinity-selected with GST fusion proteins to 14 different WW domains. To the right of each peptide sequence, numbers indicate the number of times each clone was isolated and letters denote that the sequence was isolated by more than one WW domain. Peptides in each group are arranged, from top to bottom, along with the frequency each sequence was isolated. All sequences are aligned to illustrate the motif, PxY, with the P and Y residues boldface. The two peptide ligands that did not have PxY are denoted with #. Peptides isolated from the  $x_6PPx_6$  library have their fixed prolines underlined. The consensus sequence is defined as positions displaying at least 50% conservation. The symbols  $\Theta$ ,  $\Psi$ ,  $-$ , and  $\Delta$  represent aromatic, hydrophobic, acidic, charged and leucine or proline residues, respectively. B: Aligned consensus sequences derived from WW domain-selected peptides. Consensus sequences derived from affinity-selected peptides fall into two classes based on the relative position of the PxY core within the sequence as well as the similarities between the sequences. The proline and tyrosine residues of each core are shown in boldface, while the symbols  $\Theta$ ,  $\Psi$ ,  $-$ , and  $\Delta$  represent aromatic, hydrophobic, acidic, charged and leucine or proline residues, respectively. The consensus sequence for the peptide ligands of the mNedd4.3 WW domain was deduced elsewhere [23].

tides, when fused to alkaline phosphatase [28], both of these peptides did not retain their affinity for their WW domain targets (data not shown). It may be that avidity effects inherent in the potentially pentavalent display with protein III on bacteriophage M13 permitted these two weakly binding peptides to be selected. In examining the 'x' position within the PxY motifs, certain amino acids are statistically over- or under-represented from random. After normalizing the observed frequency of this position based on the codon frequency of the combinatorial peptide library, G, P, S, and T all appeared at roughly twice their expected rate, while C, D, F, G, H, I, K, L, N, Q, R, V, W, and Y occurred at a frequency lower than expected (data not shown).

It is interesting to note that WWP1.4 and WWP2.4 selected PxY ligands, even though both domains contain phenylalanine at position 39. When this position is mu-

tated to phenylalanine in the hYAP WW domain, binding of its peptide ligand is destroyed [29,30]. Since W39 in the hYAP WW domain contacts the prolines within the PPxY motif of the ligand, presumably there are compensatory changes within WWP1.4 and WWP2.4 domains that permit binding to Class I peptide ligands.

Examination of the optimal ligand preferences for the WW domains suggested that the motifs could be grouped into two classes (Fig. 2B). While the physiological significance of the grouping is unknown, the alignments serve to highlight potentially important ligand residues. In addition, comparison of the optimal ligand specificity revealed that colinear WW domains (i.e., domains found in the same relative position within proteins of similar overall structure), tended to have similar ligand preferences. For example, the consensus sequence selected by WWP1.1 was remarkably similar to that selected by WWP2.1, and so forth. Thus, it seems colinear WW domains, which are present in related proteins, have evolved to recognize similar peptide ligands.

## 2.2. Protein database searches reveal many WW domains with a highly conserved primary structure

Upon testing the specificities of several selected peptides against WW domains shown in Fig. 1, we observed that in general the peptides exhibited a broad range of cross-reactivity. In contrast, they showed no apparent binding (data not shown) to WW domains from the *S. cerevisiae* proteins Ess1p [31,32], Ykb2p/Prp40; [33] and Yfb0p [34], overexpressed in bacteria as GST fusion proteins. The WW domains of these three yeast proteins appear to have very different ligand preferences as they select peptides that do not contain a PxY motif (data not shown). Thus, we then considered if there was any sequence conservation, beyond that shared by all WW domains, which may account for the high degree of cross-reactivity among this set of PxY binding WW domains.

As seen in Fig. 3A, alignment of the primary structures of these domains revealed amino acid conservation at several positions, in addition to those residues originally noted to define WW domains [1–3]. Positions displaying > 90% identity include position 14 (which lies within the first turn), residues 16–18 (which appear in the N-terminal  $\beta$ -sheet of the domain), position 32 (which lies in the second  $\beta$ -sheet), positions 37 and 39 (present within the C-terminal  $\beta$ -sheet), and position 42 (which lies in the final turn of the domain). Positions exhibiting > 90% similarity include residue 22 (within the N-terminal  $\beta$ -sheet), residues 28–30 (lying in the middle  $\beta$ -sheet), residues 35–36 (located within the C-terminal  $\beta$ -sheet) and residue 41 (located in the final turn).

Subsequent examination of other WW domains in GenBank identified several that also contained the same set of conserved residues. These include the three WW domains of the *Schizosaccharomyces pombe* protein PUB1, the four

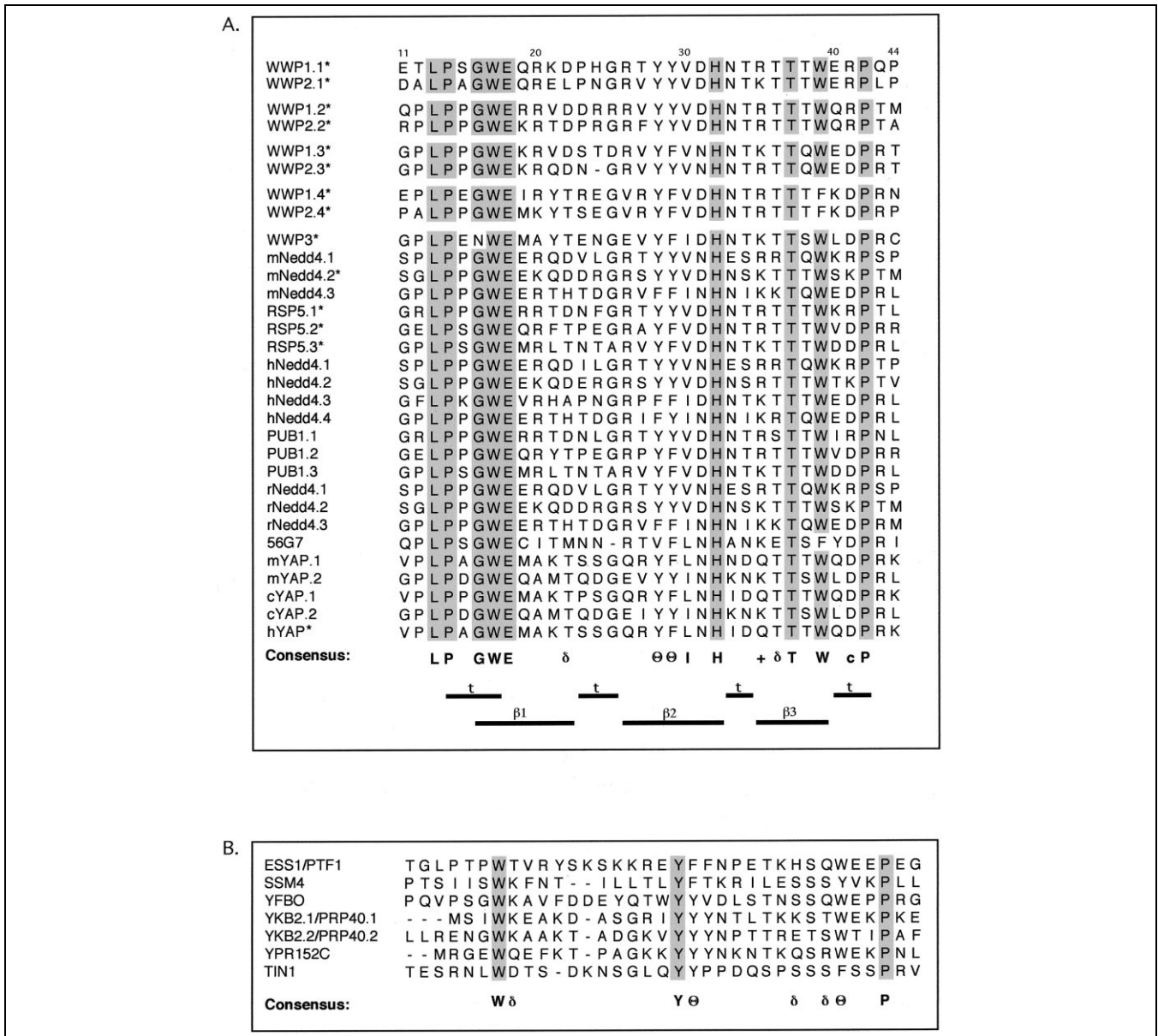


Fig. 3. A: Aligned Nedd4-like WW domains. The names used for each WW domain are as described in Figs. 1 and 3. PUB1.1, PUB1.2 and PUB1.3 denote the three WW domains of PUB1, from N- to C-terminus, respectively. cYAP refers to the YAP homolog found in chicken, and mYAP.1 and mYAP.2 refer to the two WW domains found in the mouse homolog of YAP. Shaded residues are  $>90\%$  conserved within each group. Consensus sequences indicate identities and similarities  $>90\%$  conserved, where  $\delta$ =polar,  $\Theta$ =aromatic, +=positively charged, c=charged, l=aliphatic and  $\Psi$ =hydrophobic residues.  $\beta$ 1– $\beta$ 3 denote the locations of the three  $\beta$ -sheets and t represents the turns present in WW domains. Asterisks denote WW domains shown in this study to bind (L/P)PxY peptides. Colinear WW domains from WWP1 and WWP2 are aligned to highlight mutual primary structure conservation. Alignments and shading were generated using SeqVu (Version 1.1, The Garvan Institute of Medical Research, Sydney, N.S.W., Australia). Consensus residues were created at the URL: <http://www.bork.embl-heidelberg.de:8080/Alignment/consensus.html>. Numbering corresponds to that used by Macias et al. [7] in describing amino acid positions in the hYAP WW domain–ligand complex. GenBank accession numbers are as follows: WWP1 (U96113), WWP2 (U96114), WWP3 (U96115), mNedd4 (P46934), RSP5 (P39940), human Nedd4 (P46935), PUB1 (Q92462), rat Nedd4 (U50842), 56G7 (Z46793), mouse YAP (P46938), chicken YAP (P46936), hYAP (P46937). B: The remaining WW domains in the *S. cerevisiae* genome. GenBank accession numbers are as follows: ESS1/PTF1 (P22696), SSM4 (P40318), YFBO (P43582), YKB2/PRP40 (P33203), YPR152C (U440829), TIN1 (Z72749).

WW domains of the human and rat Nedd4 homologs, and the most C-terminal WW domain of the *Caenorhabditis elegans* protein 56G7. These four proteins, along with human WWP1 and WWP2, share a similar overall modular structure with three N-terminal WW domains and a C-terminal domain homologous to the E6-AP C-terminus

(HECT). YAP orthologs in chicken, mouse and human also shared the residues highlighted in Fig. 3A. This pattern of conserved residues is absent from the seven remaining WW domains of the *S. cerevisiae* proteome (Fig. 3B). The peptide ligand preferences of these yeast WW domains are currently under investigation.

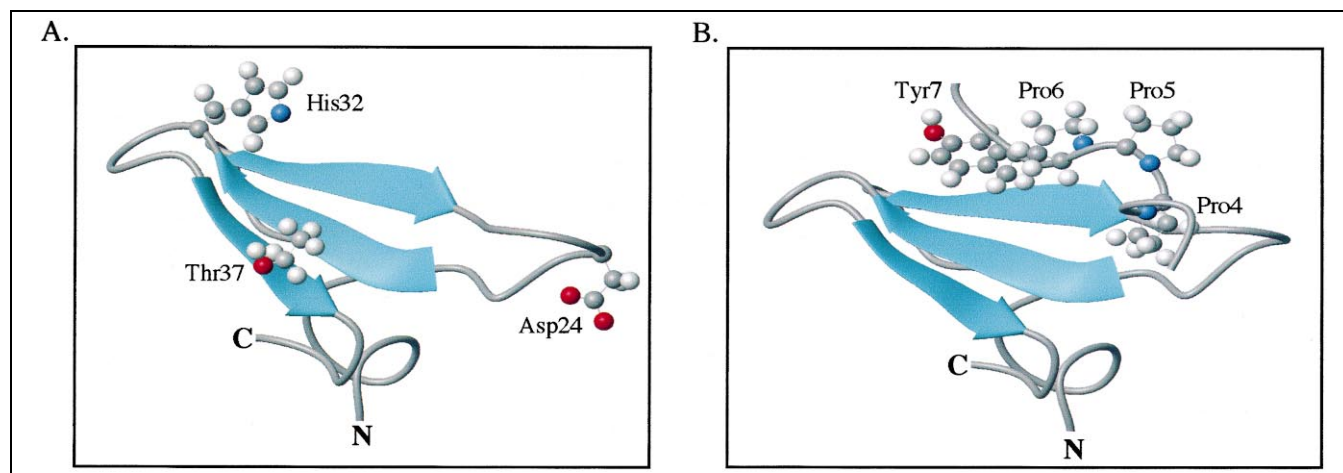


Fig. 4. A three-dimensional model of the mNedd4.3 WW domain highlighting amino acid positions subjected to mutational analysis. The structure of the hYAP WW domain, defined by NMR [7], was used as a reference as its primary structure more closely resembled that of the mNedd4.3 WW domain than other WW domains whose three-dimensional structure has been determined. Within the WW domain, light blue arrows denote  $\beta$ -sheets. The N- and C-termini of the domain are also noted. A: Amino acid positions subjected to mutational analysis are highlighted in ball-and-stick. B: The hYAP WW domain is shown bound to a peptide ligand [7]. The central proline and tyrosine residues are highlighted in ball-and-stick. Atoms are colored as follows: blue: nitrogen, white: hydrogen, gray: carbon and red: oxygen.

### 2.3. WW domain mutagenesis

Based on the alignment of the WW domains in Fig. 3A, we hypothesized that the conserved residues contribute to the preference for PxY peptides. To evaluate this possibility, we tested the functional importance of three residues, D24, H32 and T37, within the mNedd4.3 WW domain. An H32A mutation has previously been shown to destroy binding of the hYAP WW domain [7], so this residue was selected as a control, where substitution would likely effect binding. While T37 has not been previously shown to effect binding, it is absolutely conserved, in contrast to position 24, which varies between the domains (Fig. 3A). The positions of D24, H32 and T37 within a model of the three-dimensional structure of the mNedd4.3 WW domain are highlighted in Fig. 4.

To test the importance of the amino acids at the positions 24, 32, and 37 in the C-terminal WW domain of mNedd4, degenerate codons were introduced separately at each of the three positions and the mutant WW domains were expressed as N-terminal fusions to the capsid protein, pIII, of bacteriophage M13. Recombinant phage particles were then assessed for their ability to bind a specific peptide ligand ( $\text{NH}_2$ -biotin-LKLPDYWESSAS-COOH) which had been immobilized on the surface of microtiter plate wells. The results of the mutagenesis experiments are summarized in Fig. 5. Of the WW domains with random substitutions at the H32 position, only phage with histidine at that position retained binding. Amino acid replacements of another conserved residue, T37, showed similar results: only WW domains with threonine at this position retained binding. This is in contrast to position D24, where all the phage retained at least some degree of binding to the type I ligand, although the rela-

tive binding was reduced for some amino acid substitutions (data not shown). These data suggest that certain positions within Class I WW domains are critically important for interacting with PxY ligands.

### 2.4. Peptides affinity-selected by mutant WW domains

One caveat of the mutational analysis was that some of the amino acid replacements might have prevented proper folding of the domain, rather than alter its ligand specificity. To discern which possibility was correct, several mutant WW domains were expressed as GST fusion proteins

SNDLGPLPPGWEERTHTDGRVFFINHNKKITQWEDPRLQN											
↓			↓			↓					
+	-		+	-		+	-		+	-	
A			H	D		T	A				
D			F			D	E				
E			G			G	H				
I			L			I	K				
K			N			K	L				
M			P			L	N				
N			R			N	P				
P			S			P	R				
R			V			R	S				
S			W			S	V				
T						V	W				
V											

Fig. 5. Amino acid substitutions within the mNedd4.3 WW domain and their effects on binding a PxY containing ligand. The primary structure of the mNedd4.3 WW domain is shown with the substituted positions highlighted in boldface. Amino acid substitutions and their effects on binding are noted, where '+' and '-' refer to residues which, when substituted separately into the WW domain, allow or destroy binding, respectively. Amino acids present in the '+' column bind 15–100% as well as the wild type WW domain, whereas amino acids appearing in the '-' column bind at background levels (i.e., < 15%).

<b>A</b>	
<b>D24E</b>	SVVKAGPPSYEECC DGAQQPPSYYSLF PYGEGDPPSYASLF LTGESAPPDYGTLF KTTPPELPPSYESLF LDAKGGEPTTYEELF
Consensus:	PPSYESLF
<b>T37V</b>	RMDSDRGTRADL VRGLPDPFWHAE
Consensus:	RxxxDxxxxxA-
<b>T37V</b>	GWTGPRDLVDYI HWNQWTLPSDFI GWTGPRDLVDFI EWSGPPPELVWDY
Consensus:	WxGPx-LVDΨI
<b>T37S</b>	TCGYPDPPCPEPLL GCGYPNPCCSGHST TWGYPDPPCPEPLL WAGPDPPPAWQEEW* WGIMDDPPTYWEAV
Consensus:	GYP-PP
<b>H32R</b>	WAGAYSEPPAWHHHW WAGPDPPPAWQEEW*# WAWPDPPPAWQEEW#
Consensus:	WAXxxxPPAWxxxW
<b>B</b>	
<b>mNedd4.3</b>	<b>PPxYESΨΘ</b>
<b>D24E</b>	<b>PPSYESLF</b>
<b>T37V</b>	RxxxDxxxxxA- WxGPx-LVDΨI
<b>T37S</b>	GYPDPP
<b>H32R</b>	WAXxxxPPAWxxxW

Fig. 6. A: The peptide ligand preferences of several amino acid variants of the mouse Nedd4.3 WW domain. Mutant domains used as targets for phage display are noted in boldface by the particular substitution present in that domain, while the alignment of peptides selected by each domain is listed below. Phage displayed either  $x_{12}$  or  $x_6PPx_6$  combinatorial peptides; the two fixed prolines are underlined in the peptides shown. Residues that occur at a frequency of greater than or equal to 50% are shaded and listed in the underlined consensus sequences below each alignment; within the consensus sequences, ‘-’ represents negatively charged residues while ‘Ψ’ represents hydrophobic residues. An ‘\*’ denotes two peptides with the same sequence. In peptides selected by the H32R mutant, the ‘#’ sign denotes two sequences that are too similar to count as separate sequences, since they are identical except for one residue and there are only three total sequences within the alignment. T37V selected two populations of peptides as revealed by two distinct motifs. B: Consensus sequences derived from peptides selected by mutant WW domains. The consensus sequences representing preferred residues selected by the mutant forms of the mNedd4.3 WW domain are shown. In addition, the consensus sequence derived from wild type mNedd4.3 is present as a comparison, where the residues which are identical or similar between the wild type- and D24E-derived sequences are highlighted in boldface. Ψ, Θ and - represent hydrophobic, aromatic and negatively charged residues, respectively.

and their binding preferences were assessed with combinatorial peptides. Peptides affinity-selected by four mutant WW domains (D24E, T37V, T37S and H32R) have been aligned in Fig. 6A. Mutant WW domains containing T37V, T37S or H32R amino acid replacements demonstrated novel peptide ligand preferences. The T37V mutant domain selected one predominant (WxGPx-LVDΨI) and one minor motif (RxxxDxxxxxA-) from the  $x_{12}$  library, where - and Ψ represent negatively charged and hydrophobic residues, respectively. The T37S mutant domain only selected peptides from the  $x_6PPx_6$  library, preferring a GYP-PP motif. Two of these peptides are likely cyclized due to a disulfide bond between two cysteine residues and thus adopt a conformationally constrained structure [35]. Interestingly, the H32R mutant domain selected peptides with the motif PPAW, which is similar, yet distinct from, the original PPxY. Conversely, the D24E mutant WW domain selected peptides which all contained the PxY core motif. Furthermore, the consensus sequence derived from the D24E-selected peptides is nearly identical to the consensus selected by the wild type WW domain, Nedd4.3 (Fig. 6B). Thus, these substitutions allowed proper folding of the WW domain, but imparted new ligand specificities, while substitution at a non-conserved position had little effect on the original ligand preference of the domain.

### 3. Discussion

Through affinity selection experiments with bacteriophage M13 displaying combinatorial peptides, we have defined the peptide ligand specificity of 13 different WW domains from one mouse and four human proteins. All 13 domains selected peptides containing the motif (L/P)PxY and can thus be classified as Class I WW domains, in contrast to Class II, III, IV and V WW domains which select PPLP [36–38] PxxGMxPP [39], (phospho-S/T)P [40] and RxPPGPPPxR [41] containing peptide ligands, respectively.

Computer searches of the Swiss-Prot online protein database with the peptide ligand motifs selected by the 13 WW domains characterized in this study have suggested a number of potential interacting proteins. For example, a search with the optimal ligand sequence for the Nedd4.3 WW domain yielded a match with a sequence present in the cytoplasmic C-terminus of ENaC [23]; a number of experiments have confirmed that this interaction is physiologically significant [11,12]. In fact, mutations in the PPPY sequence at the C-terminus of any of the three subunits of the ENaC leads to Liddle’s syndrome, a form of hypertension [42,43]. Examples of such correspondence between the primary structure of phage display-derived peptide ligands and naturally occurring ligands have been referred to as ‘convergent evolution’ [23].

Interestingly, among the predicted interacting proteins



were some of the WWP's themselves. The WW domains of WWP1.1, WWP1.4, WWP2.4 and Nedd4.2 all selected the same peptide sequence, LKLPDY, of which the consensus LxLPxY is present within the HECT domains present in these proteins. This motif is adjacent to a conserved catalytic cysteine residue in the HECT domain that functions to transfer ubiquitin to a protein substrate during ubiquitin ligation. Furthermore, the peptide ligand sequence, LKLPDY, occurs within the HECT domains of the *S. cerevisiae* protein YGO1 (GenBank accession number P53119) and human TR12 (Q14669). These observations suggest that some HECT domains may bind in an intramolecular fashion to WW domains within the same protein and serve to regulate the catalytic activity of the domain through steric hindrance. To test this notion, peptides corresponding to the sequence flanking the catalytic cysteine were synthesized and tested for binding; these peptides bound quite well, whereas peptides synthesized with a Tyr to Ala replacement did not (data not shown). Thus, E3 ubiquitin ligases [44], which contain both WW and HECT domains, may adopt a 'closed', inactive state due to intramolecular interactions, much like that observed for the Tek and Src tyrosine kinases [45,46].

Recently, a common mechanism whereby WW and SH3 domains select proline containing peptide ligands was elucidated [47–49]. Conserved aromatic residues form one or two grooves, termed 'X–P' grooves, on the surfaces of the WW and SH3 domains, respectively, which accommodate ligand dipeptides consisting of one random, C $\alpha$ -substituted residue and one proline, an N-substituted residue. Thus, in Class I ligands any residue may appear N-terminal of the PxY core, as part of the dipeptide accommodated in the X–P groove on the WW domain surface. In our study, either leucine or proline is the preferred residue that flanks the PxY core on its N-terminus. The WW domains of Rsp5 and hYAP bind (L/P)PxY containing ligands as well [30,50]. However, the central WW domain of Rsp5 can also interact with the C-terminal domain of the RNA polymerase II large subunit via a repeated SPSY sequence. Thus, the peptide ligand preference of certain Class I WW domains may be broader than (L/P)PxY.

In comparing the primary structures of the 13 WW domains that selected PxY peptides, we noted that they shared a number of additional residues. Of these conserved residues, we have confirmed in mutagenesis experiments the importance of H32 within the domain in binding PxY-type peptide ligands [7]. We have gone on to demonstrate that T37 within the WW domain is also essential for PxY ligand recognition, in contrast to D24, which is not present at the domain–ligand interface and which can be replaced with other amino acids with little to no effect on binding. This suggests that there are a set of conserved positions within Class I WW domains that serve to direct binding to PxY containing ligands.

Recent three-dimensional structural studies of WW do-

main complexes with peptide ligands provide insight into the role of many of the residues we have identified to be conserved amongst Class I WW domains. Analysis of the complex between the dystrophin WW domain and a peptide segment of  $\beta$ -dystroglycan has revealed that amino acids I30, H32 and Q35 of the WW domain form a pocket in which the tyrosine residue of the PxY peptide ligand lies. Additionally, residues Y28, T37, and W39 contact the two prolines of the peptide ligand. Amino acids occurring at position 30 typically include valine, leucine or isoleucine in Class I WW domains. In Class II WW domains, which bind PPLP peptide ligands, this residue is commonly tyrosine or phenylalanine. The importance of this position is underscored by the observation that when the leucine at position 30 in the WW domain of the hYAP protein is replaced with a tyrosine the ligand specificity of the WW domain changed from Class I to Class II [51]. While the three-dimensional structure of a Class II domain remains to be determined, structural studies of a Class IV domain from PIN1 have revealed that the tryptophan side chain at position 30 faces the proposed ligand interface [52]. Thus, the type of residue at position 30 contributes significantly toward specifying its ligand preference.

Some of the residues conserved among Class I WW domains are likely conserved because they play a role in maintaining the tertiary structure of the domain. For example, P42, which is conserved in all WW known to date, makes no direct contact with the ligand and when it is replaced by a leucine in the WW domain of FBP28 it causes unfolding of the domain [53]. Similarly, residue P14, which is absolutely conserved in the alignment of Class I domains (Fig. 3A) has been observed to form a structurally important 'hydrophobic buckle' with P42 on the backside of the hYAP WW domain [7]. In addition, E18, a position absolutely conserved in Class I WW domains (Fig. 3A), was shown in structural studies to hydrogen bond to H32, thereby promoting the interaction of the histidine with the hydroxyl group of the tyrosine in the peptide ligand [48].

Although the dystrophin WW domain can bind a PxY ligand present in  $\beta$ -dystroglycan [48], this WW domain does not exactly fit the consensus defined by other Class I WW domains, instead containing L13V, P14Q and G16P substitutions. It is interesting to note that the dystrophin WW domain requires the structural stability supplied by adjacent EF hand-like regions to fold and function properly [48]. We predict that replacement of these three residues with those conserved among Class I WW domains would restore the ability of the dystrophin WW domain to fold and function independently.

Data involving other protein interaction domains also support the usefulness of functional primary structure alignments. Alignment of the primary structures of PH domains, which bind phosphoinositides, was useful in identifying additional PH domains with similar ligand preferences [54]. In another example, two destabilizing



mutations in the Nck SH3 domain were found to be conserved in nearly all SH3 domains [55]. In another case, alignment of SH3 domains was found to more accurately predict the significance of amino acid substitutions in the Fyn SH3 domain than a crystal structure of the same domain [56]. Furthermore, a conserved aspartic acid residue in the Src SH3 domain was found to be important in binding the arginine of Class I SH3 peptide ligands [57]. In SH2 domains, Group I domains are distinguished from Group III domains by the presence of a bulky hydrophobic residue at position  $\beta$ D5 [58]. Substitution of a Tyr at this position in a Group III SH2 domain altered its ligand preference to that of Group I SH2 domains [59]. SH2 domains also contain variable regions which influence specificity. In one case, a single substitution altered the specificity from that of the Src SH2 domain to the Grb2 SH2 domain [60].

Thus, it appears that it may be possible to discern a molecular recognition code for protein interaction modules based on their primary structures [24]. It should also be possible, as demonstrated in the mutagenesis experiments with the Nedd4.3 WW domain, to design new optimal ligand preferences. Similar experiments are ongoing with the generation of designer zinc fingers proteins that can bind specific DNA sequences [61–63].

## 4. Significance

Protein–protein interactions studies are a critical part of research in the signal transduction field. Screening phage-displayed peptide libraries has proven quite effective for delineating the optimal ligand preferences of many protein interaction modules and predicting potential protein–protein interactions. Alignment of peptides selected by 13 different WW domains demonstrated that each WW domain selected Class I peptide ligands, defined by a conserved PxY core motif. Comparison of the primary structures of these WW domains revealed that they shared a number of residues that likely contribute to the preference of Class I ligands. Mutation of two absolutely conserved residues within the prototypic mNedd4.3 WW domain abrogated binding to a PxY ligand, while mutation of a non-conserved residue did not. These substitutions did not prohibit proper folding of the domain, but instead altered the specificity of the domains. It may be possible to exploit WW domains as versatile affinity reagents and develop unique WW domain–ligand pairs.

## 5. Materials and methods

### 5.1. GST fusions of WW domains

Oligonucleotides were designed to flank individual WW domains from the following proteins: mNedd4 (GenBank accession

number L11119), WWP1 (U96113), WWP2 (U96114) and WWP3 (U96115). Upstream primers contained a *Bam*HI cleavage site and the downstream primers contained an *Eco*RI cleavage site. After estimating the size of the PCR products on a 2.5% Meta-phor agarose gel, (FMC, Rockland, ME, USA), the remaining material was digested with both restriction enzymes and ligated into the pGEX-2T vector (Pharmacia, Piscataway, NJ, USA). DH5 $\alpha$ F' *Escherichia coli* cells were transformed by electroporation and the resulting GST fusion proteins were overexpressed and purified as described [64]. A GST fusion to the WW domain of hYAP was derived in the same manner from a template kindly supplied by Dr. Marius Sudol (Mt. Sinai Medical Center, New York, NY, USA).

### 5.2. Screening of phage-display combinatorial peptide libraries

Two libraries displaying  $x_{12}$  or  $x_6$ PP $x_6$  or combinatorial peptides, with a complexities of  $\sim 10^9$  recombinant clones, were screened by three rounds of affinity selection with GST fusion proteins immobilized on the surface of a 96 well microtiter plate (Costar, Cambridge, MA, USA). After the third round of affinity selection, phage were plated out on a lawn of *E. coli* DH5 $\alpha$ F' to yield individual plaques. The binding of phage isolates to the GST fusion proteins was verified by ELISA, and the nucleotide sequences of the positive phage were determined at the University of Wisconsin Biotechnology Center sequencing facility.

### 5.3. Construction of phage-displayed, mutant WW domain libraries

Using primers designed to flank the C-terminal WW domain from mNedd4, we amplified the WW domain by PCR and cloned the resulting fragment into the M13 bacteriophage vector as a fusion to the N-terminus of mature protein pIII. The recombinant clone was confirmed by sequencing. To introduce point mutations into this WW domain, forward and reverse mutagenic primers were synthesized, each complementary to the WW domain except for a single NN(G/T) codon at the position to be mutated. Two different polymerase chain reactions were performed, each using one mutational primer described above and another primer adjacent to the inserted DNA. The two amplified products were purified, annealed and extended using *Pfu* DNA polymerase I (Promega, Madison, WI, USA). The extended DNA fragment was digested with *Xba*I and *Xho*I, ligated into a bacteriophage M13 vector, and introduced into *E. coli* JS5 cells by electroporation.

### 5.4. ELISA testing phage displayed WW domains

Streptavidin (100 ng/well in 100 mM sodium bicarbonate, pH 8.3) were incubated in each well of a microtiter plate for 45 min at room temperature and blocked for 1 h with phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 4 mM Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>). After washing the wells three times with PBS containing 0.1% Tween 20, 10 nmol of biotinylated peptide ligand (biotin-LKLPDYWESSAS; Research Genetics, Huntsville, AL, USA) were incubated in the wells for 1 h at

room temperature. Wells were washed again and the binding of phage displaying the individual mutant WW domain domains was assessed by ELISA.

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