Biochimica et Biophysica Acta 1834 (2013) 1632–1641

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Structure and dynamics of human Nedd4-1 WW3 in complex with the αENaC PY motif

Romel Bobby [a], Karima Medini [a,b,c], Philipp Neudecker [d,e], Tet Verne Lee [b], Margaret A. Brimble [a,b,c], Fiona J. McDonald [f], J. Shaun Lott [b,c], Andrew J. Dingley [a,b,c,*]

[a] School of Chemical Sciences, The University of Auckland, Private Bag 92 019, Auckland 1142, New Zealand
[b] School of Biological Sciences, The University of Auckland, Private Bag 92 019, Auckland 1142, New Zealand
[c] The Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland, Private Bag 92 019, Auckland 1142, New Zealand
[d] Institut für Physikalische Biologie, Heinrich-Heine-Universität, 40225 Düsseldorf, Germany
[e] ICS-6 (Strukturbiochemie), Forschungszentrum Jülich, 52425 Jülich, Germany
[f] Department of Physiology, University of Otago, PO Box 913, Dunedin 9054, New Zealand

ARTICLE INFO

Article history:
Received 4 March 2013
Received in revised form 26 April 2013
Accepted 29 April 2013
Available online 8 May 2013

Keywords:
E3 ubiquitin ligase
Epithelial Na^+ channel
NMR
Peptide–protein complex
Protein dynamics
WW domain

ABSTRACT

Nedd4-1 (*n*euronal precursor cell *e*xpressed *d*evelopmentally *d*ownregulated gene 4-1) is an E3 ubiquitin ligase that interacts with and negatively regulates the epithelial Na^+ channel (ENaC). The WW domains of Nedd4-1 bind to the ENaC subunits via recognition of PY motifs. Human Nedd4-1 (hNedd4-1) contains four WW domains with the third domain (WW3*) showing the strongest affinity to the PY motif. To understand the mechanism underlying this binding affinity, we have carried out NMR structural and dynamics analyses of the hNedd4-1 WW3* domain in complex with a peptide comprising the C-terminal tail of the human ENaC α-subunit. The structure reveals that the peptide interacts in a similar manner to other WW domain–ENaC peptide structures. Crucial interactions that likely provide binding affinity are the broad XP groove facilitating additional contacts between the WW3* domain and the peptide, compared to similar complexes, and the large surface area buried (83 $Å^2$) between R430 (WW3*) and L647′ (αENaC). This corroborates the model-free analysis of the ^{15}N backbone relaxation data, which showed that R430 is the most rigid residue in the domain ($S^2 = 0.90 \pm 0.01$). Carr–Purcell–Meiboom–Gill relaxation dispersion analysis identified two different conformational exchange processes on the μs–ms time-scale. One of these processes involves residues located at the peptide binding interface, suggesting conformational exchange may play a role in peptide recognition. Thus, both structural and dynamic features of the complex appear to define the high binding affinity. The results should aid interpretation of biochemical data and modeling interfaces between Nedd4-1 and other interacting proteins.

1. Introduction

Ubiquitination of cellular proteins describes the covalent attachment of ubiquitin onto specific substrate proteins through the actions of three enzymes: an E1 ubiquitin activating enzyme, an E2 ubiquitin conjugating enzyme and an E3 ubiquitin ligase. Classically, a chain of ubiquitin proteins promotes protein degradation. However, more recently it has become clear that diverse ubiquitin chains exist that promote a variety of outcomes, including endocytosis, translocation or change in the activity of the substrate protein [1,2]. Part of the diversity of the ubiquitin signal is because of the large number of E3 ubiquitin ligases that both catalyze ubiquitin chain formation and dictate substrate specificity. There are two major classes of E3 ligase: those that contain a RING domain and those that contain a HECT domain.

Nedd4-1 is a member of the Nedd4-family of HECT domain E3 ubiquitin ligases [3] and was originally discovered as a developmentally down-regulated gene in mouse brain [4]. The first substrate identified for Nedd4-1 was the human epithelial Na^+ channel (hENaC) that is located in the apical membranes of epithelial cells of the kidney, lung, colon and sweat gland [5,6], where it functions to regulate fluid and electrolyte homeostasis as well as blood pressure [7]. The hENaC channel is composed of three homologous subunits, α, β and γ, which each have their N- and C-termini located in the cytosol [8–10]. The activity of hENaC is suppressed when ubiquitinated by the Nedd4 ubiquitin ligases (Nedd4-1, Nedd4-2 and WWP2) [11–15], and ubiquitination leads to a decrease in the cell surface population of hENaC due to endocytosis and degradation [12,15,16].

The structural architecture of human Nedd4 proteins includes a C-terminal HECT ubiquitin ligase domain, a central protein–protein

Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill; ENaC, epithelial Na^+ channel; Nedd4-1, neuronal precursor cell expressed developmentally downregulated gene 4-1; RDC, residual dipolar coupling

* Corresponding author at: School of Chemical Sciences, The University of Auckland, Private Bag 92 019, Auckland 1142, New Zealand. Tel.: +64 9 923 6801; fax: +64 9 373 7422.
E-mail address: a.dingley@auckland.ac.nz (A.J. Dingley).

1570-9639/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbapap.2013.04.031

interaction region composed of four WW domains and an N-terminal membrane-targeting C2 domain [17]. The three subunits of hENaC have two short proline-rich sequences (P1 and P2) in their cytosolic C-terminal tails. The P2 sequence includes a conserved consensus sequence (PPXY), termed the PY motif, which is recognized by the Nedd4 family of proteins [5,18]. Deletion or mutation of the PY motif in the β or γ subunits of hENaC gives rise to Liddle's syndrome; a severe genetic form of arterial hypertension [18–20], in which endocytosis and degradation of the channel is severely disrupted [11,21].

Nedd4 proteins interact with ENaC through their WW domains [5,22]. WW domains are small interaction modules that contain 38–40 residues, including two conserved tryptophans and an invariant proline [23–26]. Structures of WW domains in complex with their cognate peptides have revealed that the first tryptophan and proline are important for the stability of the domain, whereas the second tryptophan is essential for ligand recognition [27–30]. Human Nedd4 WW domains have been designated as Class I WW domains because of their binding affinity to the PPXY motif in hENaC subunits. The Class I WW domains also include those that recognize the LPXY motif [31]. In contrast, Class II, III, IV and V WW domains select PPLP, PXXGMXPP, phospho-S/TP and RXPPGPPXR, respectively [31,32]. The number of WW domains in Nedd4 proteins varies. Nedd4-2 contains four WW domains (WW1, WW2, WW3 and WW4) in all animals. In contrast, Nedd4-1 lacks the WW3 domain in rat and mouse, though not in human and *Drosophila*. The WW domains of human Nedd4-1 bind the ENaC PY motif with differing affinities [22,33]. The strongest binding was reported for the third WW domain (WW3) in hNedd4-1 with a dissociation constant (K_d) of ~5 μM, whereas WW1 showed no affinity, and WW2 and WW4 showed binding that was 20–30-fold weaker than WW3 [22]. A construct encompassing WW domains 2, 3 and 4 bound only slightly weaker than the isolated WW3 domain, suggesting that the WW3 domain alone is sufficient for high-affinity recognition of the PY motif. In comparison, binding studies with rat Nedd4-1 WW4 and βENaC peptides revealed a K_d one order of magnitude weaker than the K_d reported for human WW3 [34], whereas the K_d value for the *Drosophila* Nedd4 (dNedd4) WW3 domain is similar to human WW3 [35]. WW domains that show high affinity for their targets are designated with an asterisk [34], and that nomenclature is followed here.

The solution structure of the dNedd4 WW3*–PY motif complex revealed crucial determinants involved in ligand-protein binding [35]. However, the peptide used in that study contained a 'LPXY' motif from the Commissureless protein with a consensus sequence $^{228'}$GLPSYDEA$^{235'}$ that differs from the ENaC PPPXY consensus sequence (peptide sequences are indicated with a prime (′) to distinguish from the WW domain sequences). Although both leucine and proline residues are tolerated N-terminal extensions of the PPXY core recognition motif for Class I WW domains, hENaC contains only the PPPXY core sequence. In addition, a leucine residue C-terminal of the PPPXY core is also conserved in many mammalian ENaCs, hence a consensus sequence of 'PPPXYXXL' appears to mediate high affinity binding [33,34]. The C-terminal leucine in the extended consensus sequence has been reported as a major contributor to the binding of the rNedd4-1 WW4 domain to rENaC, because a substitution to alanine leads to a six-fold decrease in affinity [33].

In order to further understand the high-affinity binding of WW3* domains to their cognate peptides, we have determined the structure and characterized the intrinsic dynamics of the complex of hNedd4-1 WW3* with the PY-motif peptide comprising the sequence $^{638'}$TA**PPP**A**Y**AT**L**G$^{648'}$ derived from α-hENaC.

2. Materials and methods

2.1. NMR sample preparation

The WW3* domain of hNedd4-1 (G416–A458) was expressed and purified as described previously [22,27], using M9 minimal media for incorporation of ^{13}C and ^{15}N isotopes. A uniformly ^{13}C/^{15}N-labeled peptide corresponding to the extended PY motif in the α-hENaC subunit ($^{638'}$TAPPPAYATLG$^{648'}$) was synthetically produced using standard Fmoc solid-phase chemistry [36]. Labeled Fmoc protected amino acids were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Following cleavage from the resin under standard acidic conditions, the peptide was dissolved in 5% (v/v) acetonitrile with 0.1% (v/v) TFA in H_2O. The crude peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Dionex UltiMate® 3000 Binary semi-preparative system (Thermo Scientific, Vic, Australia). Samples were purified on a Gemini C-18 column (10 × 250 mm, 5 μm, 110 Å; Phenomenex, CA, USA). The mass of the pure peptide was confirmed by LC–MS. An unlabelled version of the α-hENaC peptide was purchased from Auspep (Parkville, Vic, Australia).

For structure determination, two sets of samples with different labeling schemes were prepared in 20 mM sodium phosphate buffer, 0.1% (w/v) NaN_3, 1 mM TSP, pH 6.5 in 93%/7% (v/v) H_2O/D_2O. One sample was 1.1 mM of the uniformly ^{13}C/^{15}N-labeled hNedd4-1 WW3* domain with 2.2 mM of the unlabeled α-hENaC peptide. The second sample contained 1.5 mM of the uniformly ^{13}C/^{15}N-labeled α-hENaC peptide with 3.0 mM of the unlabeled hNedd4-1 WW3* domain.

2.2. NMR measurements used for structure determination

NMR spectra were recorded at 298 K on a Bruker AV600 spectrometer equipped with a 5-mm z-gradient TCI cryoprobe. Backbone and aliphatic side chain ^{1}H, ^{15}N and ^{13}C resonance assignments for the human Nedd4 WW3* domain and the α-hENaC peptide were obtained from multi-dimensional heteronuclear NMR experiments (Table S1). [37]. $H_\alpha C_\alpha N$ and $(H_\beta)C_\beta C_\alpha(CO)NC_\alpha H_\alpha$ experiments [38] were carried out to complete $^1H_\alpha$, ^{15}N, $^{13}C_\alpha$ and $^{13}C_\beta$ resonance assignments for the proline-rich αENaC peptide. Assignments for aromatic resonances were obtained from $(H_\beta)C_\beta(C_\gamma C_\delta)H_\delta$ and $(H_\beta)C_\beta(C_\gamma C_\delta C_\varepsilon)H_\varepsilon$ [39] spectra, as well as a ^{13}C-edited NOESY with a short mixing time of 50 ms. Proton chemical shifts were referenced to TSP, whereas the ^{15}N and ^{13}C chemical shifts were indirectly referenced according to the ratios given by Wishart et al. [40]. Data sets were processed using the program nmrPipe [41] and analyzed using the program CcpNmr Analysis [42].

Distance restraints were obtained from ^{15}N- and ^{13}C-edited NOESY spectra with mixing times ranging between 120–180 ms. Intermolecular distance restraints were also obtained from 250 ms half-filtered NOESY spectra [43] recorded on the labeled peptide sample. Hydrogen bond restraints for the WW3* domain were obtained by recording the long-range HNCO experiment [44]. Backbone dihedral restraints for Φ and Ψ were derived from backbone chemical shifts using TALOS + [45].

^{1}H–^{15}N residual dipolar couplings (RDCs) were obtained from the analysis of gradient-enhanced 2D ^{15}N–^{1}H IPAP-HSQC spectra [46]. The sample for measuring one-bond RDCs of the amide ^{1}H–^{15}N groups was weakly aligned in a radially compressed 5–6% polyacrylamide gel with a final protein:peptide ratio of 0.3 mM:0.6 mM.

2.3. Structure calculations and analysis

Chemical shift assignments and the ^{15}N- and ^{13}C-edited NOESY spectra were used for assigning the NOE cross peaks using the automated assignment software UNIO'10 [47,48] for the WW3* domain and the α-hENaC peptide. The tolerances for automatic assignments were 0.03 and 0.4 ppm in the ^{1}H and heteronuclear dimensions, respectively. Distance restraints were generated from the NOE cross peak volumes determined by UNIO'10. CYANA 2.1 [49] was used by UNIO'10 for the torsion angle molecular dynamics simulations. For all NOE restraints r^{-6} sum-averaging was used. The protein–peptide complex was calculated using CYANA 2.1 and the program's built-in NOE assignment routine. The half-filtered NOESY spectra were used for assignments and