NMR studies of tandem WW domains of Nedd4 in complex with a PY motif-containing region of the epithelial sodium channel

Voula Kanelis, Neil A. Farrow, Lewis E. Kay, Daniela Rotin, and Julie D. Forman-Kay

Abstract: Nedd4 (neuronal precursor cell-expressed developmentally down-regulated 4) is a ubiquitin-protein ligase containing multiple WW domains. We have previously demonstrated the association between the WW domains of Nedd4 and PPxY (PY) motifs of the epithelial sodium channel (ENaC). In this paper, we report the assignment of backbone $^{1}H\alpha$, ^{1}HN, ^{15}N, $^{13}C'$, $^{13}C\alpha$, and aliphatic ^{13}C resonances of a fragment of rat Nedd4 (rNedd4) containing the two C-terminal WW domains, WW(II+III), complexed to a PY motif-containing peptide derived from the β subunit of rat ENaC, the βP2 peptide. The secondary structures of these two WW domains, determined from chemical shifts of $^{13}C\alpha$ and $^{13}C\beta$ resonances, are virtually identical to those of the WW domains of the Yes-associated protein YAP65 and the peptidyl–prolyl isomerase Pin1. Triple resonance experiments that detect the $^{1}H\alpha$ chemical shift were necessary to complete the chemical shift assignment, owing to the large number of proline residues in this fragment of rNedd4. A new experiment, which correlates sequential residues via their ^{15}N nuclei and also detects $^{1}H\alpha$ chemical shifts, is introduced and its utility for the chemical shift assignment of sequential proline residues is discussed. Data collected on the WW(II+III)-βP2 complex indicate that these WW domains have different affinities for the βP2 peptide.

Key words: WW domain, PY motif, Nedd4, ENaC, NMR.

Résumé : La protéine Nedd4 (précurseur neuronal 4 exprimé dans les cellules et réprimé au cours du développement) est une ubiquitine–protéine ligase ayant plusieurs domaines WW. Antérieurement, nous avons démontré une association entre les domaines WW de Nedd4 et les motifs PPxY (PY) du canal sodique épithélial (ENaC). Dans cet article, nous décrivons l'attribution des résonances des $^{1}H\alpha$, ^{1}HN, ^{15}N, $^{13}C'$, $^{13}C\alpha$ et ^{13}C aliphatiques du squelette d'un fragment de la protéine Nedd4 de rat (rNedd4) ayant les deux domaines WW C-terminaux, WW (II+III), formant un complexe avec un peptide à motif PY dérivé de la sous-unité β du ENaC de rat, le peptide βP2. Les structures secondaires des deux domaines WW, déterminées à partir des déplacements chimiques des résonances de $^{13}C\alpha$ et $^{13}C\beta$, sont pratiquement identiques à celles des domaines WW de la protéine YAP65 associée à Yes et de la peptidyl-prolyl isomérase Pin1. À cause du grand nombre de résidus proline dans ce fragment de la rNedd4, des expériences de triple résonance détectant le déplacement chimique de $^{1}H\alpha$ ont été nécessaires pour compléter l'attribution des déplacements chimiques. Une nouvelle expérience de corrélation de résidus séquentiels grâce à leurs noyaux ^{15}N et qui détecte également les déplacements chimiques de $^{1}H\alpha$, est présentée et son utilité pour l'attribution des déplacements chimiques d'une séquence de résidus proline est discutée. Les données obtenues concernant le complexe WW(II+III)-βP2 indiquent que ces domaines WW ont des affinités différentes envers le peptide βP2.

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Abbreviations: HSQC, heteronuclear single quantum coherence; ENaC, epithelial sodium channel; Hect, homology to the E6-AP C-terminus; HSQC, heteronuclear single quantum coherence; Nedd4, Neuronal precursor cell-expressed developmentally down-regulated 4; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; PY motif, PPxY; WBP1(2), WW domain binding protein 1(2); YAP65, Yes-associated protein 65.

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Mots clés : domaine WW, motif PY, Nedd4, canal sodique épithélial, ENaC, RMN.

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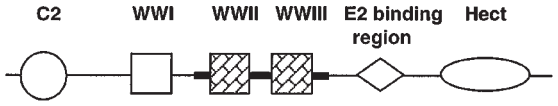
Introduction

WW domains, also known as WWP or Rsp5 domains, are protein–protein interaction modules approximately 40 amino acids in length (André and Springael 1994; Bork and Sudol 1994; Hofmann and Bucher 1995; Staub and Rotin 1996). These domains contain two highly conserved tryptophans and an invariant proline, hence the name WW or WWP domains. WW domains are present in a variety of cytoskeletal and signaling proteins such as the Yes-associated protein YAP65, the peptidyl–prolyl isomerase Pin1, dystrophin, the transcriptional activator FE65, formin binding proteins, and the ubiquitin-protein ligate Nedd4–Rsp5–Pub1. Similar to other modular binding domains including SH2, SH3, and PTB domains (Pawson 1995), WW domains mediate protein interactions in cellular processes.

WW domains bind proline-rich regions of their target proteins. The WW domain from YAP65 has been shown to interact in vitro with the sequence PPPPY, found in the WW domain binding proteins WBP1 and WBP2 (Chen and Sudol 1995). Through site-directed mutagenesis of this sequence in WBP1, the PY motif (PPxY) was proposed as the consensus sequence for YAP65 WW domain binding. The association of other WW domains with PY motifs has also been demonstrated. The WW domain from dystrophin binds a PY motif found in β-dystroglycan (Jung et al. 1995). PY motifs found in the β and γ subunits of the epithelial sodium channel, ENaC, interact with the WW domains of Nedd4 (Staub et al. 1996). The PY motif found in the α-subunit binds to the Nedd4 WW domains and the spectrin SH3 domain (Rotin et al. 1994; Staub et al. 1996). The PY motif differs from the SH3-binding consensus sequence, PxxP (Pawson 1995). However, there are cases where this sequence also interacts with WW domains. The SH3-binding sequence in formin (PPLP) interacts with both the Abl SH3 domain and WW domains from formin binding proteins (Bedford et al. 1997; Chan et al. 1996). Similarly, the PPLP sequence in Mena binds the FE65 WW domain (Ermekova et al. 1997).

Currently, the structures of two WW domains have been determined. The solution structure of the YAP65 WW domain (Macias et al. 1996) was solved in complex with a peptide derived from WBP1, which includes the PPPPY sequence and the crystal structure of the WW domain from the peptidyl–prolyl isomerase Pin1 was solved, within the context of the whole protein (Ranganathan et al. 1997). In both cases, the WW domain structure consists of a compact three-stranded antiparallel β-sheet, such that the N- and C-termini of the sheet are close in space, typical of modular binding domains. Conserved hydrophobic residues, including the first tryptophan and the invariant proline, are found in the core of the domain. The second conserved tryptophan is located in the binding surface along with other conserved hydrophobic residues, including a conserved tyrosine. NMR studies performed on the YAP65 WW domain indicate that this tryptophan contacts the second conserved proline residue in the PY motif. Since experimental data were not able to define the structure of the WBP1 peptide in this complex, the PY motif portion of the peptide was modeled as a polyproline type II (PPII) helix in the ligand binding site of the YAP65 WW domain, a conformation adopted by PxxP motif-containing ligands bound to SH3 domains (Lim et al. 1994). The peptide binding site of SH3 domains, like that of WW domains, also consists of a flat surface composed of aromatic residues (Pawson 1995), suggesting that a PPII helix is a reasonable model for the conformation of a PY motif-containing peptide in the WW domain binding site. This similarity in binding surface may also explain why some WW and SH3 domains can bind the same ligands.

Fig. 1. A schematic representation of the modular structure of the rat homologue of Nedd4, rNedd4, showing the C2 domain, three WW domains, the E2 binding region, and the ubiquitin-protein ligase (Hect) domain. The second and third WW domains are hatched and the region of Nedd4 around these domains that was included in the expressed construct is shown by the thick line. The sequence of the WW(II+III) construct with the WW domains underlined is given below. The sequence in lowercase letters corresponds to vector sequences. Numbering corresponds to the recombinant construct as described in the Materials and methods section.

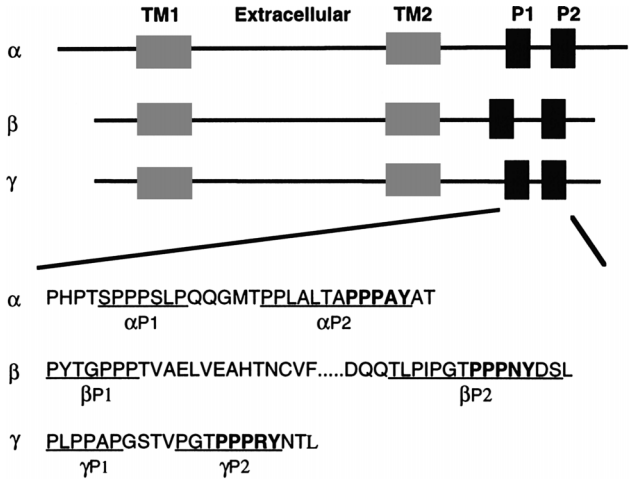


1 mghhhhhhhhhhssghiddddkhmledPTLPVLLPTSSGLPPGWEEKQDD
51 RGRSYYVDHNSKTTTWSKPTMQDDPRSKIPAHLRGKTPVDSNDLGPLPPG
101 WEERTHTDGRVFFINHNIKKTQWEDPRMQNVAITGPAEPYS

Extensive studies of WW domain–ligand interactions are still lacking. An experimental description of the structure of a WW domain ligand is currently not available, since only a model from the YAP65 WW domain–peptide structure exists and no ligand is present in the case of the Pin1 WW domain structure. Also, in contrast to some other protein–protein interaction modules, most notably SH2 domains, determinants for binding specificity have not been established for WW domains. However, there is some evidence for distinct ligand preferences for different WW domains. We have demonstrated the association of the PY motif-containing regions in ENaC with WW domains from rNedd4 and hYAP65, but not with dystrophin (O. Staub and D. Rotin, unpublished results). Recently, Fowlkes and coworkers showed selective binding in vitro of multiple WW domains in two Nedd4-like proteins (WWP1 and WWP2) to PY motifs from various proteins including WBP1, WBP2, β-dystroglycan, and RSV-1 Gag (Pirozzi et al. 1997). Whether any of these interactions have physiological relevance remains to be determined.

The biological importance of WW domain – PY motif interactions is evident from studies of Nedd4 and ENaC. Nedd4 is a modular protein consisting of a C2 domain, an E2 binding region, and a ubiquitin protein ligase Hect domain, in addition to three or four copies of the WW domain depending on the species (Fig. l) (Kumar et al. 1992; Staub

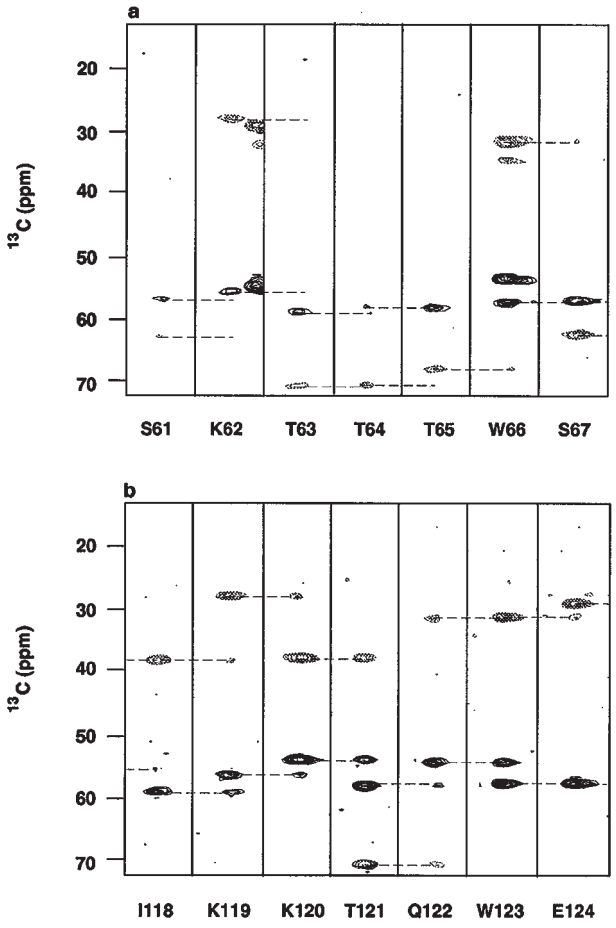
Fig. 2. A schematic representation of the α, β, and γ subunits of the rat homologue of ENaC (rENaC). TM1 and TM2 represent transmembrane segments. P1 and P2 are proline-rich regions. PY motifs (in boldface) are found in the P2 regions of each subunit.



and Rotin 1996). The association between Nedd4 and ENaC has been demonstrated both in vitro and in living cells, and this interaction is mediated by the WW domains of Nedd4 and PY motif-containing regions of ENaC (Staub et al. 1996). ENaC is located on the apical membrane of polarized epithelia and is responsible for vectoral transport of Na^+ from the lumen to the blood. Thus, proper regulation of ENaC is necessary to maintain blood volume, blood pressure, and sodium balance. ENaC is composed of three structurally similar subunits, α, β, and γ (Canessa et al. 1993, 1994), which have recently been shown to associate with a stoichiometry of $\alpha_2\beta\gamma$ (Firsov et al. 1998). Each subunit contains two transmembrane segments, a large extracellular region, and short N- and C-terminal cytoplasmic tails. The PY motifs of ENaC are found in the second of two proline-rich regions (P2) located in the C-terminus of each subunit (Fig. 2) (Schild et al. 1996; Straub et al. 1996). Deletions or mutations within PY motifs in the β or γ subunits cause Liddle's syndrome (Hansson et al. 1995*a*, 1995*b*; Shimkets et al. 1994; Snyder et al. 1995; Tamura et al. 1996), an autosomal dominant form of hypertension characterized by excessive Na^+ reabsorption in the distal nephron (Liddle et al. 1963). Mutations that cause Liddle's syndrome result in increased channel activity, which is thought to be caused by an increase in channel number at the cell surface (Schild et al. 1995, 1996) and an increase in channel opening (Firsov et al. 1996). The same mutations also abrogate binding to Nedd4 WW domains (Staub et al. 1996). Thus, the PY motifs appear to be involved in regulating channel activity.

We have proposed a model by which Nedd4 may regulate channel function by binding ENaC through a WW domain – PY motif interaction and subsequently ubiquitinating the channel through the action of the Hect domain (Staub et al. 1996). Indeed, we have recently demonstrated that ENaC stability and function are regulated by ubiquitination (Staub et al. 1997). The C2 domain likely provides the mechanism by which Nedd4, a cytosolic protein, is targeted to the plasma membrane where ENaC is located (Plant et al. 1997). Since the binding of Nedd4 WW domains to the β-subunit of ENaC is abrogated in Liddle's syndrome mutants (Staub et al. 1996), we speculate that channel ubiquitination and degradation may be impaired in the disease, leading to an increase in the number of active channels at the plasma membrane and hence to hypertension.

Fig. 3. Strip plot from the HNCACB experiment taken at the ^{15}N and HN chemical shifts of residues (*a*) Ser-61 to Ser-67 from WWII and (*b*) Ile-118 to Glu-124 from WWIII. $^{13}C\alpha\beta$ chemical shifts are on the vertical axis. Broken lines reflect sequential connectivities.



The above findings highlight the importance of understanding the interactions of Nedd4 WW domains with the PY motifs of ENaC at the molecular level. We have therefore focused our studies on solving the three-dimensional structure of Nedd4 WW domains in complex with a PY motif-containing peptide from ENaC. In this paper, we report the backbone (^1HN, $^1H\alpha$, $^{13}C\alpha$, $^{13}C'$, and ^{15}N) and aliphatic ^{13}C resonances of a fragment of rNedd4 containing the closely spaced second and third WW domains, WW(II+III), in the presence of a peptide from the βP2 region of rat ENaC (rENaC). We describe the application of a new experiment that connects sequential residues via their ^{15}N nuclei and discuss its utility for backbone resonance assignment of sequential prolines. We have also determined the secondary structures of rNedd4 WW domains on the basis of $^{13}C\alpha$ and $^{13}C\beta$ chemical shifts and have compared them with the YAP65 and Pin1 WW domains. The available NMR data have enabled us to identify the WWIII domain as a higher affinity binding domain for the βP2 peptide, demonstrating ligand specificity in the WW domains of Nedd4 and

Fig. 4. (*a*) Schematic representation of a polypeptide chain showing nuclei correlated (circled) in the HBCBCA(CO)_N_CAHA experiment. (*b*) Strips from the HBCBCA(CO)_N_CAHA experiment taken at the $^{1}H\alpha(i)$ and $^{15}N(i + 1)$ chemical shifts for residues Asp-74 to Pro-80 of WW(II+III). $^{13}C\alpha\beta$ chemical shifts are on the vertical axis. Arrows indicate negative crosspeaks.

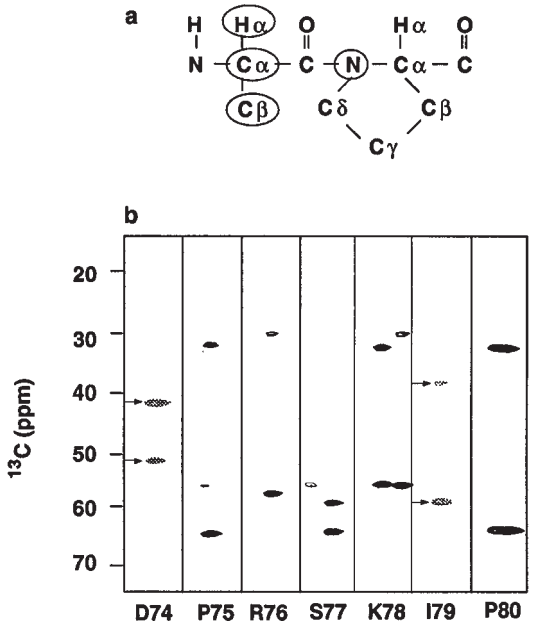
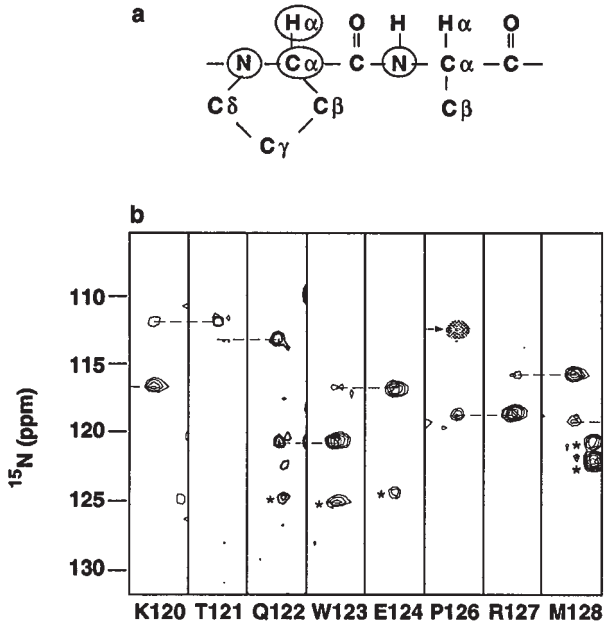


Fig. 5. (*a*) Schematic representation of a polypeptide chain showing nuclei correlated (circled) in the HACA_N experiment. (*b*) Strips from the HACA_N experiment taken at $^{13}C\alpha$ and $^{1}H\alpha$ chemical shifts for residues Lys-120 to Met-128 of WW(II+III). Resonances for Asp-125 were not observed. ^{15}N resonances of Pro-75 are aliased in this spectrum. An arrow indicates the negative crosspeaks and broken lines demonstrate sequential correlations. Asterisks represent unrelated crosspeaks.



providing a foundation for future structural studies of the βP2 peptide–WWIII complex.

Materials and methods

Sample preparation

A 10 histidine tagged fragment containing the second and third WW domains (residues Pro-397 to Ser-510) of rNedd4 was generated by PCR from cDNA (Staub et al. 1996) within a pET19b vector (Studier et al. 1990). Numbering of the residues in this paper is from the beginning of the recombinant construct (Fig. 1), with Pro-28 starting the Nedd4 sequence owing to 27 extra N-terminal residues for the histidine tag and a proteolytic cleavage site; these extra sequences were not removed. Isotopically enriched WW(II+III) was expressed in *Escherichia coli* BL21 (DE3) cells grown in 2 L of M9 minimal medium containing 3 g/L [^{13}C]glucose and 1 g/L [^{15}N]NH_4Cl as the sole carbon and nitrogen sources, respectively. After induction with 1 mM isopropyl β-D-thiogalactoside (IPTG), the overexpressed protein (16.1 kDa) was purified to homogeneity using Ni^{2+} affinity chromatography, followed by anion exchange and size exclusion chromatography. Purity and identity of the WW(II+III) protein fragment were confirmed by SDS-PAGE, electrospray mass spectrometry, and amino acid analysis. A synthetic 15-residue βP2 peptide, derived from the βP2 region of rENaC (TLPIPGTPPPNYDSL), was titrated into an NMR sample containing 1.0 mM ^{13}C- and ^{15}N-labeled WW(II+III) in 10 mM sodium phosphate, pH 7.4, 90% H_2O 10% D_2O. Following the peptide titration, the pH of the sample was lowered to pH 6.0 by the addition of concentrated HCl.

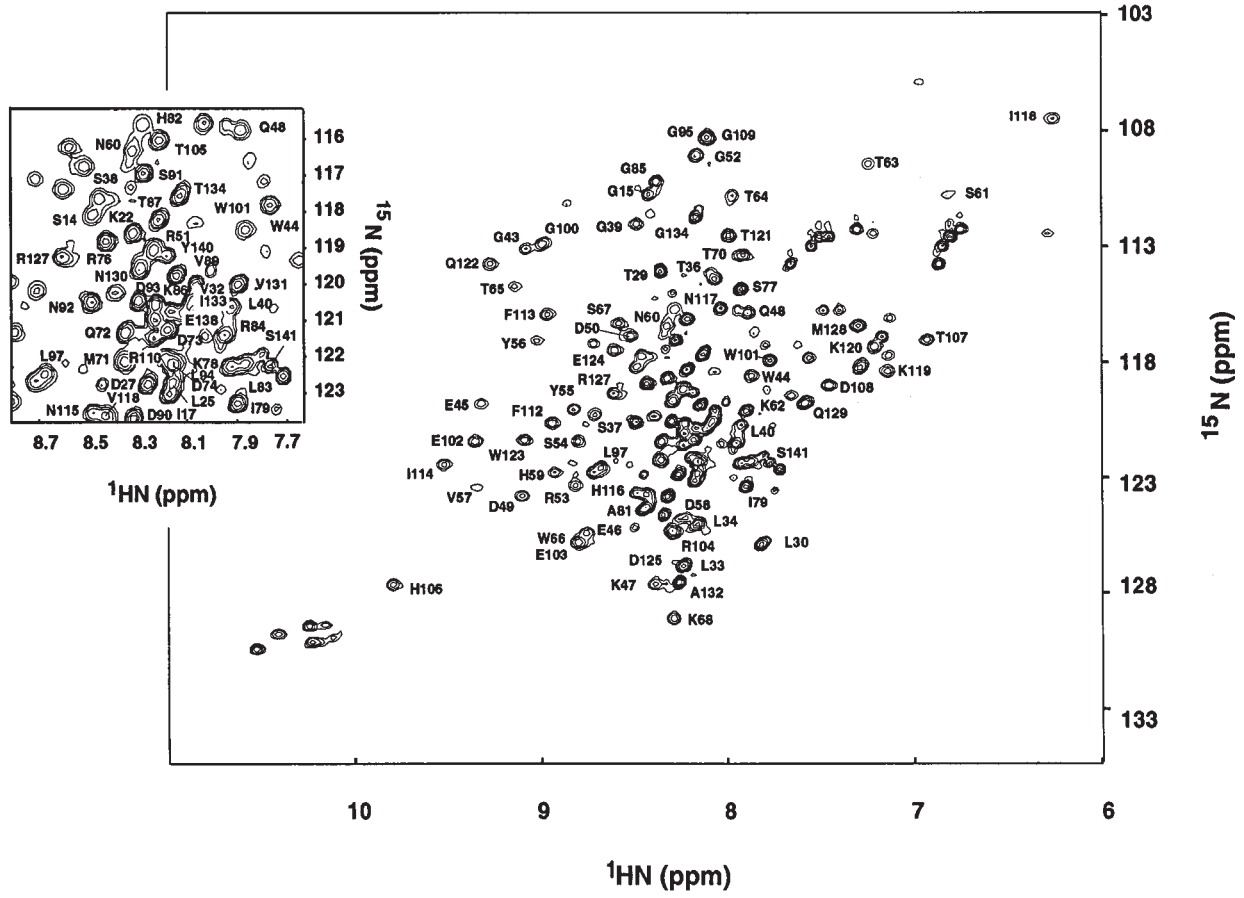
NMR spectroscopy

NMR experiments were performed at 30°C on a Varian INOVA 500-MHz spectrometer equipped with a pulsed field gradient unit and a triple resonance probe. NMR spectra were processed and analyzed using VNMR, nmrPipe/nmrDraw (Delaglio et al. 1995), and NMRView (Johnson and Blevins 1994) software.

A titration of the WW(II+III) fragment with βP2 peptide was monitored by recording gradient sensitivity enhanced ^{1}H–^{15}N heteronuclear single quantum coherence (HSQC) spectra (Zhang et al. 1994). HSQC spectra were recorded with 128 complex t_1 increments, using 16 scans. Spectral widths were 8008 Hz (^{1}HN) and 1650 Hz (^{15}N). The titration was stopped after the addition of 8.0 mM βP2 peptide, although the spectra were still changing (see below). Triple resonance experiments were performed on the WW(II+III)–βP2 complex in l0 mM sodium phosphate, pH 6.0, 90% H_20 10% D_20, 30°C.

The three-dimensional HNCO (Kay et al. 1994), HNCACB (Wittekind and Mueller 1993), HBCBCACONNH (Grzesiek and Bax 1992), and HNCAHA (Kay et al. 1992) experiments were performed to assign the ^{15}N, $^{13}C'$ (carbonyl), $^{13}C\alpha$, $^{13}C\beta$, ^{1}HN, and $^{1}H\alpha$ nuclei. These spectra were recorded with spectral widths of 8000 Hz (^{l}HN) and 1164 Hz (^{15}N). For the HNCO experiment, 64 complex t_1 and 28 complex t_2 increments were recorded, using 16 scans and a spectral width of 1541 Hz ($^{l3}C'$). The HNCACB and HBCBCACONNH experiments were recorded with 60 complex t_1 and 28 complex t_2 increments, using 16 scans and a spectral width of 7650 Hz ($^{l3}C\alpha/\beta$). The $^{1}H\alpha$ chemical shifts were obtained from the HNCAHA experiment recorded with 48 complex t_1 and 28 complex t_2 increments, using 48 scans and a spectral width of 2000 Hz ($^{1}H\alpha$). The HBCBCACOCAHA experiment, which correlates chemical shifts of intraresidue $^{13}C\alpha$, $^{13}C\beta$, $^{1}H\alpha$, and $^{13}C'$ nuclei (Kay 1993), was used to confirm the backbone assignments. In addition, the chemical shifts of $^{13}C'$ nuclei of residues preceding proline were obtained using this experiment. The HBCBCA(CO)_N_CAHA experiment (to be described elsewhere by L. Kay and coworkers), which correlates the $^{13}C\alpha(i)$, $^{13}C\beta(i)$, and $^{1}H\alpha(i)$ chemical shifts of a given residue (i) with the $^{15}N(i + 1)$ chemical shift of the next residue, was used to

Fig. 6. ^{15}N–^{1}HN HSQC spectrum of the WW(II+III)–βP2 complex. Spectra were recorded at 30°C with a WW(II+III) concentration of 1.0 mM and a βP2 peptide concentration of 8.0 mM. Backbone amide cross peaks have been labeled. For clarity, backbone resonances for amino acids in the histidine tag have not been labeled. Side-chain glutamine and asparagine amide groups and tryptophan indoles have not been assigned or labeled.



assign the ^{15}N nuclei of proline residues. The HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments were recorded with 54 complex t_1 and 64 complex t_2 increments, using 16 scans and spectral widths of (7650, 1245, 7500 Hz) and (7650, 2226, 7500 Hz) in (F_1, F_2, F_3), respectively. A new experiment, the HACA_N, was designed to correlate the chemical shifts of ^{1}Hα(*i*), ^{13}Cα(*i*), and ^{15}N(*i*) with the ^{15}N(*i* + l) chemical shift (to be described elsewhere by L. Kay and coworkers). This experiment was recorded with 32 complex t_1 and 58 complex t_2 increments, using 16 scans and spectral widths of 8008 Hz (^{1}Hα), 1375 Hz (^{13}Cα), and 3200 Hz (^{15}N). The side-chain assignments for aliphatic ^{13}C nuclei were obtained with the CCC-TOCSY(CO)NNH experiment (Grzesiek et al. 1993; Logan et al. 1992) using the same parameters as for the HNCACB and HBCBCACONNH experiments.

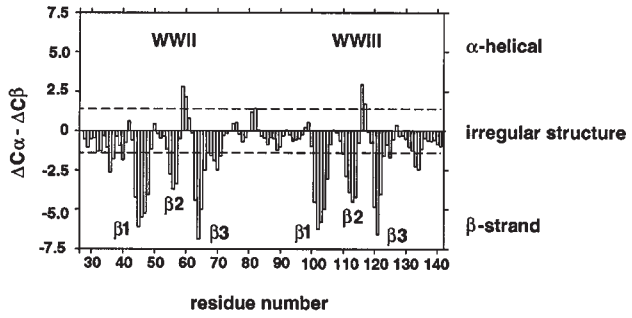
Results

NMR assignment strategy and resonance assignments of WW(II+III)

Using the HNCO, HNCACB, HBCBCACONNH, and HNCAHA experiments, we were able to assign 80% of all backbone resonances. A strip plot from the HNCACB experiment with data taken at the ^{15}N and ^{1}HN chemical shifts of residues Ser-61 to Ser-67 and Ile-118 to Glu-124 in the WWII and WWIII domains, respectively, is presented in Fig. 3. The spectrum shows quite weak crosspeaks for the WWII domain but clear sequential connectivities for residues of the WWIII domain and some residues of the WWII domain. The residues in the YAP65 WW domain corresponding to the regions of WW(II+III) indicated in the figure were shown to interact with a PY motif-containing peptide. Although crosspeaks for residues in the WWII binding region were broadened in the HNCACB, these crosspeaks were observed in the HBCBCACONNH experiment (data not shown).

The WW(II+III) protein contains 15 proline residues, including two regions with sequential prolines. Therefore, a complete backbone assignment of WW(II+III) was not possible using experiments that detect information on the ^{1}HN nucleus of amino acids only. The backbone assignments mentioned above were augmented using the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments to assign ^{13}C′ nuclei in residues preceding proline and ^{15}N nuclei of proline residues, respectively. Since the ^{1}Hα chemical shift is detected, the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments also connect sequential proline residues. In the HBCBCA(CO)_N_CAHA experiment, an Xaa-Pro connectivity is identified using two pieces of information. The chemical shifts of proline ^{15}N nuclei (134–140 ppm) are significantly downfield of the chemical shifts of non-proline ^{15}N nuclei (105–132 ppm). In addition,

Fig. 7. Secondary structure of WW(II+III) for residues Pro-28 to Ser-141. $\Delta C\alpha - \Delta C\beta$, the deviations of $^{13}C\alpha$ and $^{13}C\beta$ chemical shifts (Metzler et al. 1993) observed in WW(II+III) from those for residues in a random coil conformation (Spera and Bax 1991), is plotted as a function of residue number. Positive values indicate α-helical structure, whereas negative values denote β or extended structure.

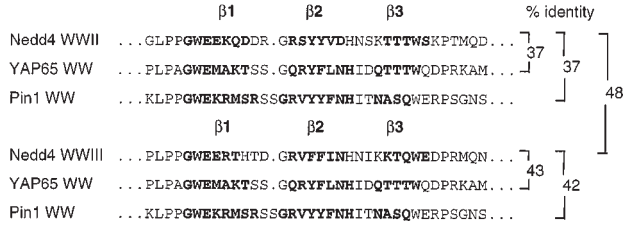


the $^{13}C\alpha$ and $^{13}C\beta$ resonances of residues preceding proline are opposite in sign to those of residues preceding non-proline amino acids. Once the $^{13}C\alpha$, $^{13}C\beta$, and $^{1}H\alpha$ chemical shifts are known, assignment of the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA spectra is straightforward. Data from the HBCBCA(CO)_N_CAHA experiment are shown in Fig. 4 with strips taken at the $^{1}H\alpha(i)$ and $^{15}N(i + 1)$ chemical shifts for residues Asp-74 to Pro-80, demonstrating the high quality of the spectrum and the negative correlations for residues N-terminal to proline.

In the regions of WW(II+III) with sequential proline residues a complete chemical shift assignment of the $^{13}C\alpha$, $^{13}C\beta$, and $^{1}H\alpha$ nuclei was not obtained and, therefore, the $^{13}C'$ and ^{15}N resonance assignment could not be completed from the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments alone. The HACA_N experiment was designed to facilitate assignment of sequential proline residues. Figure 5 shows data from the HACA_N experiment with strips taken at the $^{13}C\alpha(i)$ and $^{1}H\alpha(i)$ chemical shifts of residues Lys-120 to Met-128. The chemical shifts of the ^{15}N nuclei for residues (i) and $(i + 1)$ are along the vertical axis. Because the spectral width in the ^{15}N dimension was 3200 Hz (105–132 ppm), ^{15}N chemical shifts of proline, which are all downfield of 132 ppm, were aliased in these spectra. No correlations were observed at the $^{1}H\alpha$ chemical shift of Asp-125, perhaps owing to inefficient ^{13}C decoupling at this residue, so only a Pro-Xaa connectivity was seen for Pro-126. Both Xaa-Pro and Pro-Xaa connectivities were seen for other prolines. As a result of weak resonances and spectral overlap in the $^{1}H\alpha$ dimension with H_2O, data recorded on WW(II+III) did not confirm the proline–proline connectivities. This problem should be overcome by recording spectra on a sample in 100% D_2O. However, the other sequential connectivities observed (for Xaa-Pro, Pro-Xaa, and Xaa-Xaa) were useful in confirming previous assignments.

Figure 6 shows an HSQC spectrum of the WW(II+III)–βP2 complex, labeled with the assignments. Resonance assignments were obtained for 96% of the ^{15}N, 98% of ^{1}HN, 96% of $^{13}C\alpha$, 95% of $^{13}C'$, and 95% of $^{1}H\alpha$ positions. In addition, 90% of aliphatic ^{13}C chemical shifts were assigned.

Fig. 8. Sequence alignment of WW(II+III) of Nedd4 with WW domains of YAP65 and Pin1 and comparison of their secondary structures (Macias et al. 1996; Ranganathan et al. 1997). Residues in boldface represent those amino acids assigned to the three β-strands of the WW domain structure.



These assignments will be deposited in the BioMagResBank (Seavey et al. 1991).

Secondary structure of WW(II+III)

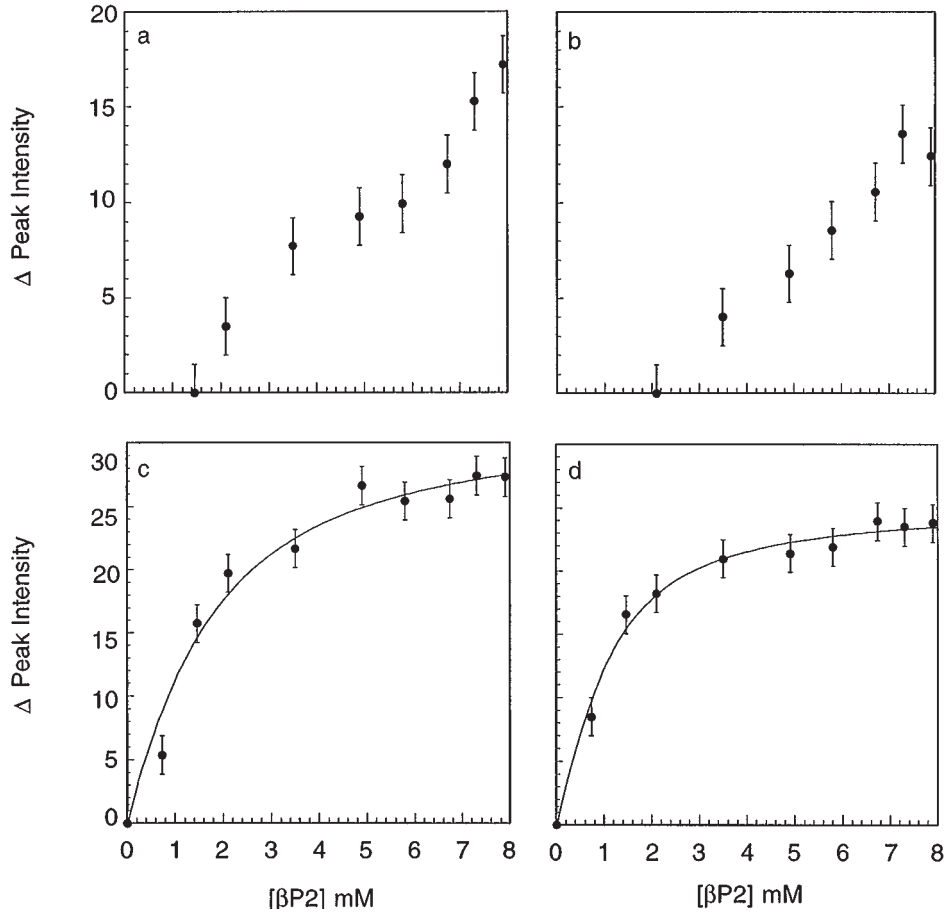
$^{13}C\alpha$ and $^{13}C\beta$ chemical shifts were used to determine the secondary structure of WW(II+III) (Metzler et al. 1993). The WWII and WWIII domains include residues 38–75 and 95–130, respectively, separated by a linker that does not contain regular secondary structure (Fig. 7). Each domain consists of three β-strands, where the second and third β-strands are separated by a helical turn. As expected on the basis of greater than 30% sequence identity of the individual rNedd4 WW domains (WWII and WWIII) to the YAP65 and Pin1 WW domains, the secondary structures of the rNedd4 WW domains are virtually identical to those of YAP65 and Pin1 (Fig. 8).

Of interest, $^{1}H\alpha$ chemical shifts of Lys-68 and Asp-125 were 2.77 and 2.83 ppm, respectively, significantly upfield of typical values. The homologous residues in the YAP65 and Pin1 WW domains are in close proximity to the aromatic rings of the first conserved tryptophan. Thus, these obviously ring current shifted values for Lys-68 and Asp-125 are consistent with an overall similar tertiary structure of the WWII and WWIII domains of Nedd4 to previously published WW domain structures.

Binding of βP2 to WWII and WWIII

As mentioned above, HSQC spectra of WW(II+III) were still changing after the addition of 8 mM βP2 peptide at which point the titration was stopped. An 8:1 ratio of βP2 peptide to WW(II+III), without full binding of the WW domains, suggests a low affinity for the βP2 peptide for at least one of the WW domains. The WW(II+III) resonances could be grouped into two sets on the basis of their behavior during the βP2 peptide titration (Fig. 9). Resonances from WWII continued to change throughout the titration, whereas those from WWIII reached a maximal intensity. The titration curves shown in Fig. 9 for WWIII were fit to the following equation: $I = I_o + (I_{max} - I_o)[\beta P2]_{free}/(K_{1/2} + [\beta P2]_{free})$, where I_o is the initial intensity, I_{max} is the maximal intensity, $[\beta P2]_{free}$ is the concentration of free peptide, and $K_{1/2}$ is the concentration of βP2 peptide required for maximal saturation of the intensity for that resonance. $[\beta P2]_{free}$ is calculated by subtracting the concentration of added peptide from the concentration of the complex as estimated from the intensity change. From these plots, a range in $K_{1/2}$ values from 0.55 to

Fig. 9. Intensity change for crosspeaks of WW(II+III) upon titration of βP2 peptide as a function of peptide concentration for residues (*a*) Asp-49 and (*b*) Ser-54 in WWII and (*c*) His-106 and (*d*) Met-128 in WWIII. Solid circles represent the intensity of the crosspeaks for the given residues. Error bars shown represent the noise level in the HSQC experiment.



3.00 mM was observed for the 10 residues with cross peaks in the titration spectra that could be clearly quantified.

Discussion

A complete chemical shift assignment for the Nedd4 WW(II+III) fragment was not possible using traditional triple resonance experiments owing to the number of proline residues in the polypeptide. The HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments are useful for assigning proline-rich sequences since magnetization is detected on the ^{1}Hα rather than ^{1}HN nucleus. The HBCBCACOCAHA experiment is also useful for confirming assignments using the ^{13}C′ chemical shift. An HCC-TOCSY(CO)NNH experiment (Logan et al. 1992) was performed to assign aliphatic proton resonances. The spectra were of high quality and yielded ^{1}Hα chemical shifts for all residues that did not precede a proline. We attempted to assign these remaining ^{1}Hα resonances using the ^{13}Cα and the ^{13}Cβ chemical shifts obtained from the HNCACB in combination with the HBCBCA(CO)_N_CAHA, which distinguishes residues preceding proline from those preceding non-proline amino acids. However, for many cases the chemical shifts of the ^{13}Cα and ^{13}Cβ nuclei in residues preceding proline were degenerate. Thus, without recording the HNCAHA experiment, the ^{1}Hα nuclei of these residues could not be assigned unambiguously and the ^{13}C′ and ^{15}N chemical shifts could not be obtained from the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments, respectively.

The WW(II+III) polypeptide contains two regions with the sequence Xaa(i – 1)-Pro(i)-Pro(i + 1)-Xaa(i + 2), making it difficult to obtain complete assignments using only the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments. The chemical shifts of ^{13}Cαβ and ^{1}Hα nuclei of the Pro(i) residues cannot be obtained independently of the HBCBCACOCAHA and HBCBCA(CO)_N_CAHA experiments. Therefore, if there is more than one region with this sequence in a protein or there are regions with more than two sequential proline residues, unambiguous chemical shift assignments of the ^{13}C′ of residue Pro(i) and the ^{15}N of residue Pro(i + l) are not possible using only these experiments. The HACA_N experiment has the advantage in that sequential residues are correlated by the same type of nucleus, ^{15}N. Therefore, this experiment can be used to assign backbone resonances in sequential proline residues as is done with the HNCACB experiment for non-proline-containing regions.

Experiments designed to assign sequential proline sequences are extremely important because proline-rich sequences play a critical role in protein recognition. In ad-

dition to WW domains, SH3 and EVH1 domains also recognize proline-rich regions in their targets. The EVH1 domain of Mena, a protein implicated in cytoskeletal organization, interacts with a region in profilin containing long stretches of prolines (Gertler et al. 1996). SH3 domains, found in a variety of proteins including nonreceptor tyrosine kineses and cytoskeletal components, recognize ligands with the consensus sequence PxxP (Alexandropoulos et al. 1995; Pawson 1995; Yu et al. 1994), such as the VPPPVPPRRR ligand for the Grb2 SH3 domains in Sos1 (Rozakis-Adcock et al. 1993). Several structures of SH3 domain–peptide complexes have been solved by NMR. Sequential assignments of the peptide have been obtained in most cases using a strategy based on nuclear Overhauser effect (NOE) (Teresawa et al. 1994; Wittekind et al. 1994), which involves the identification of residue type using ^{1}H–^{1}H or ^{1}H–^{13}C correlation spectra followed by NOE spectroscopy (NOESY) experiments to place the residues in sequential order. The triple resonance HACA_N experiment uses ^{15}N–^{15}N connectivities to obtain sequential assignments and analysis of the data is less complicated than for other correlation and NOE-based approaches. Therefore, this experiment will greatly facilitate backbone resonance assignment of sequential proline residues, particularly in larger complexes.

The peptide titration of WW(II+III) provided evidence for a difference in the binding affinities of each WW domain for the βP2 peptide (Fig. 9). However, the calculated $K_{1/2}$ value, the concentration of peptide at which half-maximal protein resonance intensity is observed, is not reflective of a binding event alone. Results from gel filtration and native gel electrophoresis experiments indicate that the WW(II+III) polypeptide is aggregated in the absence of the ligand. ^{15}N-^{1}HN HSQC spectra recorded on the free protein consist of few peaks with large line widths, confirming these results (data not shown). These observations are consistent with studies of the YAP65 WW domain in the absence of the WBP1 ligand (H. Oshkinat, European Molecular Biology Laboratory, Heidelberg, Germany personal communication). Titration curves obtained by monitoring peak intensity as a function of increasing peptide concentration thus represent both the binding of the βP2 peptide to the WW(II+III) protein and the breakup of WW(II+III) oligomers. The change in oligomeric state of the WW(II+III) sample upon binding the βP2 peptide contributes significantly to the increase in resonance intensity. Therefore, the $K_{1/2}$ values cannot be thought of as binding constants because there are two processes occurring. Studies are currently being carried out to determine K_d values for these interactions using intrinsic fluorescence experiments under conditions where the protein is monomeric. Nonetheless, it is evident from the NMR titration data that the βP2 peptide has a greater affinity for WWIII than for WWII. Differences in resonances of WWII and WWIII domains in the complex are also evident from the HNCACB experiment, as noted above. Resonances for nuclei in the WWII binding site are broadened because the titration was stopped before reaching the saturation point for this site and the protein nuclei are in exchange between free and peptide-complexed states. The different binding affinities of Nedd4 WWII and WWIII for the βP2 peptide from ENaC suggest distinct ligand preferences for each domain and perhaps different in vivo targets for each domain. Thus, different WW domain – PY motif interactions may provide a mechanism to couple Nedd4 to numerous ubiquitination targets.

In summary, we have assigned backbone and aliphatic ^{13}C resonances of a fragment of rNedd4 consisting of the second and third WW domains. The completeness of this assignment was made possible using experiments that observe connectivities involving proline residues including the HBCBCA(CO)_N_CAHA and HACA_N experiments. The HACA_N experiment will be particularly useful in assignment of proline-rich regions because sequential residues are linked by ^{15}N correlations. These experiments may provide optimal information when performed on samples in D_2O to avoid overlap of the detected $^{1}H\alpha$ resonances with water. Experiments such as these that allow complete assignment through proline-rich regions of proteins are critical owing to the abundance of such sequences in protein recognition motifs, including those for WW, SH3, and EVH1 domains. Evidence was obtained from NMR experiments for different affinities of WWII and WWIII for the βP2 peptide, suggesting different ligand preferences for each domain. These results provide a foundation for future studies of a complex of βP2 of ENaC with the WWIII domain of Nedd4. Experimental structural data on the peptide conformation and the interactions with the WW domain that should be facilitated by the experiments described here will likely provide significant insights into the mechanism of specific protein recognition by WW domains.

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