Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4

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Nedd4 is a member of a growing family of ubiquitin-protein ligases which consist of a lipid-binding domain, two to four WW domains and a C-terminal ubiquitin-protein ligase domain. The Nedd4 mRNA levels are developmentally regulated and Nedd4 protein is highly expressed in many mouse embryonic tissues. In this study we have used a far-Western screen to identify embryonic proteins that interact with the WW domains in mouse Nedd4. We report here identification of eight Nedd4 WW-domain-interacting proteins from mouse embryonic cDNA expression libraries. Two of the proteins are novel, while two have been identified previously as ligands for a WW domain. All of these proteins contain one or more PY motifs. In seven of the eight proteins, these PY motifs are necessary for their interaction with the WW domains of Nedd4. Using site-directed mutagenesis, and by using individual WW domains of Nedd4 as probes for far-Western analysis, we show that the three WW domains in Nedd4 interact with varying affinities with the PY motifs present in various Nedd4-binding proteins. These results provide evidence that Nedd4 can potentially interact with multiple proteins, possibly simultaneously, through its WW domains.

Key words: embryonic development, far-Western analysis, protein–protein interaction, PY motif, ubiquitination.

INTRODUCTION

The *Nedd*4 gene was initially identified and cloned as a developmentally regulated gene in the mouse [1]. In recent years, several Nedd4-like proteins have been discovered and predicted to act as specific ubiquitin-protein ligases (reviewed in [2]). All of the Nedd4 family of proteins have a distinct modular structure consisting of an N-terminal calcium/lipid-binding domain, two to four WW domains and a C-terminal hect type of ubiquitin-protein ligase domain [2]. Hect (homologous to E6-AP C-terminus, where E6-AP is E6-associated protein) is a large domain ($\approx$ 350 residues) that was first characterized in E6-AP, a protein required for human papilloma virus E6-mediated degradation of p53 [3,4]. Ubiquitin-protein ligases comprise the substrate-specificity arm of the ubiquitin pathway [5]. Initially, ubiquitin is activated by a ubiquitin-activating enzyme (E1), transferred to a ubiquitin-conjugating enzyme (E2) and then linked to a substrate protein either directly or via a ubiquitin-protein ligase (E3). Along with several other types of ubiquitin-protein ligase, the hect-domain proteins are a major subclass of E3s [5].

The WW domain is a stretch of $\approx$ 35 amino acids with two conserved Trp residues (WW) and a conserved Pro residue [6]. This domain consists of a hydrophobic core surrounded by β-sheets containing a number of charged residues and has a preference for binding small proline-rich sequences, called PY motifs, the most common of which is PPXY [6,7]. However, WW domains from some proteins can bind alternative proline-rich motifs, e.g. the WW domains of formin-binding proteins have a preference for PPLP [8] or PPR [9] sequences. In addition, a recent report suggests that some WW domains, including those in Nedd4, can bind phosphoserine and phosphothreonine residues [10], suggesting that these domains can also potentially mediate interaction between a large repertoire of phosphoproteins. Different WW domains from the same protein possess differential substrate specificity *in vitro* [11,12]. Therefore, it is plausible that each of the Nedd4-like proteins interacts with a number of different proteins via their WW domains *in vivo*.

One known target of Nedd4 is the epithelial sodium channel (ENaC). Nedd4 binds to the proline-rich PY motifs present in ENaC subunits through its WW domains [13,14]. Mutations in the short proline-rich segment present in the C-terminus of β- and γ-subunits of ENaC lead to constitutively increased channel activity characteristic of an autosomal dominant form of hypertension called Liddle's syndrome [13,15]. Specifically, these mutations delete or alter PY motifs in the β or γ ENaC subunits that are required for interaction with Nedd4, which suggested that Nedd4 regulates the activity or abundance of ENaC [13,15]. Subsequently, we and others have shown that Nedd4 indeed mediates the ubiquitin-dependent down-regulation of ENaC [16–19].

The number of proteins, particularly membrane proteins, believed to be regulated by ubiquitination is constantly increasing [20]. Ubiquitin-stimulated endocytosis appears to be an important means of switching off signalling pathways after they have been initiated by receptor–ligand interaction. The unique domain structure of the Nedd4 family members makes them strong candidates for regulating the ubiquitin-mediated turnover of many membrane proteins. Only one membrane protein, ENaC, has been shown to be regulated by Nedd4 to date. Although Nedd4 and two Nedd4-like proteins, AIP4 (atrophin-1-interacting protein 4) and WWP2 (WW domain protein 2)/AIP2, have recently been shown to interact with the latent membrane protein 2A (LMP2A) of Epstein–Barr virus, it is as yet not clear whether Nedd4 regulates the turnover of LMP2A in Epstein–Barr-virus-infected cells [21]. We postulate that Nedd4 regulates several proteins other than ENaC by ubiquitin-mediated modification, some of which are likely to be membrane proteins. Since Nedd4 mRNA is highly expressed in mouse embryos [22], some of the Nedd4 targets may be involved in development. To identify such

Abbreviations used: ENaC, epithelial sodium channel; GST, glutathione S-transferase; PRGP-2, proline-rich γ-carboxyglutamic acid protein 2; WW1, 2, 3, WW domain 1, 2 or 3; PY1, 2, 3, PY motif 1, 2 or 3.

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proteins, we carried out far-Western screens on mouse embryonic cDNA expression libraries using Nedd4 WW domains as a probe. In the present paper we describe the identification of a number of proteins that interact directly with the WW domains of mouse Nedd4 and demonstrate that specific PY motifs in these proteins mediate their interaction with one or more WW domains in Nedd4.

MATERIALS AND METHODS

Immunohistochemistry

Formalin-fixed mouse embryos were sectioned and prepared for immunohistochemistry as described previously [23,24]. Sections were incubated sequentially with affinity purified anti-Nedd4 polyclonal antibody N4Ab1 [22], followed by biotinylated anti-rabbit IgG and ABC reagent (both from Vector Laboratories) according to the manufacturer's protocol. The specific immune complexes were detected by using either the AEC (3-amino-9-ethylcarbazole) or DAB (3,3′-diaminobenzidine) substrate kits (Vector Laboratories). Sections were counterstained with haematoxylin, mounted under coverslips and photographed.

Screening phage-expression libraries by a far-Western method

Two mouse cDNA expression libraries, derived from day-12 and day-16 total embryos, in λExLox® vector (Novagen) were screened using the three WW domains of Nedd4 fused to glutathione S-transferase (GST) as a probe. From each library ≈ 400000 pfu were plated on to 20 15-cm-diameter plates and when the plaques were visible the plates were overlayed with nylon membrane (NEN) and incubated at 37 °C for 5 h. Duplicate plaque lifts were prepared in a similar way. Filters were air dried, blocked for 1 h in Blotto (25 mM NaCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4, 0.05 % Nonidet P40, 5 % skimmed milk powder and 1 mM dithiothreitol), washed once in Hyb75 (75 mM KCl, 2.5 mM $MgCl_2$, 20 mM Hepes, pH 7.4, 0.1 mM EDTA, 0.05 % Nonidet P40, 1 % skimmed milk powder and 1 mM dithiothreitol) [25], and then hybridized with ^{32}P-labelled WW-GST protein probe (see below) for 15 h at 4 °C in Hyb75. Membranes were washed three times in Hyb75 for a total of 30 min, air dried and then exposed to X-ray film. Plaques that bound ^{32}P-labelled WW-GST protein probe on both duplicate filters were excised and phage eluted according to the protocol provided by Novagen. The eluted phage were plated, plaque lifts probed with ^{32}P-labelled WW-GST protein probe (see below) and positive plaques excised to elute phage as described above. This process was repeated a third time to obtain stocks of pure phage. Plasmids containing cDNA were rescued from the λExLox clones using the Novagen autosubcloning procedure. All cDNA clones in pExLox were sequenced from both ends using appropriate primers. Partial sequences of various Nedd4 WW-domain-binding proteins have been deposited in the GenBank database (see below for accession numbers). Mutation of PPXY motifs in the identified proteins was performed using the QuikChange™ site-directed mutagenesis protocol (Stratagene) to change the Tyr residue to an Ala residue. All wild-type and mutant cDNA constructs were induced in *Escherichia coli* BL21(DE3) cells using the protocol described below.

Expression plasmids and production of GST-fusion proteins

The expression construct used to generate protein containing all three WW domains of mouse Nedd4 fused to GST has been described previously [16]. Single WW-domain-GST constructs were generated by amplifying each WW domain individually by PCR using appropriate primers followed by cloning into the *Bam*HI/*Eco*RI sites of pGEX-2TK (Amersham Pharmacia Biotech) [14]. WW-domain mutations were produced by the QuikChange™ method, PCR amplified and cloned into the *Bam*HI/*Eco*RI sites of pGEX-2TK [14]. A plasmid for expression of Bean (brain expressed, associated with Nedd4) as a GST-fusion protein was constructed by subcloning the wild-type or the PY-mutant Bean cDNA from pExLox into pGEX-2TK. Overnight cultures of *E. coli* DH5α harbouring the appropriate GST expression plasmid were diluted 1/50, grown for 2 h at 37 °C, induced with 1 mM isopropyl β-D-thiogalactoside and grown for an additional 5 h at 37 °C. Bacterial cell pellets were resuspended in PBS, lysed by sonication and clarified by centrifugation at 10000 ***g*** for 10 min. Glutathione–Sepharose (Amersham Pharmacia Biotech) was incubated with the cleared lysate for 60 min at room temperature, and then the beads were washed three times with PBS. Fusion protein was eluted with glutathione buffer according to the manufacturer's protocol. Protein concentration was measured using a BCA kit (Pierce).

Expression of T7 gene 10-fusion proteins

E. coli BL21(DE3) cells were transformed with various plasmids rescued from the λExLox vectors. Cultures grown to A_{600} of ≈ 1 were split 1/25 in fresh medium, grown for ≈ 3 h at 37 °C, induced with 1 mM isopropyl β-D-thiogalactoside and grown for an additional 3 h at 37 °C. Cell lysates were prepared as above for GST-fusion proteins and expression of T7 gene 10-fusion proteins was analysed by SDS/PAGE and Coomassie Brilliant Blue staining. Since various proteins were expressed at vastly different levels in *E. coli*, different amounts of bacterial extracts were used in far-Western assays (see below) to normalize the concentrations of fusion proteins.

SDS/PAGE and far-Western analysis

^{32}P-Labelled protein probes were produced by direct labelling of the appropriate GST-fusion proteins using protein kinase A (New England Biolabs). Glutathione beads containing bound fusion protein were incubated with protein kinase A and [γ-^{32}P]ATP in a buffer containing 20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 12 mM $MgCl_2$ and 1 mM dithiothreitol for 60 min at 4 °C. Beads were washed five times in PBS and labelled protein was eluted with glutathione buffer. Each purified GST-fusion protein (≈ 2 μg) or 5–10 μg of *E. coli* lysates containing various WW-domain-binding proteins and corresponding PPXY mutants were resolved on SDS/polyacrylamide gels and transferred to nitrocellulose membrane (Schleicher and Schüll). Membranes were blocked in Blotto for 1 h and then hybridized with ^{32}P-labelled WW-GST protein probes for 4 h at 4 °C in Hyb75. Membranes were washed three times in Hyb75 and then exposed to X-ray film.

Accession numbers

The GenBank accession numbers of various WW-domain-binding proteins are as follows: Bean, AF240460; N4WBP1, AF220205; N4WBP2, AF220206; N4WBP3, AF220207; N4WBP4, AF220208 and N4WBP5, AF220209.

RESULTS

Nedd4 protein is highly expressed in mouse embryos

We have shown previously that Nedd4 transcript is highly expressed in many mouse embryonic tissues at various stages of development [22]. In the brain, Nedd4 mRNA expression is down-regulated in the later stages of embryogenesis and in the

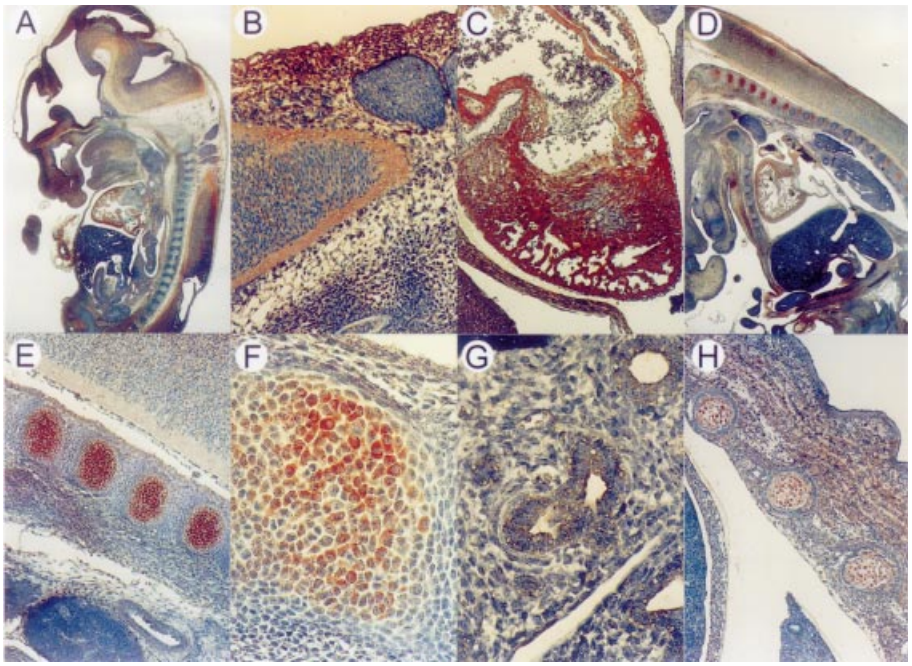

Figure 1 Expression of Nedd4 protein in mouse embryos

Affinity purified Nedd4 antibody was used to stain sagittal sections of mouse embryos. Nedd4 protein is stained red/brown and haematoxylin-stained nuclei appear blue. (**A**) A day-12 embryo showing widespread Nedd4 expression. Particularly strong expression is seen in the central nervous system and heart. (**B**) A day-11 embryo showing high Nedd4 immunoreactivity in regions of the central nervous system and skin. (**C**) An enlarged view of day-12 embryonic heart. (**D**) A part of the day-14 embryo showing high Nedd4 expression in chondrocytes and heart. (**E**, **F**) Higher magnifications of the day-14 embryo in (**D**) showing strong expression of Nedd4 protein in chondrocytes. (**G**) Higher magnification of day-14 lung showing Nedd4 expression in epithelial cells lining the ducts. (**H**) A section of the day-16 embryo showing expression of Nedd4 in skin and underlying skeletal-muscle cells.

adult animal [1,22]. To check whether Nedd4 protein is also widely expressed in mouse embryos, we used an affinity purified rabbit polyclonal antibody raised against recombinant Nedd4 protein [22] to stain mouse embryos at various stages of development. Nedd4 reactivity was evident in many embryonic tissues at various ages (Figure 1, and results not shown). The expression of Nedd4 protein was particularly high in day-11 and -12 central nervous system and heart (Figures 1A–1C). In day-14 embryos, high levels of Nedd4 immunoreactivity were noted in chondrocytes (Figures 1D–1F), in addition to expression in heart and central nervous system. At this stage, Nedd4 protein was also expressed in lung epithelial cells lining the ducts (Figure 1G). In day-16 embryos, expression of Nedd4 was particularly evident in skin and skeletal-muscle cells. In later stages of development, Nedd4 expression was somewhat lower, but more widespread (results not shown).

Identification of proteins that interact with the WW domains in mouse Nedd4

Since Nedd4 expression was high in embryos, we postulated that Nedd4 may target proteins that are expressed during development. Therefore, we screened two mouse cDNA expression libraries derived from day-12 and -16 embryonic mRNA using the three WW domains of mouse Nedd4 as a probe by a far-Western approach. After three cycles of screening we identified a total of eight unique proteins that interact with the three WW domains of Nedd4 (Table 1 and Figure 2). From the day-12 library, we isolated three independent clones. Sequencing of these clones revealed that two, containing identical cDNA inserts of 1.4 kb, were derived from the same novel mRNA that encoded a putative protein with two PY motifs. Because of its restricted expression in brain (B. P. Haines and S. Kumar, unpublished work), we have named this protein Bean (described in the Materials and methods section). The third clone contained a partial cDNA for WBP-2 encoding the three PY motifs (see below).

From the day-16 library, we identified 22 clones that interacted with the three WW domains of Nedd4 (Table 1). These clones belonged to seven unique genes. Two of these, WBP-1 and WBP-2, were previously identified as ligands for the WW domain of the Yes-associated protein (YAP) [26]. Interestingly, of the 22 clones, six represented WBP-2, whereas only one represented WBP-1 (Table 1). The WBP-1 clone had an insert of ≈ 0.7 kb corresponding to the 3′ end of the murine WBP-1. This region encodes both the PY motifs that are present in this protein. Three of the WBP-2 clones (≈ 1.6 kb) contained the entire coding region, while the other three contained coding region from the C-terminus. All clones contained coding region for the three PY motifs of WBP-2.

One clone, named N4WBP1, contained an insert of ≈ 0.9 kb. TBLASTN searches showed that this sequence represented a partial sequence of the murine homologue of the human proline-rich γ-carboxyglutamic acid protein 2 (PRGP-2) [27]. Both murine and human counterparts of this protein contain a single conserved PY motif towards their C-termini. Another protein interacting with the Nedd4 WW domain, N4WBP2, was represented by two clones, both of which contained a cDNA insert of ≈ 0.8 kb, corresponding to the entire coding region of Rnf11. Rnf11 is a 154-amino-acid putative RING-finger protein of unknown function [28]. RING fingers are found in a diverse group of proteins and are shown to function as ubiquitin-ligases

Table 1 A summary of the various clones identified by far-Western screening of day-12 (E12) and day-16 (E16) murine embryo libraries using Nedd4 WW domains as a probe

The number of representative clones for each protein isolated from day-12 and -16 cDNA libraries, the length of the cDNA, sequences of various PY motifs and known homologues, if any, are shown. Some proteins contain more than one PY motif. These have been numbered (e.g. PY1–PY3) from the N- to C-termini of the protein. Partial nucleotide sequences for N4WBP1, N4WBP2, N4WBP3, N4WBP4, N4WBP5 and Bean can be obtained from the GenBank database (see the Materials and methods section). References or accession numbers for various proteins that show homology to WW-domain-binding proteins are cited in the text.

Protein	No. of clones (library)	cDNA insert	PY motif(s)	Homology
Bean	2 (E12)	1.4 kb	PPGY (PY1) PPPY (PY2)	None
WBP-1	1 (E16)	0.7 kb	PPAY (PY1) PPPY (PY2)	Murine WW-domain-binding protein 1 (WBP-1)
WBP-2	1 (E12) 6 (E16)	0.7 kb 1.6 kb	PPGY (PY1) PPPY (PY2) PPPY (PY3)	Murine WW-domain-binding protein 2 (WBP-2)
N4WBP1	1 (E16)	0.9 kb	PPPY	Human proline-rich γ-carboxyglutamic acid protein 2 (PRGP-2)
N4WBP2	2 (E16)	0.8 kb	PPPY	Murine RING-finger protein (Rnf11)
N4WBP3	1 (E16)	1.1 kb	PPTY (PY1) PPSY (PY2)	Rat oestrogen-responsive mRNA, human LITAF/PIG7
N4WBP4	1 (E16)	0.7 kb	PPPY (PY1) PPTY (PY2)	88% with human chromosome-20 protein (acc. no. CAB55862), 65% with human putative protein C18orf1
	2 (E16)	0.25 kb	PPTY (PY2)	
N4WBP5	8 (E16)	1.8 kb	PPPY (PY1) PPSY (PY2)	None

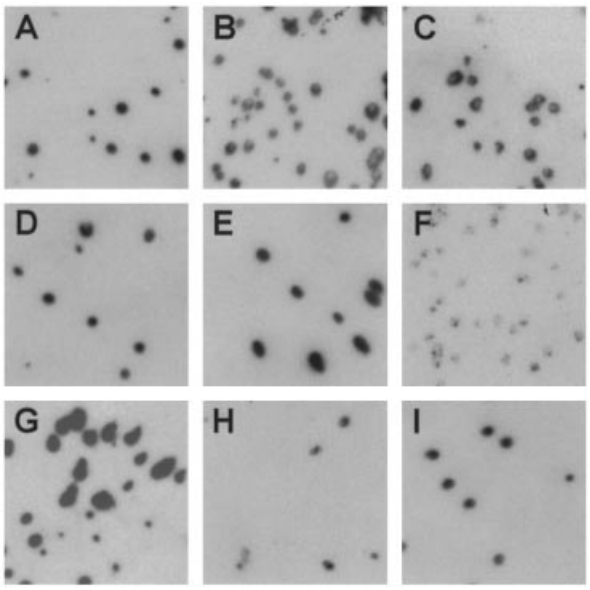

Figure 2 Tertiary screen filters showing that murine Nedd4 WW domains bind specifically to plaques expressing proteins later identified to contain PPXY motifs

(**A**) N4WBP1; (**B**) N4WBP2; (**C**) N4WBP3; (**D**) N4WBP4; (**E**) clone containing the C-terminal region of N4WBP4 with one PY motif; (**F**) N4WBP5; (**G**) Bean; (**H**) WBP-1; (**I**) WBP-2.

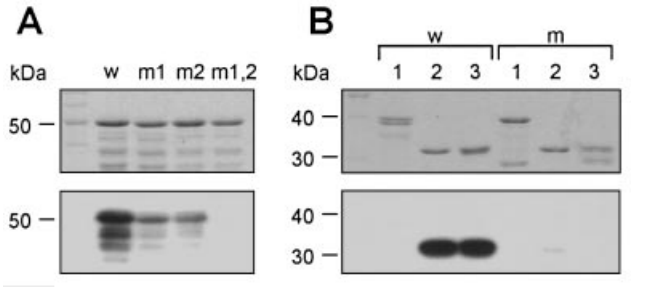

Figure 3 Bean and Nedd4 interaction is mediated via the two PY motifs present in Bean

(**A**) Wild-type GST-Bean (w) or GST-Bean fusion proteins carrying mutations of PY1 (m1), PY2 (m2) or both PY motifs (m1,2) were immobilized on nitrocellulose filters and hybridized to the ^{32}P-labelled Nedd4 WW domains. (**B**) GST-fusion proteins carrying individual WW domains 1–3, in either wild-type (w) or mutant (m) configuration, were hybridized with ^{32}P-labelled GST-Bean protein. In both (**A**) and (**B**) the upper panels show Coomassie Brilliant Blue-stained gels, whereas the lower panels show corresponding far-Western blots. The left-most lanes in (**A**) and (**B**) contain protein-size markers.

in some cases [5]. There is a single PY motif in the N-terminal region of Rnf11, which is conserved in both mouse and human orthologues [28].

A single clone was isolated for N4WBP3 that contained a cDNA insert of $\approx$ 1.1 kb. The N4WBP3 protein was 96% identical to the putative product of a rat oestrogen-responsive uterine mRNA [29], and 81% identical to human LITAF/PIG7, a lipopolysaccharide- and p53-induced transcription factor that regulates tumour necrosis factor α gene expression [30,31]. N4WBP3 contains two PY motifs in its N-terminal half. Interestingly, both of these PY motifs are conserved in the rat and human proteins. N4WBP4 was represented by three clones, two of which had small inserts of 0.25 kb that were identical to the 3′ end of the larger clone of 0.7 kb. N4WBP4 shares $\approx$ 88% identity with a putative human chromosome-20 protein (accession no. CAB55862) and $\approx$ 65% identity with a chromosome-18-specific putative protein, C18orf1 [32]. N4WBP4 contains two PY motifs, which are conserved in both CAB55862 and C18orf1. N4WBP5 was represented by eight of the 22 clones isolated from the day-16 library. The longest cDNA clone for N4WBP5 contained an insert of $\approx$ 1.8 kb. N4WBP5 does not share any significant homology with the known proteins in the database,

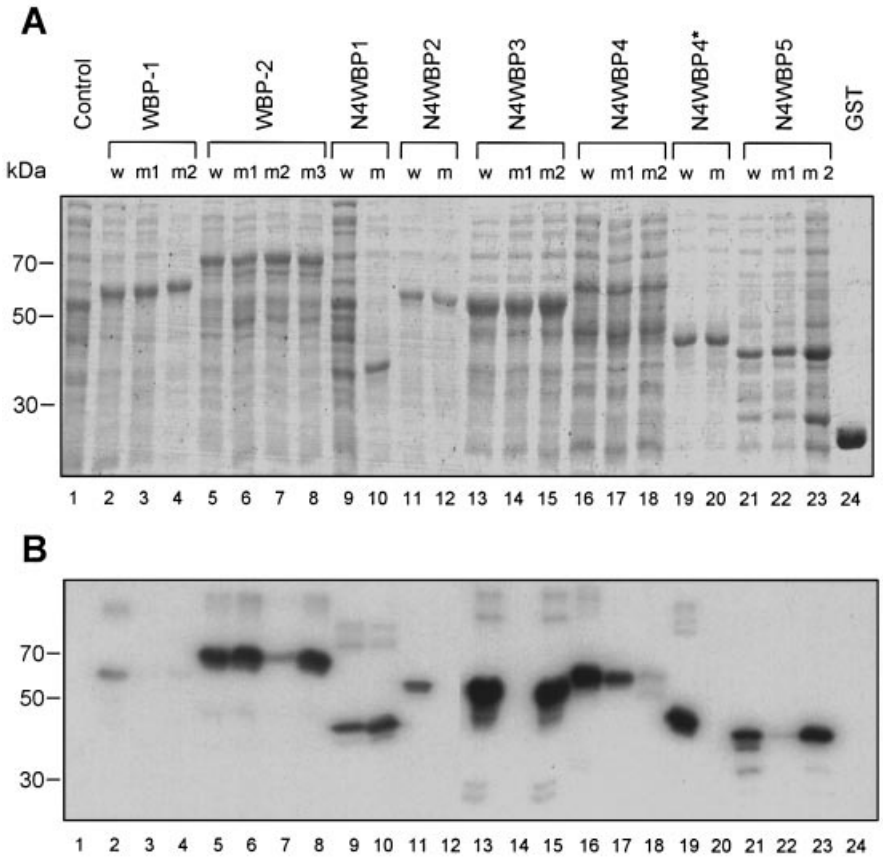

Figure 4 The three WW domains of Nedd4 interact with various proteins

E. coli BL21(DE3) lysates containing approximately equal amounts of T7 gene 10-fusion proteins were electrophoresed on SDS/polyacrylamide gels, transferred to nitrocellulose membranes and probed with ^{32}P-labelled GST-fusion protein containing all three WW domains of mouse Nedd4. For each WW-domain-interacting protein, we electrophoresed the wild-type (w) and mutant (m) proteins carrying a single mutated PY motif. For proteins that contain more than one PY motif, the mutant PY motif is indicated (m1, m2 or m3). (**A**) Coomassie Brilliant Blue-stained gel of fusion proteins. (**B**) A far-Western blot hybridized with Nedd4 WW domains. The exposure time for this blot was 45 min. Lanes 1 (control) contain extract from untransformed *E. coli* BL21(DE3). Lanes 1–24 in (**A**) correspond to lanes 1–24 in (**B**).

although ESTs from several species corresponding to N4WBP5 are present in GenBank. The conceptual translation of the N4WBP5 cDNA revealed two PY motifs, located towards the putative N-terminus of the protein.

Several of the Nedd4 WW-domain-binding proteins contain putative transmembrane regions

Since Nedd4 is likely to target membrane proteins, we analysed the available protein sequences for the proteins in Table 1 for possible membrane-spanning regions by Kyte Doolittle hydropathy analysis. The human homologue of N4WBP1, PRGP-2, has been predicted previously to contain a single transmembrane region [28]. This region is more than 95% identical in mouse N4WBP1. N4WBP4, N4WBP5 and Bean also contained putative transmembrane regions (results not shown). N4WBP2, N4WBP3, WBP-1 and WBP-2 did not contain any possible membrane-spanning regions.

WW domains in Nedd4 interact with the PY motifs in various Nedd4 WW-domain-binding proteins

All proteins identified in the far-Western library screens contained at least one PY motif (Table 1), suggesting that the interaction between Nedd4 and the binding partners may be mediated by WW domain/PY motif contacts. To confirm this, we rescued the plasmids containing various cDNA inserts from the λExLox vectors and either transformed *E. coli* BL21(DE3) cells to generate T7 gene 10-fused proteins, or subcloned the cDNA released from the clone into a GST-fusion construct. When purified GST-fused Bean protein immobilized on nitrocellulose membrane was probed with the three WW domains of Nedd4, clear binding of the probe was evident (Figure 3A). When either of the PY motifs in Bean was mutated, the binding of Nedd4 WW domains to the Bean protein was significantly reduced (Figure 3A). When both PY motifs in Bean were mutated, the binding of WW domains was completely abolished. These results suggest that the interaction between Nedd4 WW domains and Bean is mediated via the PY motifs in Bean and that both PY motifs in Bean bind the WW domains of Nedd4 with equal efficiency.

To analyse the interaction between other putative Nedd4 partners and the WW domains of Nedd4, the lysates prepared from transformed *E. coli* BL21(DE3) cells, which contained easily visible amounts of fusion proteins, as detected by Coomassie Brilliant Blue staining of electrophoresed proteins (Figure 4A), were subjected to far-Western analysis using the three WW domains of Nedd4 (Figure 4B). As expected, all

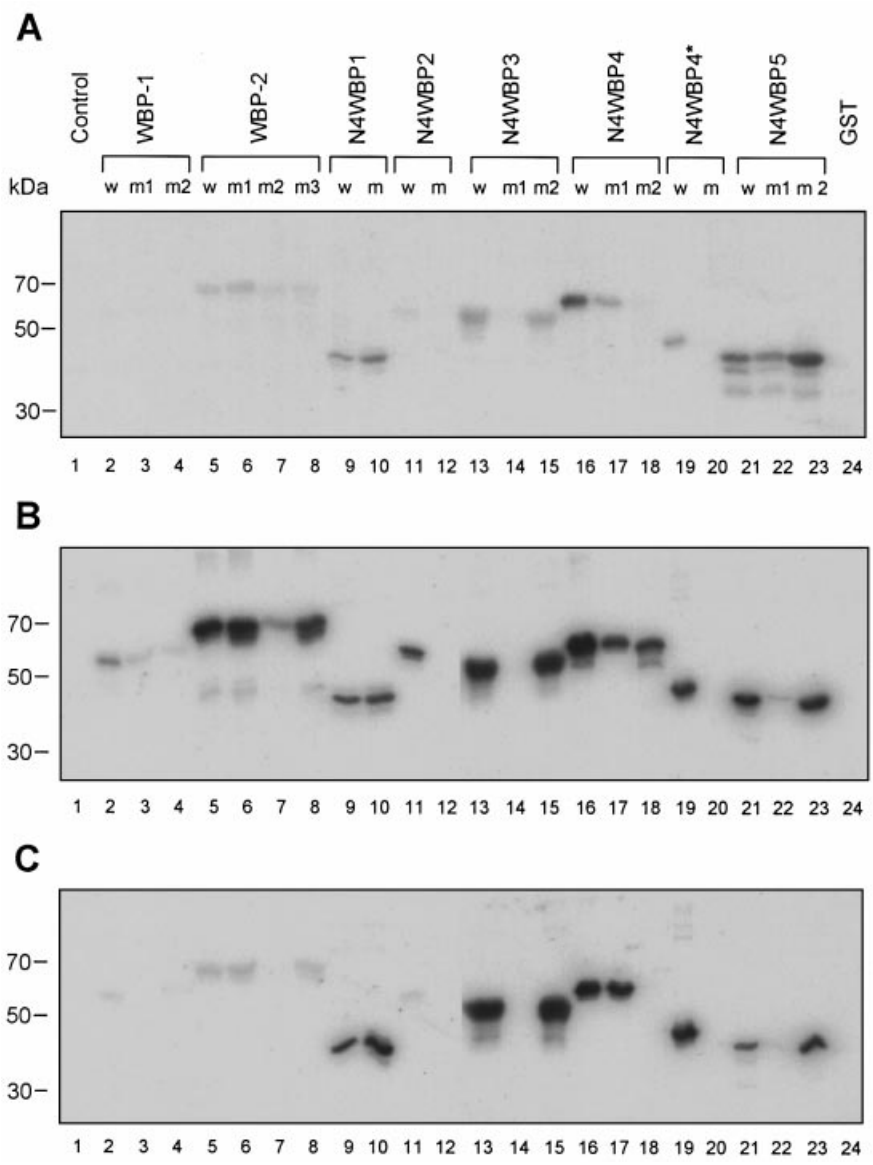

Figure 5 Different WW domains of Nedd4 interact with specific PY motifs

E. coli BL21(DE3) lysates containing T7 gene 10-fusion proteins were electrophoresed and blotted exactly as in Figure 4. Blots were then probed with ^{32}P-labelled GST-fusion protein containing Nedd4 WW1 (**A**), WW2 (**B**) or WW3 (**C**). The relative exposure time for the blot in (**A**) was 18 h, whereas for blots in (**B**) and (**C**) it was 3 h. For each WW-domain-interacting protein, we blotted the wild-type (w) and mutant (m) proteins carrying a single mutated PY motif. For proteins that contain more than one PY motif, the mutant PY motif is indicated (m1, m2 or m3). Lanes 1 (control) contain extract from untransformed *E. coli* BL21(DE3). Lanes 1–24 in Figure 4(A) correspond to lanes 1–24 in panels **A**–**C**.

proteins showed binding to the three WW domains, although binding by WBP-1 was relatively weaker than by others. Mutations in the single PY motif in N4WBP2 completely abolished its interaction with the Nedd4 WW domains (Figure 4B), suggesting that the binding is mediated via the PY motif. However, mutation of the only PY motif in N4WBP1 had no effect on interaction with the WW domains, indicating that this motif is not required to mediate binding (Figure 4B).

WBP-1 contains two PY motifs, both of which appeared to bind Nedd4 WW domains with equal efficiency, as mutations in either one reduced binding by approximately half (evident in longer exposures of the blots in Figure 4, not shown). WBP-2, which contains three PY motifs, showed strong interaction with Nedd4 WW domains. Mutations of PY motif 1 (PY1) or motif 3 (PY3) did not influence this binding significantly; however, mutation of the second PY motif drastically abrogated its binding with the three WW domains (Figure 4B), suggesting that this motif is crucial for mediating interaction. N4WBP3 contains two PY motifs, but only the first one of these is required for interaction with the WW domains of Nedd4, since mutation of this PY motif completely abolished interaction, whereas mutation of the second PY motif had no effect (Figure 4B). Mutation of both PY motifs in N4WBP4 affected binding to the Nedd4 WW domains; however, the second PY motif appeared to be the main ligand for binding, as its mutation had a much more dramatic effect on binding than the mutation of the first PY motif (Figure 4B). In the shorter version of this protein (shown as N4WBP4*), which contains only the second PY motif, mutation of the PY motif

completely abolished binding to Nedd4 WW domains (Figure 4B). Finally, mutation studies suggested that in N4WBP5, the first PY motif, but not the second one, is crucial for interaction with the WW domains of Nedd4. These results suggest that, with the exception of N4WBP1, the interactions between various proteins and Nedd4 WW domains are mediated through the PY motifs and that the PY motifs of Nedd4 WW-domain-binding proteins have varied affinities for the three WW domains of Nedd4.

Different WW domains of Nedd4 bind specific PY motifs in Nedd4-binding proteins

Murine Nedd4 protein contains three WW domains. To establish which of these WW domains interact with the PY motifs in various proteins isolated in the screens above, we used single WW domains of Nedd4 as probes in far-Western experiments. In the case of Bean, a reverse experiment was performed where GST-fused single wild-type and mutant WW domains were immobilized on nitrocellulose and probed with Bean protein (Figure 3B). When the first WW domain of Nedd4 was used, very weak binding to some proteins was observed in long exposures of the blots (Figure 5A). Bean, WBP-1 and N4WBP2 did not bind Nedd4 WW domain 1 (WW1; Figures 3B and 5A). PY-mutation studies similar to those in Figure 4 demonstrated that Nedd4 WW1 binds to WBP-2 via PY motifs 2 and 3, to N4WBP3 via the first PY motif and to N4WBP4 via the second PY motif (Figure 5A). WW1 also showed interactions with N4WBP1 and N4WBP5; however, these interactions were independent of the PY motifs, as mutations of PY motifs did not significantly alter binding.

Bean interacted with equal efficiency with Nedd4 WW domains 2 (WW2) and 3 (WW3; Figure 3B). Under identical conditions, WW2 showed stronger binding to WBP-1, WBP-2, N4WBP2 and N4WBP5, when compared with binding by WW3 (Figure 5B compared with 5C). This was particularly the case with WBP-1, WBP-2 and N4WBP2, all of which showed very weak interactions with WW3. N4WBP1 interacted with both WW2 and WW3 with equal efficiency and in both cases, as with the data in Figure 4(B), the interactions were independent of the PY motif. N4WBP3 also bound WW2 and WW3 with equal affinity, but in this case the interaction was dependent on the first PY motif of N4WBP3 (Figures 5B and 5C). In N4WBP4, both PY motifs showed equal binding to WW2; however, only the second motif bound to WW3, as mutation of this PY motif, but not the first PY motif, completely abolished binding. On the other hand, N4WBP5 binding to both WW2 and WW3 was mediated by the first PY motif in the protein and mutation of the second PY motif did not influence interactions (Figures 5B and 5C). These results clearly suggest that the three WW domains of Nedd4 have different preferences for PY motifs.

DISCUSSION

In this paper we have described the identification of eight new proteins that interact with the Nedd4 WW domains. As all of these proteins are expressed in mouse embryos at the time when Nedd4 expression is very high, it is likely that at least some of these proteins are authentic Nedd4-binding proteins *in vivo*. Our preliminary co-immunoprecipitation experiments also suggest that the two WW-domain-binding proteins (Bean and N4WBP5) tested so far can interact with Nedd4 *in vivo* upon transient overexpression in mammalian cells (B. P. Haines, K. F. Harvey and S. Kumar, unpublished work). Except for N4WBP1, in all cases, the interaction of Nedd4 WW domains was mediated via one or more PY motifs. Since PY motifs are *bona fide* ligands for WW domains, the results validate the authenticity of our far-Western screens.

Two of the proteins identified in the screens, Bean and N4WBP5, are novel, although EST sequences corresponding to these have recently appeared in the database. For the remainder of the proteins, there is little functional information currently available. WBP-1 and WBP-2 had previously been identified as proteins that interact with the WW domain of Yes-associated protein (YAP) [26]; however, the cellular and biochemical functions of these proteins are obscure. PRGP-2, the human homologue of N4WBP1, was identified as a vitamin-K-dependent protein with an N-terminal γ-carboxyglutamic acid-containing domain [27]. PRGP-2 is predicted to contain a transmembrane domain [27], suggesting that it may interact with Nedd4 at the membrane. Rnf11 (N4WBP2) is a predicted RING-finger protein of unknown function [28], whereas LITAF/Pig7 (N4WBP3) is a transcription factor that is up-regulated in response to lipopolysaccharide treatment of cells and promotes tumour necrosis factor α gene expression [30]. Although it is not clear how Nedd4, a cytoplasmic protein [22], might regulate the activity of a nuclear transcription factor, human Nedd4 and its yeast homologue Rsp5 have been shown to be involved in the potentiation of hormone-dependent activation of transcription by progesterone and glucocorticoid receptors [33]. Nedd4 has also been shown to interact with the haematopoietic transcription factor p45/NF-E2 (an erythroid-specific nuclear factor) *in vitro* [34], although the physiological significance of this interaction has yet to be demonstrated.

Our results show that in all cases except N4WBP1, interaction with Nedd4 WW domains is mediated through PY motifs. However, not all PY motifs appear to be required for binding to the Nedd4 WW domains. For example, while both PY motifs in Bean, WBP-1 and N4WBP4 appear to bind Nedd4 WW domains, only PY2 in WBP-2, PY motif 1 (PY1) in N4WBP3 and PY1 in N4WBP5 are crucial for interaction with Nedd4 WW domains (Table 2). Interestingly, the sequence of the PY motif itself does not appear to dictate its binding affinity to Nedd4 WW domains. For instance, while the PPPY motifs in N4WBP1 and in WBP-2 (PY3) do not appear to be essential for interaction with the WW domains, PPPY motifs in Bean (PY2), N4WBP2, N4WBP4 (PY1), N4WBP5 (PY1), WBP-1 and WBP-2 (PY2) bind Nedd4 WW domains (Table 2). In addition, the Nedd4 WW domains efficiently bind divergent PY motifs, such as PPGY in Bean (PY1), PPTY in N4WBP3 (PY1) and N4WBP4 (PY2), and PPAY in WBP-1. In mouse and human ENaC, Nedd4 WW domains interact with the PPAY motif in the α-subunit, the PPNY motif in the β-subunit and PPR/KY in the γ-subunit [14]. These observations suggest that the context of a given PY motif in the surrounding sequence, rather than the sequence of the third residue in a PPXY motif, may determine its binding affinity to the WW domains.

It is of interest that the only PY motif present in N4WBP1 is not required for the interaction with Nedd4 WW domains, suggesting that binding is mediated by an alternative sequence present in N4WBP1. It will be interesting to characterize the WW-domain-binding motif in N4WBP1, as it may define a novel ligand for WW domains. In addition to PY motifs, some WW domains are known to bind PPLP, PPR and PGR sequences [8,9,35], and phosphoserine/threonine residues [10]. A PPLP motif is present in mouse N4WBP1, but not in its human counterpart PRGP-2, which may mediate interaction with the WW domains of Nedd4. This seems unlikely however, as the WW domains of Nedd4 appear to belong to the group-I subclass, which prefer PPXY, and not the group-II subclass of WW

Table 2 The three WW domains in Nedd4 bind various PY motifs in Nedd4 WW-domain-binding proteins with different affinities

PY motifs (shown in bold) and 10 amino acids on either side of the PY motifs are shown. In the case of the PY3 motif of WBP-2 and the PY motif of N4WBP1, there are only 9 and 7 carboxyl residues, respectively, before the termini of these proteins (shown by *). The data on binding of various WW domains and PY motifs are derived from the far-Western assays in Figures 4 and 5. The binding data for WW1 are not directly comparable with WW2 and WW3 data as WW1 binds very weakly to its ligands (see Figure 5 above). The number of + symbols represent relative strength of interactions, whereas — indicates that no binding was seen in far-Western assays.

		PY-motif/WW-domain interactions			
Protein	PY motif and surrounding sequence	WW1–3	WW1	WW2	WW3
Bean	TVLWELYPDS**PPGY**EECMGPGATQ (PY1)	+++++	−	+++	+++
	ATQLYVPTDA**PPPY**SMTDSCPRLN (PY2)	+++++	−	+++	+++
WBP-1	LDLRLLSAFK**PPAY**EDVVHHPGTP (PY1)	++	−	+	+
	EDVVHHPGTP**PPPY**TVGPGYPWTT (PY2)	++	−	+	+
WBP-2	PPVANGMYPC**PPGY**PYPPPPPEFY (PY1)	−	−	−	−
	DGAMGYVQPP**PPPY**PGPMEPPVSG (PY2)	++++	−/+	+++	+
	NVYMPTSQPP**PPPY**YPPEDKKTQ* (PY3)	−/+	−/+	−	−
N4WBP1	LAASGVHDAP**PPPY**SSLRRPH*	−	−	−	−
N4WBP2	GEGTEPDQEP**PPPY**QEQVPVPIYH	++	−	++	−
N4WBP3	AAGPSVVPTA**PPTY**EETVGVNSYY (PY1)	+++++	+	+++	+++
	ITGPDGKGMN**PPSY**YTQPVPVPNA (PY2)	−	−	−	−
N4WBP4	PTISLSDGEE**PPPY**QGPCTLQLRD (PY1)	+++	+/−	+++	−
	YSSGGRMEGP**PPTY**SEVIGHYPGS (PY2)	+++++	+	+++	+++
N4WBP5	GEPEQTAGDA**PPPY**SSITAESAAY (PY1)	+++++	+	+++	++
	DYKDESGFPK**PPSY**NVATTLPSYD (PY2)	−/+	+	−	−

domains, which prefer the PPLP motif [36]. Also, since our far-Western assays utilized proteins expressed in *E. coli*, it is unlikely that the interaction between Nedd4 WW domains and N4WBP1 is mediated by phosphoserine or phosphothreonine residues in N4WBP1. This suggests that the WW domains of Nedd4 may recognize a novel motif in N4WBP1.

Our far-Western data using individual Nedd4 WW domains as probes suggest that the three WW domains interact with different PY motifs with varying degrees of affinity. The first WW domain (WW1) only interacts very weakly with some of the PY motifs in N4WBP3, N4WBP4 and N4WBP5 (Table 2). Interestingly, WW1 also does not bind any of the ENaC subunits, p45/NF-E2 or RNA polymerase II, all of which interact with Nedd4 via either WW2 or WW3, or via both WW2 and WW3 [14,34,37]. These observations suggest that WW1 of Nedd4 may have more stringent binding specificity than WW2 and WW3. WW2 binds more PY ligands than WW1 or WW3. Thus WW3 shows more specificity of protein–protein interaction than WW2. As discussed above, the differences in interaction between WW2/WW3 and PY motifs do not appear to be dependent on the tetrapeptide PPXY sequence. For instance, PPPY motifs in Bean and N4WBP5 can bind both WW2 and WW3 with roughly equal affinities, yet PPPY motifs in WBP-1 (PY2), WBP-2 (PY2), N4WBP2 and N4WBP4 (PY1) mostly bind WW2, but not WW3. Furthermore, the PPPY motifs (PY3) in WBP-2 and N4WBP1 do not appear to interact with either WW2 or WW3. These results suggest that the context of the PY motif in the surrounding protein sequence, which presumably dictates the accessibility of the PY motif to a WW domain, may determine the specificity of its interaction with a particular WW domain.

Although the studies described in this paper provide new information on the complexity of interaction between various WW domains and their ligands, the functional significance of the interactions between Nedd4 and various proteins that have been identified in this study remains to be established. Since four of the eight Nedd4 WW-domain-binding proteins are putative membrane-associated/spanning proteins, we believe that they are strong candidates for Nedd4-mediated regulation. Our current and planned studies will address whether Nedd4 indeed mediates the ubiquitin-dependent regulation of these proteins.

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